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Sean Matthew McCauley
University of Massachusetts Medical School

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INNATE DETECTION OF HIV-1 IN MYELOID DENDRITIC CELLS

A Dissertation Presented

By

Sean M. McCauley

Submitted to the Faculty of the University of Massachusetts Graduate School of

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INNATE DETECTION OF HIV-1 IN MYELOID DENDRITIC CELLS

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Under the mentorship of:

Jeremy Luban, M.D., Thesis Advisor

MIchael Brehm, Ph.D., Member of Committee

Heinrich Gottlinger, Ph.D., Member of Committee

William Theurkauf, Ph.D., Member of Committee

Akiko Iwasaki, Ph.D., External Member of Committee

Kate Fitzgerald, Ph.D., Chair of Committee

Mary Ellen Lane, Ph.D., Dean of the Graduate School of Biomedical Sciences

July 24, 2018

ABSTRACT

Protective antiviral immune responses require priming of naïve T cells by dendritic cells (DCs) that have matured sufficiently to produce co-stimulatory cell surface molecules and cytokines. Although only low levels of productive HIV-1 infection are detected in ex vivo DCs following HIV-1 challenge, those few cells exhibit innate activation. Experimentally bypassing blocks to entry and replication leads to more efficient transduction of DCs and maturation as indicated by production of interferons and interferon stimulated genes. Furthermore, similar innate activation occurs upon transduction of macrophages or CD4+ T cells. However, the mechanism by which HIV-1 is detected to activate innate immune signaling is not clear. The purpose of this thesis is to incorporate my data and observations into the understanding of HIV-1 innate detection and attempt to resolve seemingly conflicting observations.

Reverse transcription and genomic integration are necessary for innate activation implying the need de novo transcription. Coding sequences are unnecessary save for those cis-acting sequences necessary for the HIV-1 life cycle. CRM1 dependent, HIV-1 unspliced RNA export is essential for innate activation. As intact viral sequence is unnecessary for transcription and export, defective proviruses may contribute to systemic inflammation seen in chronically infected individuals. These insights, are hoped to aid in the production of qualitatively better anti-retroviral drugs as well as in the design a protective HIV vaccine.

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CHAPTER I

Introduction

1.1 Brief Introduction

Presently, there are over 40 antiretroviral drug formulations approved by the FDA marketed specifically to inhibit HIV-1 replication (FDA.gov). These drugs are designed to obstruct various specific steps of the HIV-1 life cycle including fusion and entry, reverse transcription, integration, and HIV-1 protease processing (Arts and Hazuda, 2012). These formulations can be very effective in reducing viral load in infected individuals to near-undetectable levels, curtailing the risk of viral transmission, and preventing progression to AIDS. Unfortunately, while antiretroviral medications are highly effective in inhibiting the HIV-1 life cycle, they are neither a cure nor a protective vaccine. HIV-1 infected individuals must maintain a strict regimen of antiviral drugs or risk viral rebound and drug resistance (Davey et al., 1999; Johnson et al., 2013; Siliciano and Siliciano, 2013; Wang et al., 2011). Furthermore, it is economically and logistically difficult to distribute medication to areas of the world most impacted by HIV-1. According to the most recent data available from UNAIDS, an estimated global 36.7 million people were HIV-1 positive with approximately 1.8 million new infections and 1 million AIDS related deaths in 2016 alone (UNAIDS.org). This highlights the truth that, although modern therapies are incredibly effective in managing HIV, no cure exists and more people are infected every year underscoring the critical need for a protective vaccine that prevents establishment of infection.

1.2 HIV-1 Virology

Human immunodeficiency virus 1 (HIV-1) is an RNA virus of the genus *Lentivirus* in the family *Retroviridae*. HIV-1 is a positively stranded, RNA virus enclosing two copies of a single 9.5 kb RNA genome (Wain-Hobson et al., 1985; Watts et al., 2009). Like all retroviruses, HIV-1 encodes a single stranded RNA genome that is reverse transcribed to produce a double stranded DNA copy. This cDNA copy is transported to the nucleus where it is incorporated in the host chromosomal DNA. Once integrated, this single genome encodes for 15 viral protein products that it produces through complex alternative splicing and proteasomal processing.

Prior to cellular infection, the HIV-1 virion is contained within a lipid bilayer composed of host cellular membrane. Within the viral membrane, the RNA genomes are themselves enclosed in a conical structure derived from the viral capsid protein (CA) (Ganser et al., 1999; Li et al., 2000). The CA monomers assemble into repeating units of hexamers with critically placed pentamers to generate a curvature with conical shape. This structure, similarly termed as the viral capsid, also encloses the viral reverse transcriptase (RT) and integrase (IN) enzymes. HIV-1 infects CD4+ T cells, and to a lesser extent macrophages and dendritic cells, through the binding of a trimeric viral Env complex to cell surface CD4 and a coreceptor, generally CCR5 or CXCR4 (Arrildt et al., 2012; Munro and Mothes, 2015; Pancera et al., 2014). Following receptor association, Env

drives fusion of the viral and cellular membranes leading to release of the of the HIV-1 capsid structure into the cytoplasm.

Following entry, the RNA genome is reverse transcribed by the RT enzyme to a single, minus stranded DNA copy (Hu and Hughes, 2012). In proliferating CD4+ T cells, where cytoplasmic dNTP levels are abundant, this reaction occurs quickly following entry. However, in non-dividing cells with limited cytoplasmic dNTPs such as DCs and macrophages, reverse transcription proceeds much more slowly taking up to 48 hours (Kim et al., 2012). The RT enzyme also has an RNase H subunit which degrades the RNA genome following completion of the first complementary DNA (Driscoll et al., 2001). With the RNA copy removed, RT continues to synthesize the plus stranded DNA. At some point during this time, the viral capsid structure dissociates to release the reverse transcription complex; although whether this occurs early after entry, during cytoplasmic transport, or after nuclear pore complex binding is not well understood (Aiken, 2006; Campbell and Hope, 2015; Chin et al., 2015; Hulme et al., 2015; Peng et al., 2014). Following nuclear import, the HIV-1 integrase enzyme cleaves the host DNA to induce a strand transfer reaction. Host DNA damage complexes are then recruited to repair the lesion thereby completing viral DNA incorporation (Craigie and Bushman, 2012; Krishnan and Engelman, 2012; Lesbats et al., 2016).

Once the HIV-1 genome is integrated into the host chromosomal DNA, cellular factors including NF-κB, Sp1, TATA box binding protein (TBP), and RNA polymerase II (RNA Pol II) bind to the promoter element of the 5' long terminal repeat (LTR) (Nabel and Baltimore, 1987; Rittner et al., 1995; Wei et al., 1998). This minimal complex allows for low level transcription of HIV-1 RNAs. The majority of which are short, abortive transcripts that incompletely transcribed and, ostensibly, quickly degraded (Kao et al., 1987). However, a small number complete transcription and are subsequently spliced and processed (Das et al., 2011; Karn and Stoltzfus, 2012). This early, low level transcription allows for the the translation of the viral transactivation factor (Tat). Tat then binds to the trans-activation response element (TAR) of the HIV-1 LTR and recruits the positive transcriptional elongation factor (P-TEFb) (Dingwall et al., 1989, 1990; Tahirov et al., 2010). This, in turn, phosphorylates RNA Pol II leading to a significant enhancement of viral transcription (Bieniasz et al., 1999; Karn and Stoltzfus, 2012; Richter et al., 2002).

HIV-1 transcribes and processes its pre-mRNA into over 100 different alternatively spliced variants (Ocwieja et al., 2012). Three separate classes of HIV-1 mRNAs result from HIV-1 RNA splicing: unspliced genomic RNA encoding Gag and Gag-Pol polyproteins, partially spliced RNAs encoding Env, Vif, or Vpu, and multiply spliced RNA encoding Rev, Tat, Vpr, or Nef (Karn and Stoltzfus, 2012; Kim et al., 1989; Purcell and Martin, 1993). Multiply spliced HIV-1 RNAs are exported from the nucleus using the canonical NXF1/TAP mRNA export

pathway. In contrast, partially spliced and full length HIV-1 RNAs retain the major Env intron and are blocked from export (Cullen, 2003; Jensen et al., 2003; Rekosh and Hammarskjold, 2018; Sommer and Nehrbass, 2005; Stutz and Izaurralde, 2003). The HIV-1 protein Rev potentiates nuclear export of these RNAs by associating with the Rev response element (RRE), contained within the Env intron, and binding to the karyopherin CRM1 which drives export outside of the NXF1/TAP pathway (Cochrane et al., 1990; Cullen, 2003; Emerman et al., 1989; Fischer et al., 1995; Malim et al., 1989; Rojas-Araya et al., 2015).

Once exported from the nucleus, full length, unspliced HIV-1 RNA is translated into the Gag and Gag-Pol polyproteins. An RNA motif of "slippery sequence" (UUUUUUA) along with an upstream ACAA tetraloop induces a -1 ribosomal frameshift in around 5 percent of the translating molecules thereby producing Gag-Pol from the same RNA transcript (Bell and Lever, 2013; Biswas et al., 2004; Dulude et al., 2002; Mouzakis et al., 2013; Staple and Butcher, 2005). The Gag and Gag-Pol polyproteins then associate with a small number of membrane bound Env trimers upon the inside surface of the host cell membrane along with two copies of the unspliced HIV-1 RNA genome (D'Souza and Summers, 2005; Sundquist and Kräusslich, 2012). Gag protein is sufficient to drive viral budding from the cell membrane; however, it utilizes the host ESCRT pathway to potentiate viral release (Bieniasz, 2006, 2009; Carlton and Martin-Serrano, 2007; Göttlinger et al., 1991; von Schwedler et al., 2003; Usami et al., 2009; Votteler and Sundquist, 2013; Weiss and Göttlinger, 2011). Once released from the cell

membrane, the viral particle exists as a membrane bound, spherical array of outwardly facing Gag and Gag-Pol molecules. The viral protease enzyme (PR), itself autocatalytically processed from the Gag-Pol molecule, subsequently cleaves the Gag and Gag-Pol into its constituent elements, matrix (MA), capsid (CA), nucleocapsid (NC), p6, protease (PR), reverse transcriptase (RT), and integrase (IN). The viral MA proteins remain associated to the membrane forming a discontinuous layer. Inside, the CA monomers spontaneously assemble into the conical structure of the mature virion and enclose the genomic RNA (Ganser et al., 1999; Sundquist and Kräusslich, 2012; Zhao et al., 2013). At this point, the matured viral particle is infectious and ready to restart the cycle.

Figure 1.1
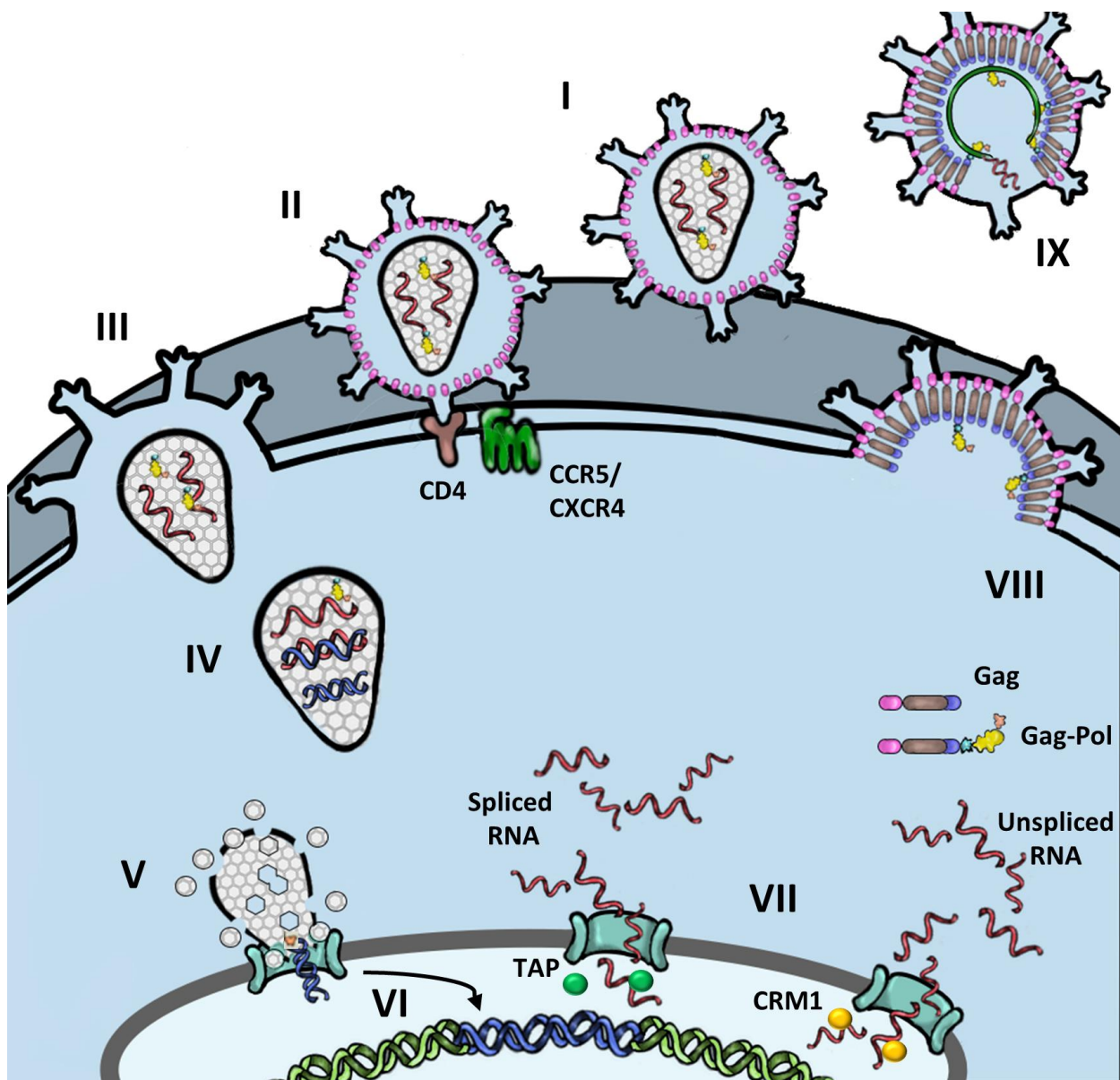
Figure 1.1 Depiction of the HIV-1 viral life cycle

I. The HIV-1 virion is contained within a lipid bilayer composed of host cellular membrane. Within the viral membrane, the RNA genomes are themselves enclosed in the viral capsid protein which also encloses the viral reverse transcriptase (RT) and integrase (IN) enzymes (Campbell and Hope, 2015). **II.** Viral entry is potentiated by Env interactions with CD4 and a coreceptor, generally CCR5 or CXCR4 (Arrildt et al., 2012; Munro and Mothes, 2015). **III.** Following entry, the viral capsid is released into the cytoplasm. **IV.** Reverse transcription of the viral RNA genome to cDNA occurs during cytoplasmic transport (Hu and Hughes, 2012). During transport, the viral capsid dissociates at an unknown rate (Campbell and Hope, 2015). **V.** Following reverse transcription, viral cDNA is transported to the nucleus. **VI.** The viral cDNA is transported to the nucleus where it is incorporated into host chromosomal DNA by integrase (Craigie and Bushman, 2012; Lesbats et al., 2016). Here, viral RNA is transcribed by host factors following transactivation by Tat (Karn and Stoltzfus, 2012) **VII.** Fully spliced viral RNAs are exported from the nucleus by the canonical TAP/NXF1 mRNA export pathway. However, partially or unspliced viral HIV-1 RNAs are exported through the CRM1 export pathway (Cullen, 2003; Pocock et al., 2016). **VIII.** Unspliced HIV-1 RNA translates the Gag and Gag-Pol polyproteins which assemble at the cell membrane and promote viral budding (Bell and Lever, 2013; Sundquist and Kräusslich, 2012). **IX.** Following Gag assembly, viral membrane fission is driven by the host ESCRT complex (Usami

et al., 2009; Votteler and Sundquist, 2013). The viral protease enzyme then cleaves the Gag and Gag-Pol polyproteins into their functional constituents and the capsid protein auto-assembles into the viral capsid hexagon lattice enclosing the viral RNA (Bell and Lever, 2013).

1.3 HIV-1 Persistence

The majority of those infected with HIV-1 mount an antigen-specific immune response against the virus with antibodies as well as CD4+ and CD8+ responses (Altfeld et al., 2002; Cao et al., 2003; Rosenberg et al., 1997; Scheid et al., 2009; Virgin and Walker, 2010). These immune responses are of varying effectiveness but will generally reduce viremia by logs, largely correlating with HIV-1-specific CD8+ T cell responses. However, those infected with HIV-1 progress to AIDS despite having measurable antiviral immune responses of varying efficacy. The important exception are the small percentage of individuals referred to as elite controllers who can constrain HIV-1 replication and resist progression to AIDS; although the exact reason for their resistance is unknown (Bello et al., 2009; Fowke et al., 1996; Porichis and Kaufmann, 2011; Saag and Deeks, 2010; Walker, 2007). As stated though, the majority of HIV-1 infected patients, without consistent access to antiretroviral medication, will progress to AIDS. The reason for the resistance to viral elimination has been the subject of intense research with the expectation that a better understanding of how HIV-1 evades immune control will lead to the design of a better antiviral therapies or a prophylactic vaccine. A simple, intuitive explanation is the simple fact that HIV-1 infects and inactivates the same CD4+ T cells tasked with antiviral defense. However, the ability to infect and replicate in immune cells is not unique to HIV-1. Furthermore, anti-HIV-1 cellular and humoral responses are readily detectable

prior to immune collapse and establishment of AIDS, indicating that CD4+ T cell activity is not wholly functionally impaired.

The HIV-1 Env glycoprotein is the only extracellular viral protein and is therefore uniquely exposed to antibody attack. In response, Env has evolved a closed conformational state that encloses and conceals receptor binding interfaces and functional centers. Furthermore, the Env protein is covered in an extensive glycan "shield" that occludes antibody access. Indeed, this glycan layer makes up half of Env's total molecular weight (Julien et al., 2013; Lyumkis et al., 2013; Munro and Mothes, 2015; Pancera et al., 2014). HIV-1's capacity for mutation and functional plasticity is another oft-cited basis for HIV-1 persistence. Indeed, HIV-1 has great capacity for mutation and immunological escape due largely to the error-prone reverse transcriptase enzyme (Abram et al., 2010; Bowman et al., 1998; Hu and Hughes, 2012; O'Neil et al., 2002). In particular, Env protein is capable of tremendous sequence and conformation divergence while still maintaining its function (Moore et al., 2012; Powell et al., 2017; Richman et al., 2003; Wei et al., 2003). However, high rates of mutation and co-infection of sequence variants referred to as quasispecies are common among RNA viruses and, alone, cannot account for the failure of the immune system to lastingly control HIV-1 infection (Andino and Domingo, 2015; Lauring and Andino, 2010).

Another important point is that, like all retroviruses, HIV-1 creates a DNA copy of its RNA genome and integrates its own sequence into the host cell's

chromosomal DNA.   At which point, regardless of its ability to replicate, HIV-1 becomes a permanent genetic element of the infected cell and all its daughter cells. The provirus may exist in a transcriptionally active state thereby contributing to the active infection. On the other hand, the provirus may, for reasons not well understood, become transcriptionally silenced. In this way, the virus creates a pool of infected cells harboring inactive proviruses that can go undetected by the immune system (Finzi et al., 1999; Hiener et al., 2017; Lorenzi et al., 2016; Mbonye and Karn, 2017; Siliciano et al., 2003). This latent reservoir of infected cells can persist despite effective immune control or antiretroviral therapy. Transcriptional reactivation of latently infected cells can then re-establish viral production upon halting of therapy or immune exhaustion.

The latent reservoir of HIV-1 infected cells is considered to be the major barrier obstructing viral eradication. Histone deacetylases, transcriptional activators, and other latency reversal agents have been used both *in vitro* and clinically to reactivate latent viral reservoirs in the hopes of promoting immune clearance of infected cells. However, *in vitro*, *ex vivo*, and humanized mice experiments have shown that HIV-1 specific cytotoxic T lymphocytes (CTL) fail to eliminate latently infected CD4+ T cell upon transcriptional reactivation unless the CTL are secondarily stimulated (Deng et al., 2015; Huang et al., 2018; Shan et al., 2012; Smith et al., 2016; Spina et al., 2013). This would suggest that the existing immune response to HIV-1 infection is in some way deficient. Indeed, a long body of evidence indicates that HIV-1 alters T cell function beyond simply

depleting them via infection. Chronic HIV-1 infection induces both CD4+ and CD8+ T cells to have an "exhausted" phenotype characterized by the expression of inhibitory receptors and of reduced effector function and cytokine responsiveness. The exact cause of T cell exhaustion is unknown but is known to result from repeated antigen stimulation and inflammation as occurs in chronic HIV-1 infection (Amu et al., 2016; Blackburn et al., 2009; Buggert et al., 2014; Day et al., 2006; Douek et al., 2003; Larsson et al., 2013; Morou et al., 2014; Wherry and Kurachi, 2015; Yamamoto et al., 2011; Zajac et al., 1998).

1.4 HIV-1 interactions with DCs

A functional adaptive immune response depends on proper communication of pathogenic antigens to T cells by antigen presenting cells. The defective immune response to HIV-1 infection may be due to insufficient priming of the adaptive immune system. Dendritic cells (DCs) are a class of highly heterogeneous immune cells tasked with the classification and presentation of antigen to T cells. The specific activation state of the DCs determines the nature and constitution of the immune response elicited by the T cells upon antigen recognition. Self antigen is presented in an inactivated context leading to a tolerogenic immune state. In contrast, DCs present pathogenic antigens in a matured stated leading to protective immunity. Dendritic cells mature upon innate immune recognition of danger associated signals (pathogen associated molecular patterns - PAMPs) by

an array of highly specific pathogen recognition receptors (PRRs). Upon PRR activation, DCs express costimulatory molecules such as CD80 and CD86 as well as secrete numerous cytokines that communicate the nature of the presented antigen to T cells.

As intracellular parasites, virus do not produce completely foreign molecules which would readily distinguish them from the host. As a result, cells often sense infection through the detection of viral nucleic acids by a broad array of PRRs. Innate detection of viral infection generally leads the induction of type 1 interferon (IFN) production as well a large number of interferon stimulated genes (ISG) that elicit extensive antiviral activities. The type 1 IFN response leads to DC maturation and is critical to the generation of an antiviral immune response. Circulating plasmacytoid dendritic cells (pDCs) readily endocytose HIV-1 particles and detect HIV-1 RNA via TLR7 and produce copious levels of type I interferon (Beignon et al., 2005; Fonteneau et al., 2004; Lepelley et al., 2011; O'Brien et al., 2013). However, pDCs have limited capacity to stimulate antigen specific T cells (Boasso et al., 2011; Hoeffel et al., 2007; Jaehn et al., 2008).

In contrast, myeloid dendritic cells (mDCs) constitute the body's primary antigen presenting cells and lack expression of TLR7 precluding maturation in response to HIV-1 (Izaguirre et al., 2003; Miller and Bhardwaj, 2013; O'Brien et al., 2013; Smed-Sörensen et al., 2004, 2005). Myeloid dendritic cells are refractory to productive HIV-1 infection with blocks to both entry and reverse transcription.

Infection of a cell by HIV-1 requires binding of cell surface CD4 and CCR5 with the HIV-1 Env glycoprotein complex. This initiates a fusion event that delivers the viral capsid core into the cytoplasm. Surface expression of CD4 on mDCs is prohibitively low and viral endocytosis is driven by alternative interactions with DC-SIGN and other c-type lectins (Chauveau et al., 2017; Granelli-Piperno et al., 2004, 2006; Piguet and Steinman, 2007). Alternatively, viral particles can be captured by surface CD169 and transferred to CD4+ T cells, further potentiating infection (Cameron et al., 1992; Izquierdo-Useros et al., 2012, 2014; Kijewski and Gummuluru, 2015; Piguet and Steinman, 2007). However, immature DCs are more likely to capture HIV-1 via c-type lectins to be endocytosed (Geijtenbeek et al., 2000; Kijewski and Gummuluru, 2015; Piguet and Steinman, 2007; Puryear et al., 2013; Turville et al., 2002). In endosomal compartments, HIV-1 is unable to reverse transcribe and is rapidly degraded. Viral antigens are then processed and presented in the absence of PRR stimulation and DC maturation (Moris et al., 2006). Paradoxically, this may lead to a more tolerogenic immune response with limited antiviral activity. Indeed, it is well documented that HIV-1 specific T cell express inhibitory molecules such as PD-1 and TRAIL (Brown et al., 2010; Day et al., 2006; Khaitan and Unutmaz, 2011; Morou et al., 2014; Trautmann et al., 2006; Wherry and Kurachi, 2015).

The entry block to infection in DCs can be experimentally circumvented when HIV-1 virions are pseudotyped with the glycoprotein of a heterologous virus; most often Vesicular Stomatitis Virus glycoprotein (VSV-G) (Granelli-Piperno et al.,

2000). This allows for the productive release of the HIV-1 capsid into the cytoplasm. However, DCs and other non-dividing myeloid cells contain another block to productive infection. The host dNTP nuclease SAMHD1 restricts reverse transcription in dendritic cells possibly due to depletion of cytoplasmic dNTPs (Goldstone et al., 2011; Lahouassa et al., 2012; Powell et al., 2011) or by an as yet unknown, nuclease independent mechanism (Lim et al., 2012; Reinhard et al., 2014; White et al., 2013). The HIV-2 and SIV specific protein Vpx has been found to rescue this block to infection by promoting the proteasomal degradation of SAMHD1 following CUL4A ubiquitination by direct binding of DCAF1 (Bergamaschi et al., 2009; Hrecka et al., 2011; Sharova et al., 2008; Srivastava et al., 2008). When provided *in trans* via SIV$_{MAC}$251 viral-like particles (SIV VLPs) the Vpx protein is able to fully facilitate HIV-1 reverse transcription in DCs (Goujon et al., 2006; Laguette et al., 2011; Nègre et al., 2000).

Figure 1.2
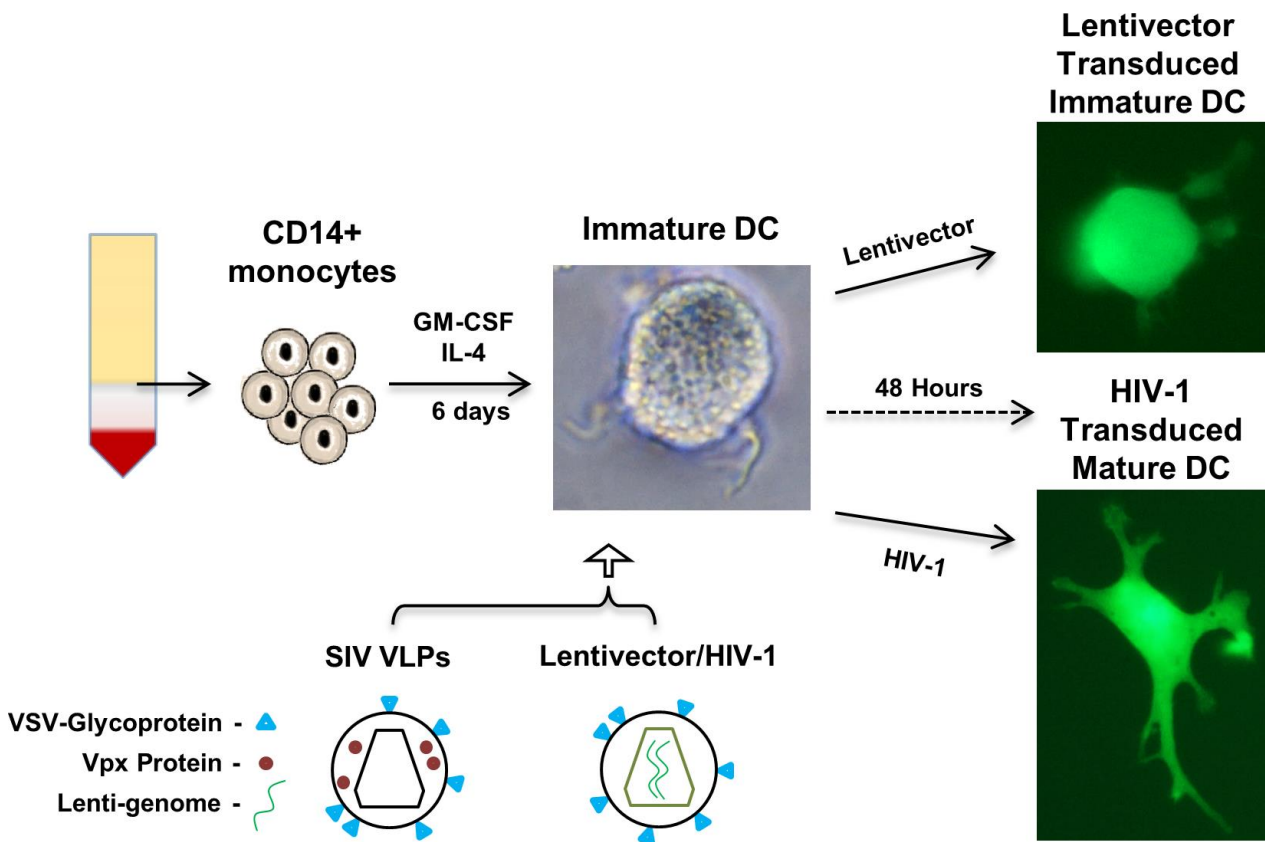
Figure 1.2 HIV-1 transduction of dendritic cells

Dendritic cells are differentiated in vitro following the positive selection of CD14+ monocytes. CD14+ cells are plated at a density of 1 to 2 x 10^6 cells/ml in RPMI-1640 supplemented with 5% heat inactivated human AB+ serum, 20 mM L-glutamine, 25 mM HEPES pH 7.2, 1 mM sodium pyruvate, and 1x MEM non-essential amino acids (RPMI-HS complete). DCs were generated by culturing monocytes for 6 days in the presence of 1:100 cytokine-conditioned media containing human GM-CSF and human IL-4 (McCauley et al., 2017; Pertel et al., 2011).

Depicted are SIV viral like particles (VLPs) that are produced to deliver Vpx protein which enhances reverse transcription by depleting host SAMHD1. Both the SIV VLPs and the HIV-1 or lentivector are pseudotyped with VSV-glycoprotein to potentiate CD4 independent entry. Dendritic cells do not detect infection from minimal lentivectors and genes can be delivered (GFP) without stimulating the cells (McCauley et al., 2017). However, full length HIV-1 infection does lead to the maturation of DCs as evident by the long extensions spreading outward from the cell body.

1.5 HIV-1 Incoming

The innate immune response to HIV-1 infection has been widely disputed among different labs with greatly varying results. Several groups have failed to detect innate detection in myeloid dendritic cells even under conditions where HIV-1 transduction is optimized using VSV-G pseudotyped virus supplemented with Vpx-containing SIV VLPs (Beignon et al., 2005; Fonteneau et al., 2004; Granelli-Piperno et al., 2004; Smed-Sörensen et al., 2005). In contrast, a growing body of evidence suggests that, depending on the experimental system used, HIV-1 infection can be detected by DCs. For example, a common route of viral detection is through the sensing of unique nucleic acid forms and locations.

Multiple cellular receptors have been shown to detect RNA in a range of viral infections. Endosomal toll-like receptors (TLR) including TLR3, TLR7, and TLR8, as well as cytoplasmic RIG-I like receptors (RLRs) have been well characterized to sense diverse forms of single and double stranded viral RNA (Brencicova and Diebold, 2013; Iwasaki and Medzhitov, 2010; Thompson et al., 2011). As a positive sense, single stranded RNA virus with extensive secondary structure, HIV-1 may be exposed to detection from several RNA sensors. As previously mentioned, HIV-1 is readily endocytosed by plasmacytoid dendritic cells (pDC) where its genomic RNA is detected by TLR7. The main result of which is the production of impressive quantities of IFNα (Beignon et al., 2005; Fonteneau et al., 2004; Haupt et al., 2008; Lepelley et al., 2011; O'Brien et al., 2013; Smed-

Sörensen et al., 2005). However, pDCs have limited capacity to present antigen to T cells and may in fact express inhibitory factors that limit the antiviral T cell response (Boasso et al., 2007, 2011; Hoeffel et al., 2007; Jaehn et al., 2008; Manches et al., 2008).   In contrast to pDCs, myeloid dendritic cells have limited expression of TLR7 and IRF7 (essential for TLR7 signaling) and do not detect HIV-1 in the same manner (Bloch et al., 2014; Izaguirre et al., 2003; Jarrossay et al., 2001; Kaisho, 2012; Smed-Sörensen et al., 2005; Wacleche et al., 2018).

Methods of HIV-1 RNA detection by other cellular receptors have been suggested. Although mDCs are impeded in TLR7 RNA detection, TLR8 signaling appears to be intact and has been suggested to detect incoming HIV-1 RNA (Gringhuis et al., 2010). In this study, sensing of HIV-1 RNA by TLR8 was shown to activate NF-κB and acted to enhance transcription of the integrated HIV-1 genome. However, neither DC maturation nor a type 1 interferon response was reported following infection. RIG-I is a cytoplasmic RNA sensor that detects 5' triphosphorylated RNAs as well as RNAs with short double stranded motifs (Reikine et al., 2014). The complex and highly regulated secondary structure of HIV-1 genomic RNA has been implicated as a substrate for RIG-I signaling in cell lines and human PBMCs (Berg et al., 2012; Solis et al., 2011). However, these experiments were completed through transfection of various HIV-1 derived RNA motifs as opposed to direct HIV-1 challenge. As such, the relevance of these observations to true HIV-1 challenge or in cells types like DCs is still unknown.

The TREX1 protein is a cytosolic DNA nuclease has been reported to degrade cytoplasmic HIV-1 cDNA following reverse transcription of the RNA genome (Yan et al., 2010). Depletion of TREX1 protein resulted in the accumulation of excess reverse transcribed viral cDNA leading to the activation of an unknown cytoplasmic DNA sensor. Supporting this model, TREX1 knockdown was shown to inhibit HIV-1 infection *in vitro* and in humanized mice through the induction of an antiviral interferon response (Wheeler et al., 2016). Furthermore, in a THP1 model of HIV-1 infection, innate activation was shown to be dependent on the expression level of TREX1 as due to its degradation of partial reverse transcription products (Kumar et al., 2018).

The resultant detection of reverse transcribed viral cDNA in dendritic cells was argued to be mediated by the cytoplasmic DNA sensor, cyclic GMP-AMP synthase (cGAS) (Gao et al., 2013; Wu et al., 2013). Double stranded DNA or single stranded DNA with Y-form secondary structure activates the cGAS enzyme to produce the secondary messenger cGAMP (cyclic GMP-AMP) to activate STING and, consequently, IFN via IRF3 (Chen et al., 2016; Herzner et al., 2015; Yoh et al., 2015). HIV-1 transduction led to cGAMP production and IRF3 dimerization in monocyte derived dendritic cells and macrophages only when reverse transcription was facilitated through the addition of Vpx (Gao et al., 2013). In similar experiments, polyglutamine binding protein 1 (PQBP1) was also found to directly bind HIV-1 cDNA (Yoh et al., 2015). This interaction led to a type 1 interferon response that was dependent on cGAS; implying a model where

the specific binding capacity of retroviral cDNA is modulated by interactions with both cGAS and PQBP1 as a complex.

In another study, HIV-1 cDNA was found not to be sensed by DCs even upon depletion of SAMHD1 by Vpx. Mutation of the HIV-1 capsid protein (CA) to increase affinity for the cytoplasmic protein cyclophilin A (CypA) led to detection and DC maturation (Lahaye et al., 2013). CypA binds to the HIV-1 capsid structure following viral entry and leads to stabilization of the capsid structure and regulation of capsid disassembly (De Iaco and Luban, 2014; Franke et al., 1994; Li et al., 2009; Luban et al., 1993; Sokolskaja et al., 2006; Thali et al., 1994). Sheltered in the capsid structure, viral cDNA is ostensibly protected from cGAS detection. Increasing the CA affinity to CypA was suggested to lead to premature capsid uncoating thereby leaving the HIV-1 cDNA exposed prior to nuclear entry (Lahaye et al., 2013; Maelfait et al., 2014).

In partial agreement with this model, HIV-1 was reported to circumvent innate detection in monocyte derived macrophages through CA interactions with both CypA and CPSF6 (Rasaiyaah et al., 2013). CPSF6, an mRNA polyadenylation factor, associates with CA to facilitate HIV-1 nuclear import through association with the importin TNPO3 (Brass et al., 2008; De Iaco et al., 2013; Price et al., 2012; Valle-Casuso et al., 2012). Mutations blocking these interactions inhibited HIV-1 infection through the induction of type 1 interferon following production of cGAMP, thereby implicating cGAS detection of cDNA (Rasaiyaah et al., 2013).

However, this interaction did not depend on Vpx promotion of reverse transcription, possibly due to differential restriction of reverse transcription by SAMHD1 in macrophages (Cribier et al., 2013; White et al., 2013). Furthermore, innate detection of HIV-1 was proposed to be promoted by reduction of CypA-CA binding as opposed to increased binding as suggested in DCs.

IFI16, another cellular DNA sensor, has also been shown to detect transfected, HIV-1 based DNA sequences and induce type 1 interferon in macrophages (Jakobsen et al., 2013). Similarly, IFI16 was implicated to sense products of incomplete reverse transcription in resting CD4+ T cells isolated from tonsil explants (Monroe et al., 2014). This led to caspase 1 activation, induction of the IL1β inflammasome, and consequently, bystander T cell death as due to pyroptosis (Doitsh et al., 2010). As CD4+ T cells constitute the primary target of HIV-1 replication, the observation that IFI16 may also detect HIV-1 cDNA and lead to indirect T cell depletion is of considerable interest. However, an analogous mechanism of IFI16 has not been observed in specialized antigen presenting such as dendritic cells and macrophages. Similarly to the role of PQBP1 in modulating cGAS activity, IFI16 has also been suggested to function as a cofactor in cGAS recognition of HIV-1 reverse transcription products (Jønsson et al., 2017). In this study, knockdown of IFI16 led to decreased production cGAMP and attenuated the cGAS-STING dependent innate immune response to Vpx-assisted HIV-1 infection in macrophages. This supports a cGAS detection model in which retroviral cDNA is detected in the cytoplasm by

cGAS with proteins including PQBP1 and IFI16 acting to enhance retroviral DNA

binding and enzymatic activity (Jønsson et al., 2017; Sumner et al., 2017).

Figure 1.3
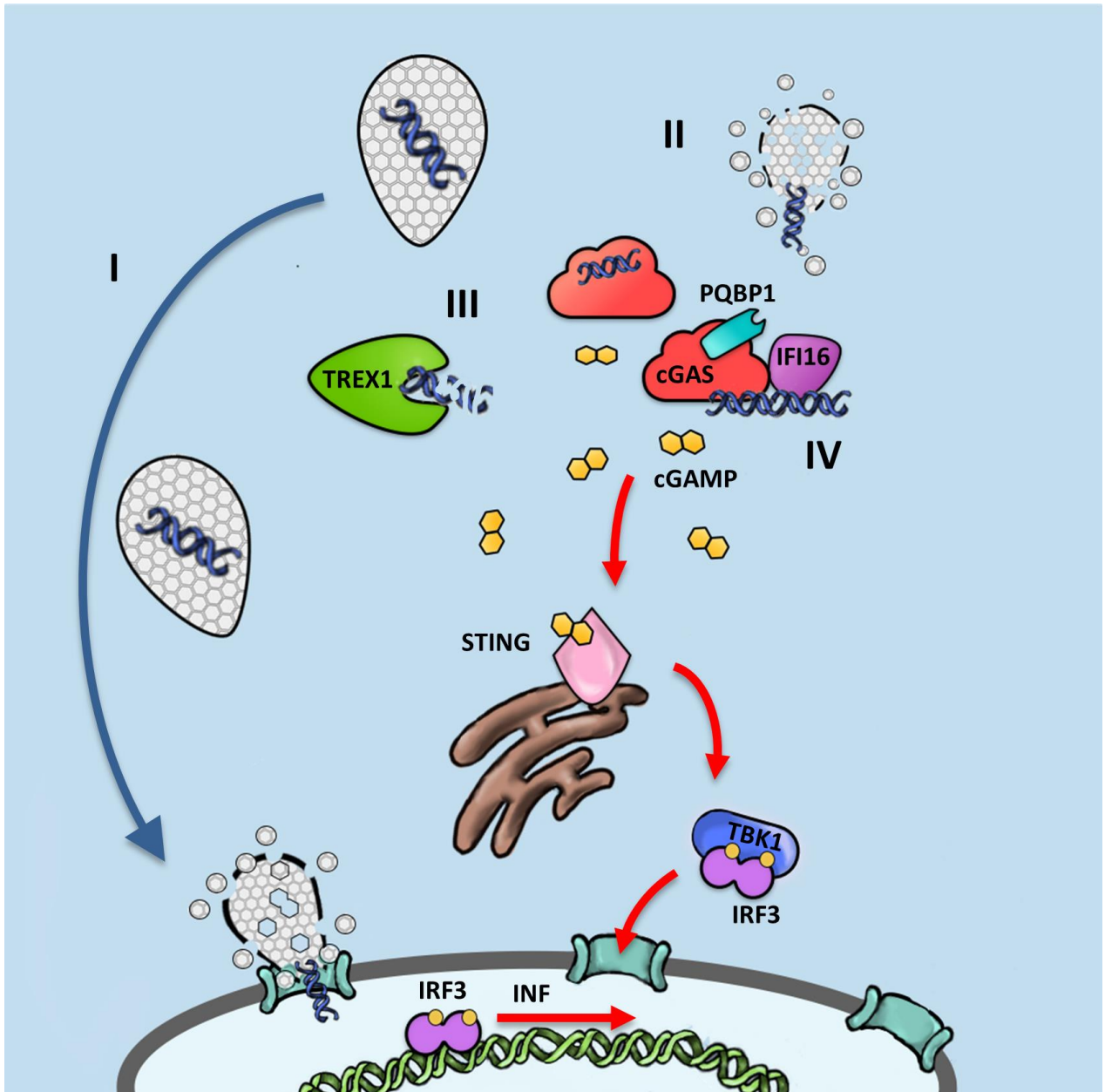
Figure 1.3 Detection of intracellular viral cDNA

I. The HIV-1 capsid complex may not sufficiently disassemble enough prior to nuclear transport to allow cytoplasmic DNA sensors access to viral cDNA, thereby bypassing cytoplasmic detection (Lahaye et al., 2013; Maelfait et al., 2014; Rasaiyaah et al., 2013). **II.** The viral capsid may also uncoat sufficiently prior to nuclear entry and allow cytoplasmic access (Campbell and Hope, 2015) **III.** Once open to the cytoplasm, viral cDNA may be degraded by the nuclease TREX1 (Kumar et al., 2018; Wheeler et al., 2016; Yan et al., 2010) **IV.** Cellular detection of HIV-1 reverse transcribed cDNA is likely driven by cyclic GMP-AMP synthase (cGAS) (Chen et al., 2016; Gao et al., 2013; Herzner et al., 2015; Lahaye et al., 2013). Although other factors such as polyglutamine binding protein 1 (PQBP1) and interferon-γ inducible protein 16 (IFI16) may also bind HIV-1 cDNA and act to enhance cGAS activity (Jakobsen et al., 2013; Jønsson et al., 2017; Yoh et al., 2015). Following detection, cGAS produces cyclic GAMP which activates ER bound stimulator of interferon genes (STING) which stimulates TBK1 to phosphorylate IRF3. Phosphorylated IRF3 then dimerizes and transports to the nucleus to drive type 1 interferon expression (Chen et al., 2016).

1.5 Detection of HIV-1 Post Integration

Sensing of reverse transcription products, be they ssDNA, dsDNA, or RNA-DNA hybrids, are events that intuitively would occur prior to HIV-1 genomic integration. Rather than detection of products of HIV-1 reverse transcription prior to incorporation into the host chromosomal DNA, evidence exists for mechanisms of detection dependent on viral integration and transcription. However, as with pre-integration sensing, these studies vary considerably in reported determinants and mechanism of innate activation.

The act of integration itself is a process that requires the insertion of a copy of foreign DNA into host chromosomal DNA. The viral pre-integration complex (PIC) associates with LEDGF/p75 to target integration site selection to chromosomal locations of high transcriptional activity (Ciuffi et al., 2005; Marshall et al., 2007; Shun et al., 2007). The enzymatic operation consists of the HIV-1 integrase enzyme cleaving target DNA phosphodiester bonds to induce a strand transfer reaction. A diverse number of host DNA damage complexes are then recruited to repair the lesion (Craigie and Bushman, 2012; Lesbats et al., 2016). The commissioning of DNA-dependent protein kinase (DNA-PK), an important enzymatic and signaling regulator of the non-homologous end joining DNA repair pathway, has also been suggested to induce innate immune activation in CD4+ T cells (Cooper et al., 2013). In this study, *in vitro* HIV-1 infection of cell lines and PBMCs led to acute CD4+ T cell death that was found to be dependent on

integration. Signaling of DNA damage by DNA-PK prompted p53 activation and induction of cell death. However, this mechanism of detection has not been identified in other cells types such as dendritic cells and still requires independent verification.

Following integration, HIV-1 transcribes its RNA in multiple forms through intricate alternative splicing through a complex process of internal regulation. In this way, HIV-1 is able to produce 15 different protein products from only a single 9 kb pre-mRNA genome (Karn and Stoltzfus, 2012; Stoltzfus and Madsen, 2006). The main product of unspliced, genomic HIV-1 RNA is the structural polyprotein Gag. In dendritic cells, produced Gag itself has been suggested to be directly detected by the innate immune system (Manel et al., 2010). In this study, HIV-1 transduction of dendritic cells induced maturation and a type 1 interferon response. This response was dependent on both Vpx facilitation of reverse transcription and on productive integration. Binding of CypA to capsid, contained within *de novo* synthesized Gag polyprotein, induced an IRF3 dependent type 1 interferon response through an unknown signaling mechanism. However, more recent work by this group has instead pursued cGAS detection of pre-integrated viral cDNA as a primary mechanism for the innate activation seen in DCs following optimized HIV-1 challenge (Lahaye et al., 2013; Silvin and Manel, 2015).

Counterintuitively, cGAS detection of HIV-1 infection has also been suggested to be dependent on prior integration (Vermeire et al., 2016). In this study, HIV-1 infection of IL2/PHA activated CD4+ T cells induced the production of type 1 interferon that was inhibited upon blocking of either viral integration or transcription. Knockdown of cGAS or its downstream effectors STING or IRF3 abrogated interferon or ISG production. Although the authors argue that *de novo* Vpr and Vpu respectively act to potentiate or suppress the antiviral response, a mechanism of cytoplasmic DNA sensing by cGAS following HIV-1 proviral integration is not elucidated. Furthermore, even though experiments verifying interferon induction and Vpu and Vpr interactions were corroborated in DCs, post integration cGAS sensing is not described in cells other than CD4+ T cells (Vermeire et al., 2016).

A recent promontory study has proposed a mechanism of innate detection of HIV-1 in dendritic cells involving determinants both before and after viral integration (Johnson et al., 2018). Infection of DCs with a myeloid tropic HIV-1, in the presence of Vpx, induced robust DC maturation as evident by CD86 and type 1 interferon expression. Inhibition of HIV-1 integration either with Raltegravir or an integrase deactivating mutation, D116, greatly reduced, but did not abolish, the innate response to Vpx-assisted HIV-1 challenge. Secondary stimulation of DCs with R848, a TLR7/8 agonist and activator of NF-κB, was shown to rescue high level interferon expression in integration deficient infections. Knockdowns in monocyte derived dendritic cells, or CRISPR

knockouts in THP1 derived DCs, of the cGAS-STING-IRF3 pathway all reduced

maturation and ISG expression. Importantly, disruptions of IFI16, MAVS, PKR,

IRF1, IRF5, IRF7, or IRF9 had no effect on sensing of HIV-1 whether or not viral

integration was inhibited (Johnson et al., 2018). The authors conclude a complex

model in which cGAS detection of HIV-1 reverse transcription products "primes"

a low-level type 1 interferon response which, following integration and

transcription, is amplified by activation of NF-κB. However, neither the

determinant by which integration and viral production is sensed nor the

mechanism for the resultant NF-κB activation is determined by this study.

However, this remains an intriguing hypothesis that may serve to clarify many of

the seeming conflicting observations reported for HIV-1 innate detection.

1.6 Sensing of HIV-1 RNA Processing

Following integration, HIV-1 transcribes its RNA and processes it through

complex alternative splicing and differential export. HIV-1 processes its pre-

mRNA into over 100 different alternatively spliced variants (Ocwieja et al., 2012).

As described previously, HIV-1 RNA motifs have been suggested to be

substrates for the cytoplasmic RNA receptor RIG-I (Berg et al., 2012; Solis et al.,

2011). The implications for these studies suggest that incoming HIV-1 genomic

RNA may be innately detected prior to reverse transcription or integration.

However, these studies are limited mainly to RNA transfections in cell lines and

would be unable to distinguish whether *de novo* HIV-1 RNAs, as opposed to incoming virion associated genomic RNA, could be detected. One study has described a mechanism in which newly transcribed HIV-1 RNA is sensed by the host helicase DDX3 (Gringhuis et al., 2016). In this study, DDX3 was found to detect abortive HIV-1 RNA transcripts in a manner dependent on HIV-1 integration. Dendritic cell transfection of short HIV-1 RNAs, found in abortive, Tat independent transcription, led to type 1 interferon induction in a manner dependent on both DDX3 and MAVS. Knockdowns of DDX3 and MAVS in DCs similarly inhibited maturation upon challenge with myeloid tropic HIV-1; however, it is unknown whether abortive transcription products constitute the determinant for detection in primary cell infection.

Transcription of the integrated HIV-1 genome follows the binding of cellular factors including NF-κB, Sp1, TATA box binding protein (TBP), and RNA polymerase II (RNA Pol II) to the promoter element of the 5' long terminal repeat (LTR). This minimal complex allows for the transcription of a low level of viral transcripts, which subsequently undergo splicing and processing (Das et al., 2011; Karn and Stoltzfus, 2012; Nabel and Baltimore, 1987; Rittner et al., 1995; Tahirov et al., 2010). Binding of Tat recruits the positive transcriptional elongation factor (P-TEFb) which phosphorylates RNA Pol II leading to a significant enhancement of HIV-1 transcription.

Three classes of HIV-1 mRNAs result from HIV-1 RNA transcription and splicing: unspliced genome RNA encoding Gag and Gag-Pol polyproteins, partially spliced RNAs producing Env, Vif, or Vpu, and multiply spliced RNA for synthesis of Rev, Tat, Vpr, or Nef (Karn and Stoltzfus, 2012; Pan et al., 2008; Purcell and Martin, 1993; Schwartz et al., 1990; Stoltzfus, 2009). Multiply spliced HIV-1 RNAs are exported from the nucleus using the canonical NXF1 mRNA export pathway. In contrast, partially spliced and full length HIV-1 RNA retain the major Env intron and are restrained to the nucleus due to the formation of splicing complexes (Jensen et al., 2003; Sommer and Nehrbass, 2005; Stutz and Izaurralde, 2003). Other retroviruses bypass this restriction by encoding constitutive transport elements (CTE) that directly bind NXF1 to potentiate export (Aibara et al., 2015; Cullen, 2003; Pilkington et al., 2014; Pocock et al., 2016; Rekosh and Hammarskjold, 2018). While experiments have shown that CTE elements are sufficient to support HIV-1 RNA export, HIV-1 naturally uses an alternative export pathway (Bray et al., 1994; Smulevitch et al., 2006; Valentin et al., 1997). The HIV-1 protein Rev, itself produced from an early, fully spliced transcript, binds to and multimerizes upon the Rev response element (RRE) contained within the Env intron. In turn, Rev binds to the karyopherin CRM1 to potentiate nuclear export, sans completion of splicing (Cullen, 2003; Nakielny and Dreyfuss, 1999).

The specialized export of intron containing RNAs is a mechanism not normally utilized in mammalian cells and could potentially be sensed as a danger signal. Indeed, Rev mediated export of intron containing RNA has been shown to induce

innate activation in DCs, macrophages, and CD4+ T cells (Akiyama et al., 2017; McCauley et al., 2017). Challenge with HIV-1 was shown to induce a type 1 interferon response that was unreliant on cGAS-IRF3 signaling in both DCs (McCauley et al., 2017) and macrophages (Akiyama et al., 2017). This response was shown to be dependent upon integration and transcription of the provirus. Furthermore, mutations or deletions of HIV-1 coding sequence failed to limit ISG induction suggesting a lack of sequence necessity. Importantly, while VSV-G pseudotyping and Vpx addition enhanced infectivity and innate activation, both were unnecessary to induce DC maturation in infected cells.

Maturation of DCs was still observed upon replacement of Tat mediated transcriptional transactivation for a doxycycline inducible HIV-1 (Tet-HIV-1) construct. Innate activation was seen upon transcriptional activation by doxycycline stimulation. Furthermore, separation of Tet-HIV-1 transduction and doxycycline addition for nine days did not limit DC or T cell activation (McCauley et al., 2017). This suggests that possible detection or priming mechanisms prior to integration and expression are unnecessary for HIV-1 transcription dependent innate activation. In contrast, inhibition of CRM1 HIV-1 RNA export with leptomycin or with Rev/RRE mutations completely abrogated innate activation. However, rescue of unspliced export using heterologous retroviral CTEs to promote canonical NXF1 RNA export failed to promote DC and macrophage activation indicating that Rev-CRM1 mediated transport of unspliced RNA is indispensable for detection.

Both groups support a model where HIV-1 export of intron containing RNA through the CRM1 pathway induces innate activation in DCs, macrophages, and T cells. Furthermore, the authors suggest that the type 1 interferon response induced upon HIV-1 transcription and RNA export may contribute to long term pathogenesis in HIV-1 infection. Chronic inflammation persists in patients on effective antiretroviral therapy (ART) regardless of having clinically undetectable viral loads (Brenchley et al., 2006; Deeks et al., 2013; Sinha et al., 2016; Taiwo et al., 2013). It is known that the HIV-1 proviral reservoir in patients on ART is composed of only a small percentage of intact, replication competent sequences with the majority harboring deactivating mutations and deletions (Barton et al., 2016; Bruner et al., 2016; Cohn et al., 2018; Huang et al., 2018; Lorenzi et al., 2016; Pollack et al., 2017). *A priori*, any cell harboring a provirus capable of Rev-CRM1 export, regardless of its ability to continue replication, could initiate transcription and induce an antiviral response contributing to systemic inflammation (Akiyama et al., 2017; McCauley et al., 2017).

1.7 Perspectives

DCs are highly refractory to productive HIV-1 infection with blocks to both CD4-Env mediated viral entry as well as reverse transcription as due to SAMHD1. HIV-1 entry into DCs can be promoted experimentally through VSV-G pseudotyping allowing for CD4-Env independent fusion (Granelli-Piperno et al.,

2000). Furthermore, the addition of the SIV/HIV-2 Vpx protein leads to SAMHD1

degradation thereby alleviating the block to reverse transcription (Bergamaschi et

al., 2009; Sharova et al., 2008; Srivastava et al., 2008). Under these *in vitro*

circumstances, DCs can be efficiently and productively infected. However, these

are artificial conditions; useful and essential for experimentation, but not

reflective of the fact that, *in vivo*, DCs are not efficiently infected with HIV-1.

Furthermore, they do not appear to detect HIV-1 in a manner leading to innate

activation (Fonteneau et al., 2004; Manches et al., 2014; Miller and Bhardwaj,

2013; Smed-Sörensen et al., 2004). Instead, HIV-1 antigen is processed and

presented to T cells in a non-activated state, very likely leading to a stunted and

ineffective antiviral state.

Understanding how dendritic cells interact with HIV-1 in both normal and

optimized systems is important in the development of rational and effective anti-

HIV-1 immunotherapies and vaccines. Studies have shown that advanced DC

function correlates with better immune function and disease control. Elite

controllers, also known as long term non-progressors (LTNP), are individuals

who have the capacity to resist progression to AIDS without antiretroviral

treatment. T cell responses in elite controllers are much more functional and

effective at controlling viral replication (Betts et al., 2006; Imami et al., 2013;

Porichis and Kaufmann, 2011). It is possible that the improvement to the antiviral

response is due to superior T cell priming by DCs more reactive to the HIV-1

virus. Indeed, dendritic cells of elite controllers have improved capacity to sense

HIV-1 and to induce type 1 interferon as a result (Manches et al., 2014; Martin-Gayo et al., 2015, 2018). As such, this stands as compelling evidence that improving DC responsiveness to HIV-1 may be effective as a functional cure or prophylactic vaccine to HIV-1 infection.

CHAPTER II

Intron-containing RNA from the HIV-1 provirus activates type I interferon and inflammatory cytokines

Adapted from the publication:

2.1 Summary

HIV-1-infected people who take drugs that suppress viremia to undetectable levels are protected from developing AIDS (Günthard et al., 2016). Nonetheless, these individuals have chronic inflammation associated with heightened risk of cardiovascular pathology (Brenchley et al., 2006; Freiberg et al., 2013; Sinha et al., 2016). HIV-1 establishes proviruses in long-lived CD4$^+$ memory T cells, and perhaps other cell types (Jiang et al., 2015; Kandathil et al., 2016; Siliciano et al., 2003), that preclude elimination of the virus even after years of continuous antiviral therapy (Davey et al., 1999). Though the majority of proviruses that persist during antiviral therapy are defective for production of infectious virions (Bruner et al., 2016), many are expressed (Wiegand et al., 2017), raising the possibility that the HIV-1 provirus or its transcripts contribute to ongoing inflammation. Here we found that the HIV-1 provirus activated innate immune signaling in isolated dendritic cells, macrophages, and CD4$^+$ T cells. Immune activation required transcription from the HIV-1 provirus and expression of CRM1-dependent, Rev-dependent, RRE-containing, unspliced HIV-1 RNA. If *rev* was provided *in trans*, all HIV-1 coding sequences were dispensable for activation except those *cis*-acting sequences required for replication or splicing. These results indicate that the complex, post-transcriptional regulation intrinsic to HIV-1 RNA is detected by the innate immune system as a danger signal, and that drugs which disrupt HIV-1 transcription or HIV-1 RNA metabolism would add qualitative benefit to current antiviral drug regimens.

2.2 HIV-1 transduction matures dendritic cells

To determine if HIV-1 proviruses activate innate immune signaling, human blood cells were transduced with single-cycle vectors, either a full-length, single-cycle HIV-1 clone with a frameshift in *env* and eGFP in place of *nef* (HIV-1-GFP) (He et al., 1997), or a minimal 3-part lentivector encoding GFP (Fig. 2.1a, Table 2.1) (Pertel et al., 2011). Monocyte derived dendritic cells (DCs) were challenged initially since HIV-1 transduction of these specialized antigen-presenting cells activates innate immune signaling (Berg et al., 2012; Gao et al., 2013; Landau, 2014; Manel et al., 2010; Rasaiyaah et al., 2013). To increase the efficiency of provirus establishment, vectors were pseudotyped with the vesicular stomatitis virus glycoprotein (VSV G) and delivered concurrently with virus-like particles (VLPs) bearing $SIV_{MAC}251$ Vpx (Fig. 2.1b) (Goujon et al., 2006; Pertel et al., 2011). Transduction efficiency, as determined by flow cytometry for GFP-positive cells, was 30-60% (Fig. 2.1b), depending on the blood donor.

DCs matured in response to HIV-1-GFP transduction, as indicated by increased mean fluorescence intensity of co-stimulatory or activation molecules, including HLA-DR, CD80, CD86, CD40, CD83, CCR7, CD141, ISG15, MX1, and IFIT (Sousa, 2006) (Fig. 2.1b, c). Maturation was evident among both GFP positive and negative cells (Fig. 2.1b), the latter resulting from activation *in trans* by type 1 IFN as several others have shown (Manel et al., 2010; Rasaiyaah et al., 2013).

Identical results were obtained with full-length, single-cycle vectors generated

from primary, transmitted/founder clones that were derived by single genome

sequencing, HIV-1-AD17 (Parrish et al., 2013), HIV-1-Z331M-TF (Deymier et al.,

2015), and HIV-1-ZM249M (Salazar-Gonzalez et al., 2009), the first virus being

clade B, the other two clade C (Fig. 2.1d). A single cycle HIV-2 vector induced

maturation, indicating that this innate response was not unique to HIV-1 (Fig.

2.1e).

DCs matured when HIV-1-GFP transduction efficiency was augmented with

nucleosides (Reinhard et al., 2014), rather than with SIV VLPs, indicating that

Vpx was not required for maturation (Fig. 2.1f). DCs were then challenged with

replication-competent HIV-1 bearing CCR5-tropic Env, either T cell-tropic or

macrophage-tropic (Granelli-Piperno et al., 1998), with or without Vpx-VLPs (Fig.

2.1g). The percent of cells transduced by vector bearing either Env increased

with Vpx, though DC maturation was observed under all conditions, even among

the very few DCs transduced by T cell-tropic *env* (see inset of Fig. 2.1g). These

results indicate that neither VSV G, nor Vpx, nor high-titer infection, was required

for DC maturation.

In response to transduction with HIV-1-GFP, steady-state *CXCL10*, *IFNB1*, and

IL15 mRNAs reached maximum levels at 48 hrs, increasing 31,000-, 92-, and

140-fold relative to mock-treated cells, respectively (Fig. 2.1h, i).

Correspondingly, IFNα2, CCL7, IL-6, CXCL10, and TNFα proteins accumulated

in the supernatant (Fig. 2.1j). In contrast to the results with HIV-1-GFP, there

were no signs of maturation after transduction with the 3-part minimal lentivector
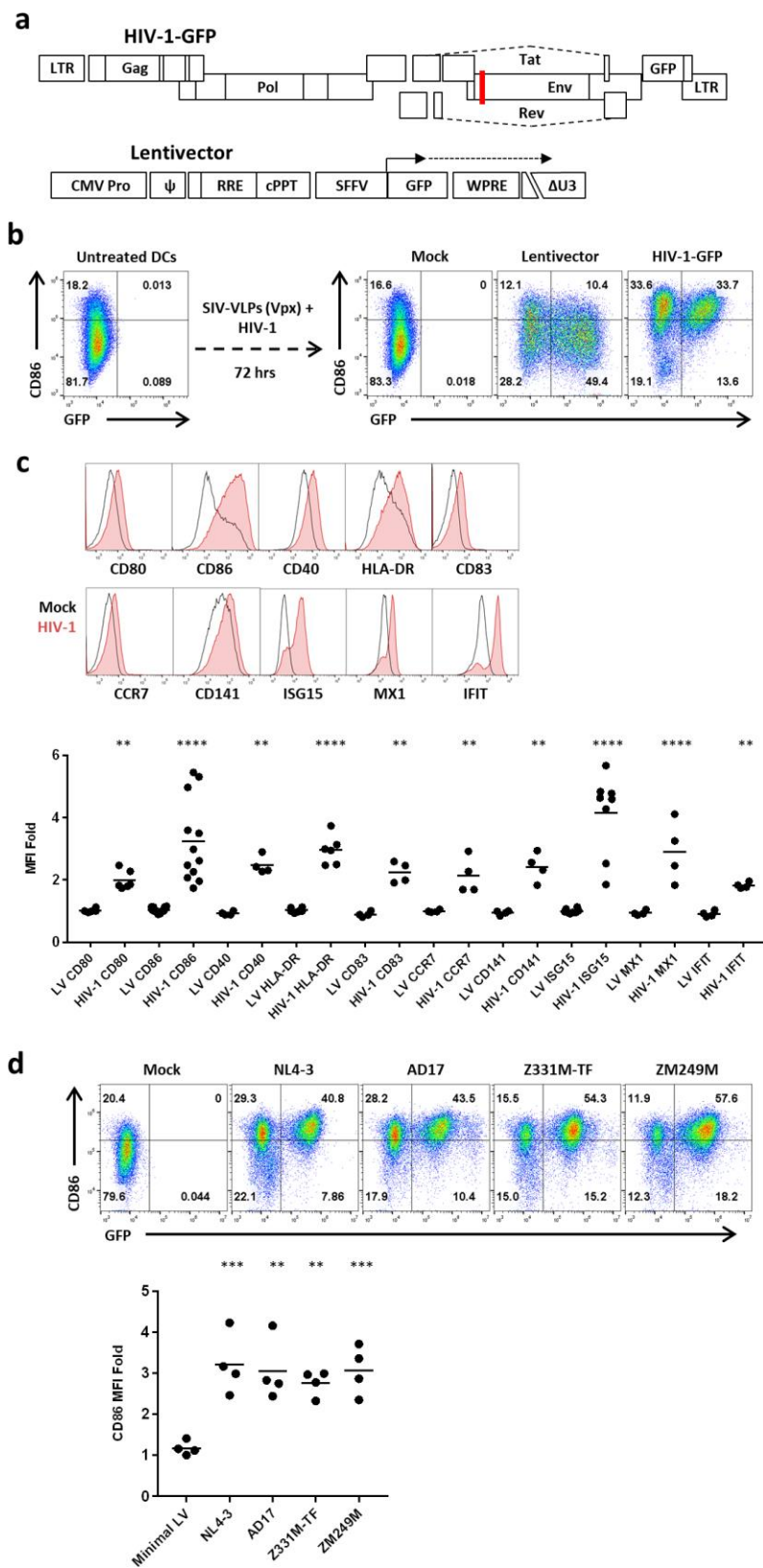
(Fig. 2.1b, h, j).

Figure 2.1

Figure 2.1
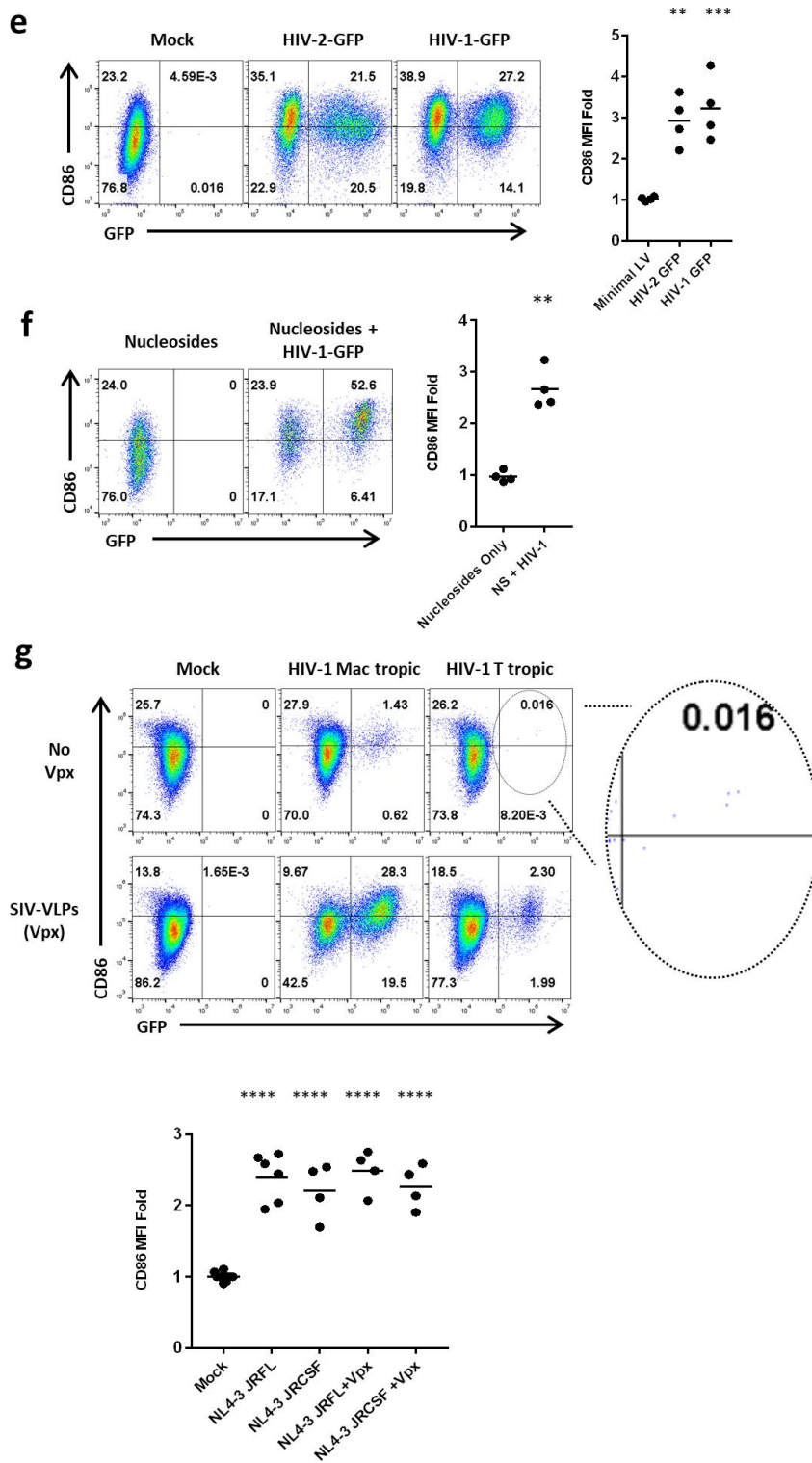
(continued)

Figure 2.1

(continued)

h

i

CXCL10

j
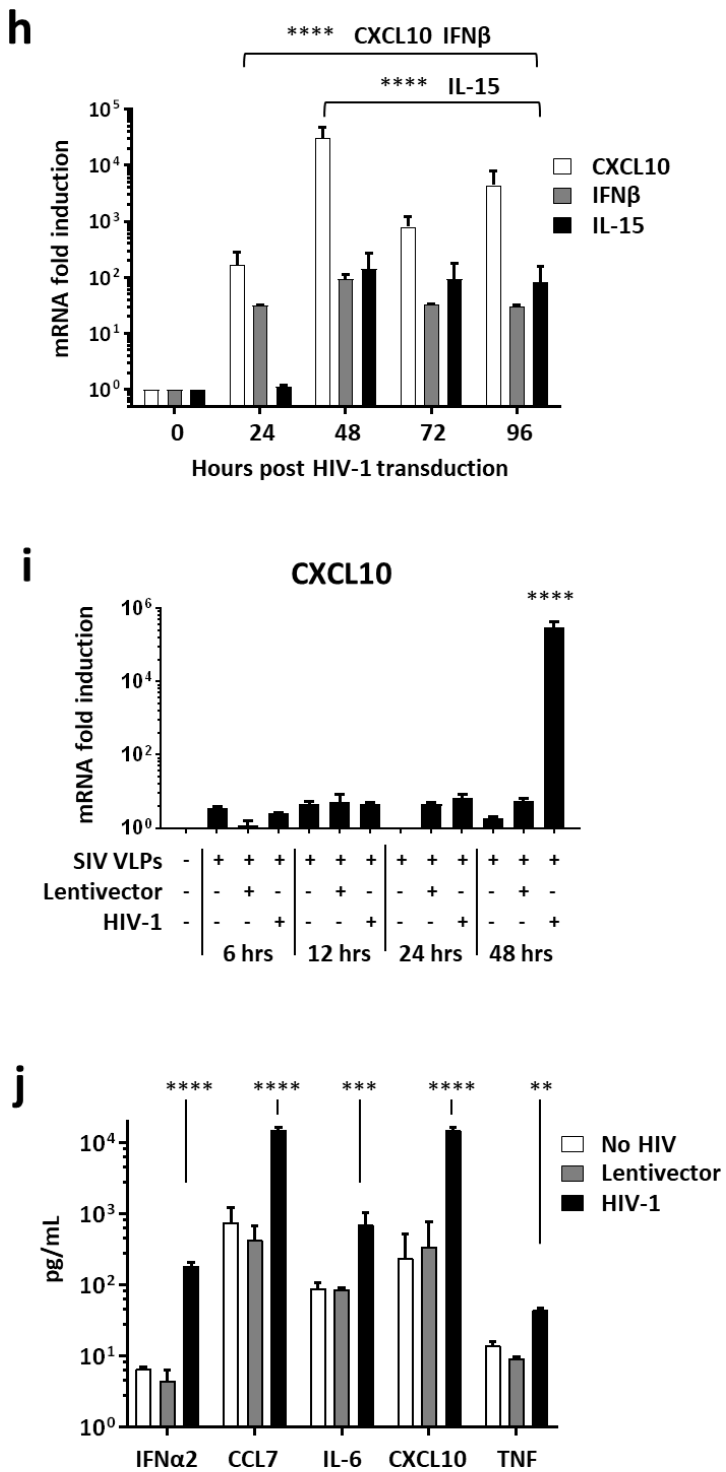
Figure 2.1 HIV-1 transduction matures DCs.

a, Schematic of HIV-1-GFP, with frameshift in *env* (red line) and *gfp* in place of *nef (He et al., 1997)*, and of the minimal lentivector, with self-inactivating ΔU3 LTR (Zufferey et al., 1998) and *gfp* driven by the SFFV promoter (Pertel et al., 2011). Unless indicated otherwise, vectors were pseudotyped with VSV G and cells were co-transduced with SIV$_{MAC}$251 VLPs bearing Vpx. **b**, Flow cytometry of DCs for GFP and CD86, after treatment as indicated. **c,** Flow cytometry histograms for the indicated markers 72 hrs after DC transduction with HIV-1 (red) or mock (black). **d,** Flow cytometry of DCs for GFP and CD86 after transduction with single-cycle clones, HIV-1-NL4-3, HIV-1-AD17, HIV-1-Z331M-TF, or HIV-1-ZM249M. **e,** Transduction of DCs with HIV-2-ROD-GFP, single-cycle vector. **f,** DC transduction with HIV-1-GFP in the absence of Vpx and the presence of 2 mM nucleosides. **g,** 12 day spreading infection on DCs, with macrophage-tropic or T cell-tropic, replication-competent HIV-1, with or without SIV VLPs. **h,** qRT-PCR quantitation of *CXCL10* (black), *IFNB1* (gray), or *IL15* (white) mRNAs from DCs transduced with HIV-1-GFP. **i,** qRT-PCR quantitation of *CXCL10* mRNA in DCs transduced with either HIV-1-GFP or minimal lentivector, assessed at the indicated times post-transduction. **j,** Cytokines in DC supernatant as assessed by luminex, 72 hrs after transduction with HIV-1-GFP (black) or minimal lentivector (gray).

Shown are blood donor data representative of n=12 (**b**), n=4 (**c, d, e, f, h, i, j**), or

n=8 (**g**). To determine significance, the MFI of all live cells for each sample was

calculated as fold-change of variable versus negative control for the indicated

marker. The exception being (**g**) where the MFI of only GFP+ cells was

compared. Combined flow cytometry MFI fold change data for all donor

replicates is represented by the associated dot plots. Significance was tested in

all flow cytometry data by one-way ANOVA with Dunnett's post-test comparing

test against minimal lentivector negative (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq$

0.001, **** = $p \leq 0.0001$). qRT-PCR and Luminex data are displayed as mean

+/- SD, with significance test against correspondingly-timed negative control by

two-way ANOVA, Dunnett's post-test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$,

**** = $p \leq 0.0001$).

2.3 Maturation requires integration, independent of coding sequence

To determine if early stages in the HIV-1 replication cycle were necessary for maturation, reverse transcription was inhibited by nevirapine (NVP) or the HIV-1 RT mutant D185K/D186L, and integration was inhibited with Raltegravir (RALT) or the HIV-1 IN mutant D116A (Table 2.1, 2.2), as previously described (De Iaco and Luban, 2011). Each of these four conditions abrogated maturation, as indicated by cell surface CD86 (Fig. 2.2a) and steady-state *CXCL10* mRNA (Fig. 2.2b). When integration was inhibited, *CXCL10* mRNA increased in response to challenge with HIV-1-GFP, but levels were nearly 1,000 times lower than when integration was not blocked (Fig. 2.2b).

HIV-1 virion RNA and newly synthesized viral cDNA are reported to be detected by RIG-I and by cGAS, respectively (Berg et al., 2012; Gao et al., 2013). Signal transduction downstream of both sensors requires TBK1 and IRF3. The TBK1 inhibitor BX795 (Table 2.1) blocked DC maturation in response to cGAMP but had no effect on maturation after HIV-1-GFP transduction (Fig. 2.2c). Moreover, IRF3 knockdown (Table 2.1) (Pertel et al., 2011) suppressed activation of CD86 or ISG15 in response to cGAMP, but not in response to HIV-1 transduction (Fig. 2.2c). Similarly, no effect on HIV-1-induced DC maturation was observed with knockdown of IRF1, 5, 7, or 9, or of STAT1 or 2, (Pertel et al., 2011), or of pharmacologic inhibition of CypA, PKR, c-Raf, IkBa, NF-kB, MEK1+2, p38, JNK, Caspase 1, pan-Caspases, ASK1, eIF2a, TBK1, IKKe, TAK1, or NLRP3. Under

the conditions used here, then, DC maturation required reverse transcription and integration but was independent of most well characterized innate immune signaling pathways.

Completion of the HIV-1 integration reaction requires cellular DNA repair enzymes (Craigie and Bushman, 2012). That DCs did not mature in response to transduction with minimal lentivectors (Fig. 2.1b, i, j) indicates that activation of the DNA repair process is not sufficient, and that transcription from the HIV-1-GFP provirus must be necessary for maturation. Indeed, *gag* expression from an integrated vector has been reported to be necessary for DC maturation (Manel et al., 2010). To determine if any individual HIV-1 proteins were sufficient to mature DCs, a minimal lentivector was used to express codon optimized versions of each of the open reading frames possessed by HIV-1-GFP (Fig. 2.2d, Table 2.1). Among these vectors was a *gag*-expression vector that produced as much p24 protein as did HIV-1-GFP (Fig. 2.2d). None of these vectors matured DCs (Fig. 2.2d).

HIV-1-GFP was then mutated to determine if any protein coding sequences were necessary for DC maturation. For these and any subsequent experiments in which an essential viral component was disrupted within HIV-1-GFP, the factor in question was provided *in trans*, either during assembly in transfected HEK293 cells, or within transduced DCs, as appropriate (see Methods). Mutations that disrupted both *gag* and *pol*, either a double frameshift in *gag*, or a mutant in

which the first 14 ATGs in *gag* were mutated, abolished synthesis of CA (p24) yet

retained full maturation activity (Fig. 2.2e, Table 2.1). Deletion mutations

encompassing *gag*/*pol*, *vif*/*vpr*, *vpu*/*env*, or *nef*/U3-LTR, each designed so as to

leave *cis*-acting RNA elements intact, all matured DCs (Fig. 2.2f, Table 2.1).

These results indicate that these HIV-1-GFP RNA sequences, as well as the

proteins that they encode, were not required for DC maturation.

Tat and Rev coding sequences were individually disrupted by combining start

codon point mutations with nonsense codons that were silent with respect to

overlapping reading frames (Table 2.1). Neither Δ*tat* nor Δ*rev* matured DCs upon

transduction (Fig. 2.2g). However, DCs matured upon co-transduction of Δ*tat* and

Δ*rev*, or when minimal lentivectors expressing codon-optimized Tat and Rev

were co-transduced *in trans* (Fig. 2.2g). These results indicate that the

maturation defect with the individual vectors was due to disruption of Tat and Rev

function, and not due to a *cis*-acting defect of the mutant RNA.

The minimal 3-part lentivector expressed GFP from a heterologous promoter and

had a deletion mutation encompassing the essential, *cis*-acting TATA box and

enhancer elements (Zufferey et al., 1998), as well as in the *trans*-acting *tat* and

rev, that inactivated the promoter in the proviral 5' LTR (Fig. 2.1a). To test the

importance of LTR-driven transcription for DC maturation by the HIV-1 provirus,

the HIV-1 LTR was restored in the minimal vector (Fig. 2.2h, Table 1); in addition,

GFP was inserted in place of *gag* as a marker for LTR expression, and the

heterologous promoter was used to drive *tat*, *rev*, or both genes separated by P2A coding sequence (Fig. 2h). None of the LTR-driven, minimal vectors matured DCs (Fig. 2.2h).
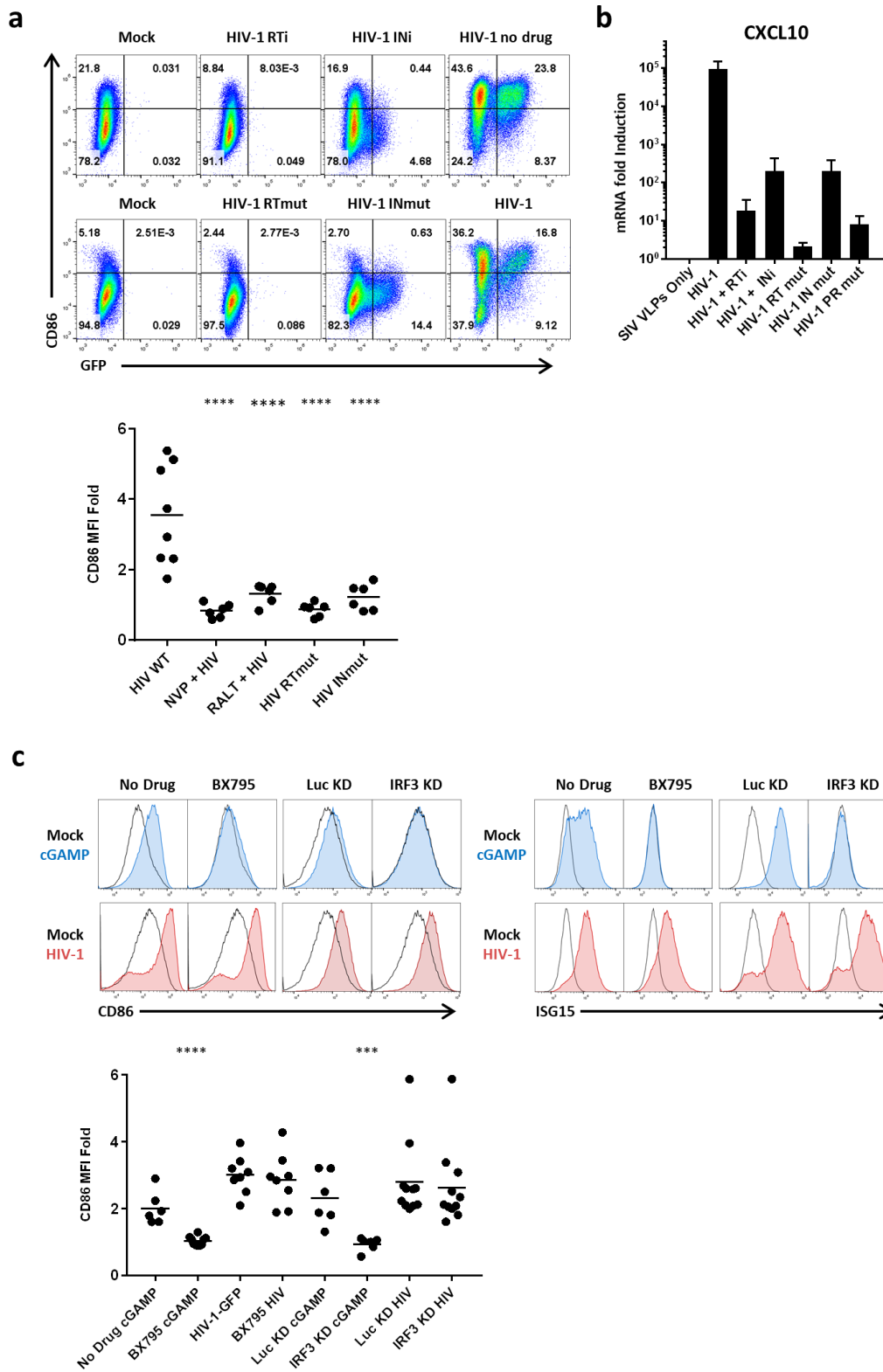
Figure 2.2
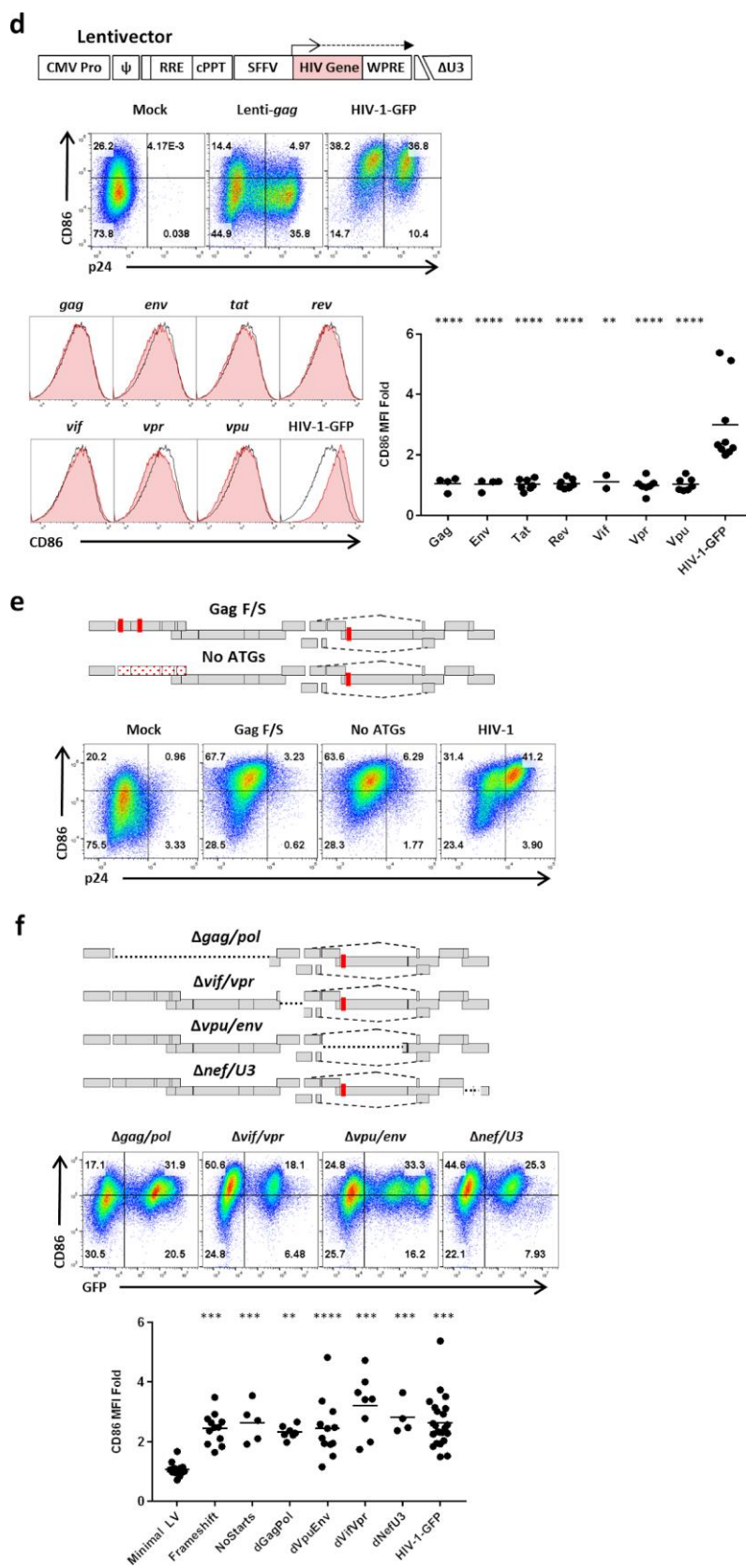
Figure 2.2 (continued)

Figure 2.2

(continued)

g

h

Lentivector-U3+

| CMV Pro | ψ | eGFP | RRE | cPPT | SFFV | *tat* and/or *rev* | +U3 LTR |
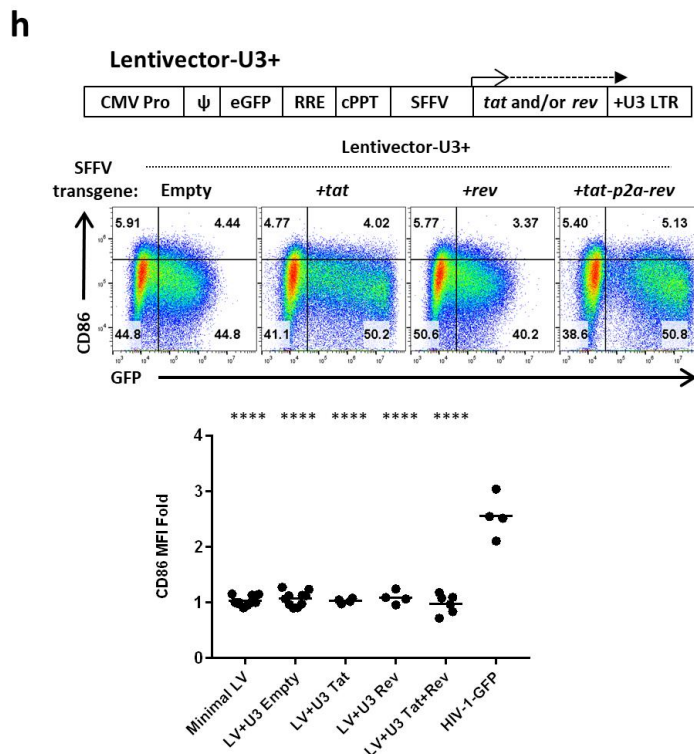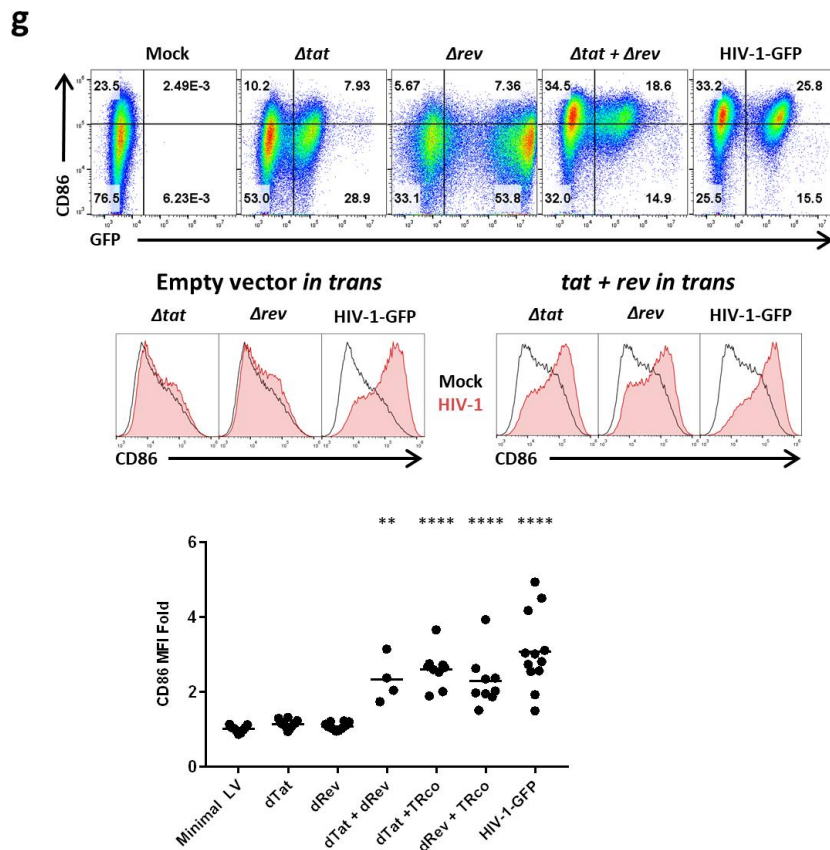
Figure 2.2 HIV-1 RNA regulation is necessary for DC maturation, but LTR-driven transcription is insufficient and coding sequences are not necessary.

a, Assessment of GFP and CD86 by flow cytometry following transduction with, top, HIV-1-GFP in the presence of 5 µM nevirapine (RTi), 10 µM raltegravir (INi), or no drug, and, bottom, HIV-1-GFP bearing mutant RT-D185K/D186L (RTmut) or mutant IN-D116A (INmut). **b**, qRT-PCR quantitation of *CXCL10* mRNA from the same DCs as in (a). **c**, DCs treated with 1 µM of the TBK1 inhibitor BX795, or expressing shRNAs targeting either IRF3 or luciferase control(Pertel et al., 2011), were challenged with 25 µg/mL cGAMP or HIV-1-GFP and assayed by flow cytometry for CD86 and ISG15. **d**, Flow cytometry of DCs after transduction with minimal lentivectors expressing codon optimized HIV-1 genes; **e**, HIV-1-GFP in which translation was disrupted by two frameshifts in *gag* or by mutation of the first 14 AUGs in *gag*; **f**, HIV-1-GFP bearing deletion mutations encompassing *gag*/*pol*, *vif*/*vpr*, *vpu*/*env*, or *nef*/U3-LTR; **g**, HIV-1-GFP bearing mutations in *tat* or *rev*, co-transduced with both mutants, or co-transduced with minimal vector expressing *tat* and *rev in trans*; or **h**, minimal lentivector with GFP in place of *gag*, SFFV promoter driving expression of *tat*, *rev,* or both, and repaired U3 in the 3' LTR; the latter restores 5'-LTR-directed transcription to the provirus as a result of the reverse transcription strand-transfer reactions. When an essential viral component was disrupted within HIV-1-GFP, the factor in question was provided *in trans*, either during assembly in transfected HEK293 cells, or within transduced

DCs, as appropriate (see Methods). Shown are blood donor data representative of n=6 (a, b, e, f), n=12 (c, g, h), n=8 (d).

To determine significance, the MFI of individual flow cytometry samples was calculated as fold-change of variable versus negative control for the indicated marker. Combined flow cytometry MFI fold change data for all donor replicates is represented by the associated dot plots. Significance was tested in all flow cytometry data by one-way ANOVA with Dunnett's post-test comparing test against minimal lentivector negative (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001). qRT-PCR data are displayed as mean +/- SD, with significance test against correspondingly-timed negative control by two-way ANOVA, Dunnett's post-test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001).

2.4 Maturation requires transcription and Rev-mediated export

To determine if *tat* was necessary for DC maturation, *tat* and TAR were mutated in HIV-1-GFP and the LTR promoter was modified to be tetracycline-inducible, as previously described (Das and Berkhout, 2016) (Tet-HIV-1 in Fig. 2.3a Table 2.1). The doxycycline-dependent reverse transactivator (*rtTA*) was delivered *in trans* by lentivector. In the presence of doxycycline (Table 2.2), Tet-HIV-1 and rtTA matured DCs when given in combination, but neither vector matured DCs when given in isolation (Fig. 2.3a). Additionally, the magnitude of cell surface CD86 was dependent on the doxycycline concentration, indicating that maturation was dependent on the level of HIV-1 transcription (Fig. 3b). These results demonstrated that *tat* was not required for maturation, so long as the provirus was expressed.

To ascertain whether *rev* was necessary for DC maturation, the RTE from a murine intracisternal A-particle retroelement (IAP), and the CTE from SRV-1, were inserted in place of *nef* (HIV-RTE/CTE in Fig. 2.3c, Table 2.1) (Smulevitch et al., 2006). Each of these elements utilizes the NXF1 nuclear RNA export pathway, thereby bypassing the need for CRM1 and *rev* (Fornerod et al., 1997). p24 levels with this construct were similar to those of HIV-1-GFP, indicating that unspliced RNA was exported from the nucleus at least as well as with Rev (Fig. 2.3c). Nonetheless, the HIV-RTE/CTE vector did not mature DCs (Fig. 2.3c), indicating that maturation was dependent upon *rev* and CRM1-mediated RNA

export. Consistent with this conclusion, the CRM1 inhibitor leptomycin B (Table 2.2) abrogated DC maturation by HIV-1-GFP (Fig. 2.3d). In contrast, leptomycin B had no effect on DC maturation in response to Sendai virus infection (Fig. 2.3e). ISG15 was used to monitor maturation in these experiments since, as previously reported for DCs, leptomycin B altered background levels of CD86 (Chemnitz et al., 2010).

Figure 2.3

a
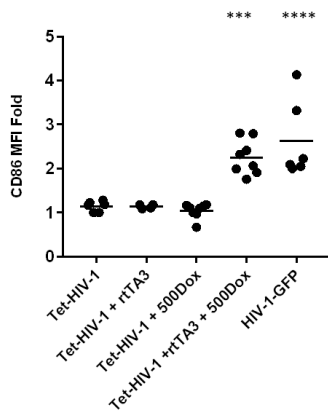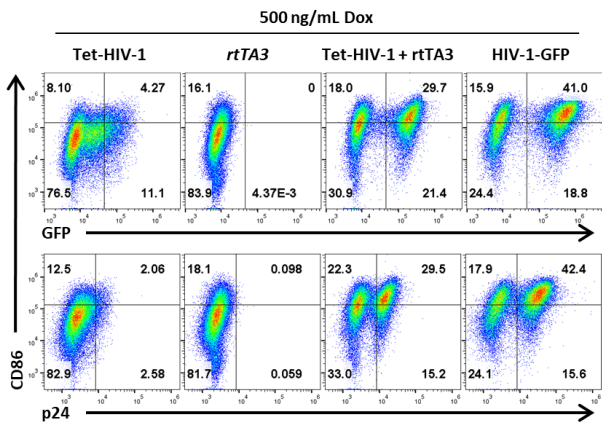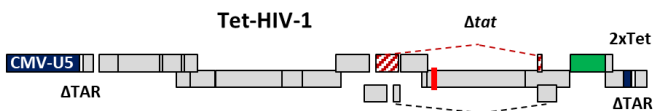
b
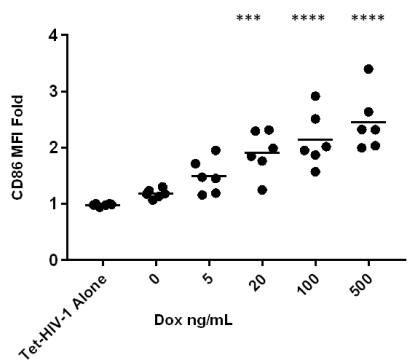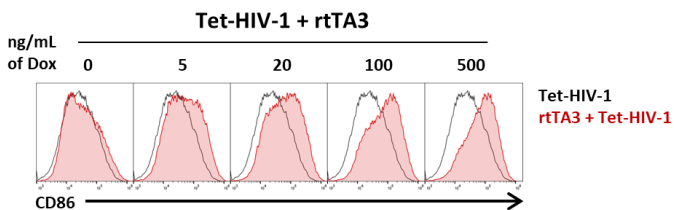
Figure 2.3

Continued
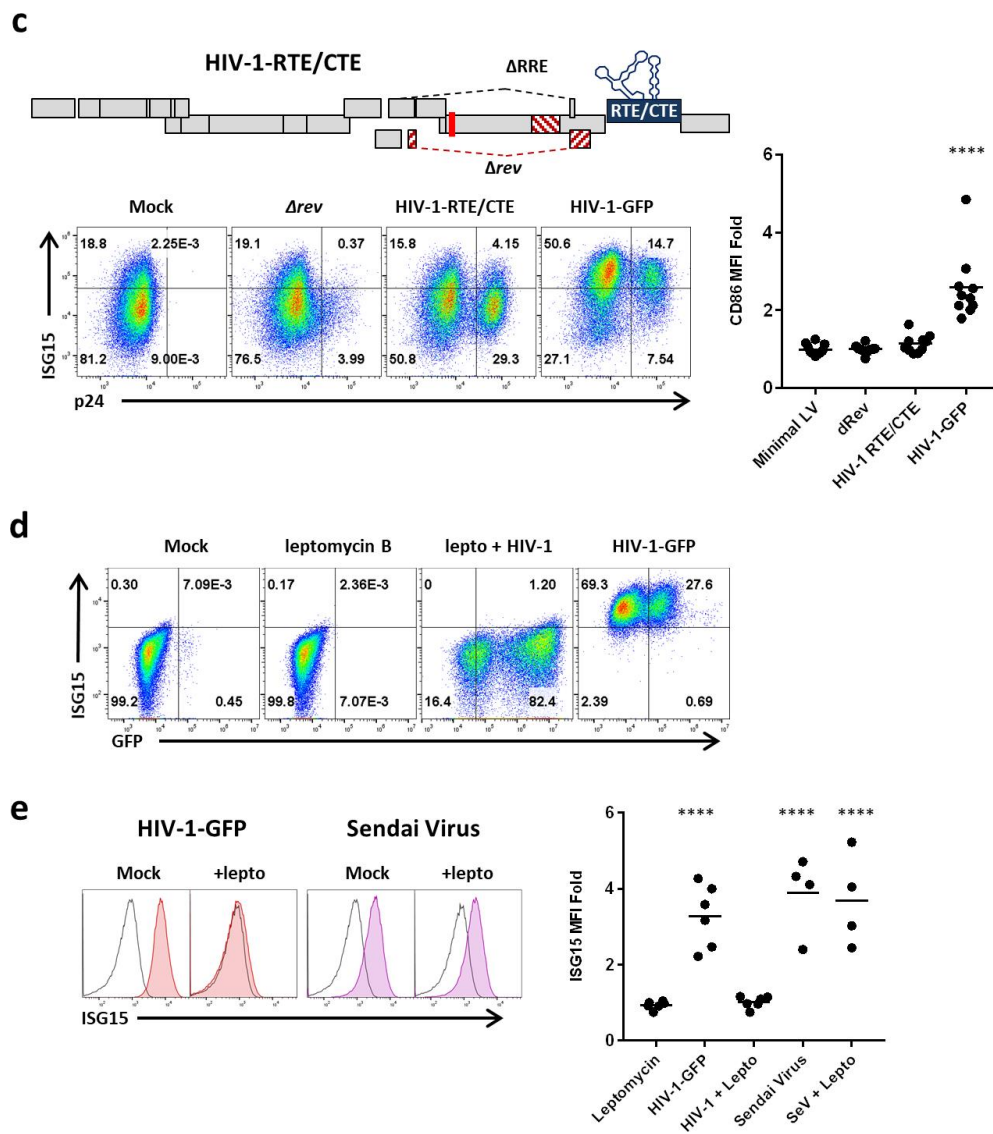
Figure 2.3 Rev-mediated RNA export is necessary for DC maturation but Tat is dispensable

a, 2xTet operator(Das and Berkhout, 2016) was cloned into the 3'LTR of HIV-1-GFPΔ*tat* to generate Tet-HIV-1; strand-transfer reactions that occur during reverse transcription generate a Tet-regulated 5'-LTR in the provirus. DCs transduced with Tet-HIV-1, rtTA3, or both, were treated for 3 d with 500 ng/mL doxycycline and assayed by flow cytometry for p24, GFP, and CD86. **b**, DCs co-transduced with Tet-HIV-1 and rtTA3 were treated with increasing concentrations of doxycycline. **c**, To generate HIV-1-RTE/CTE, the RTEm26CTE element (Smulevitch et al., 2006) was cloned in place of *nef* in HIV-1-GFPΔ*rev*/ΔRRE. DCs were transduced with the indicated vectors and assessed for p24 and ISG15 by flow cytometry. **d**, DCs were treated with 25 nM leptomycin B, transduced with HIV-1-GFP, and assessed for GFP and ISG15 by flow cytometry. **e**, DCs were treated with 25 nM leptomycin B, transduced with HIV-1-GFP or infected with Sendai virus (SeV), and assessed for ISG15 by flow cytometry. Shown are blood donor data representative of n=10 (**a, c**), n=4 (**b**), n=6 (**d, e**).

To determine significance, the MFI of individual flow cytometry samples was calculated as fold-change of variable versus negative control for the indicated marker. Combined flow cytometry MFI fold change data for all donor replicates is represented by the associated dot plots. Significance was tested in all flow

cytometry data by one-way ANOVA with Dunnett's post-test comparing test

against minimal lentivector negative (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001,

**** = p ≤ 0.0001).

2.5 HIV-1 transcription activates macrophages and CD4+ T cells

To determine if innate immune detection of HIV-1 was unique to DCs, monocyte-derived macrophages and CD4$^+$ T cells were examined. In response to transduction with HIV-1-GFP, macrophages upregulated CD86, ISG15, and HLA-DR, and CD4$^+$ T cells upregulated MX1, IFIT1, and HLA-DR (Fig. 2.4a). DCs, macrophages, and CD4$^+$ T cells were then transduced side-by-side with mutant constructs to determine if the mechanism of innate immune activation was similar to that in DCs. As with DCs, HIV-1-GFP bearing the Δ*gag/pol* deletion activated macrophages and CD4$^+$ T cells (Fig. 2.4b). Also in agreement with the DC results, neither the minimal lentivector, nor HIV-1-GFP bearing mutations in *integrase*, *tat*, or *rev*, matured any of the three cell types (Fig. 2.4b).

CD4$^+$ T cells were infected with either macrophage-tropic or T cell-tropic HIV-1 to determine whether replication-competent HIV-1 was similarly capable of innate immune activation in these cells, in the absence of VSV G. As with DCs, innate immune activation, as detected by MX1 and ISG15 upregulation, was observed in cells productively infected with HIV-1, but not with minimal lentivector (Fig. 2.4c). Finally, to test the effect of HIV-1 proviral RNA on non-activated T cells, CD4$^+$ T cells were co-transduced with Tet-HIV-1 and the rtTA3 vector, and cultured for 9 days in the absence of stimulation. Upon doxycycline treatment, T cells expressed GFP and MX1 (Fig. 2.4d). As in DCs, dose-dependent activation was observed with doxycycline (Fig. 2.4d). These data indicate that innate

immune activation by HIV-1, in all three cell types, requires integration,

transcription, and Rev-dependent, HIV-1 intron-containing RNA.

Figure 2.4

Figure 2.4

Continued

c

CD4+ T Cell Spreading Infections
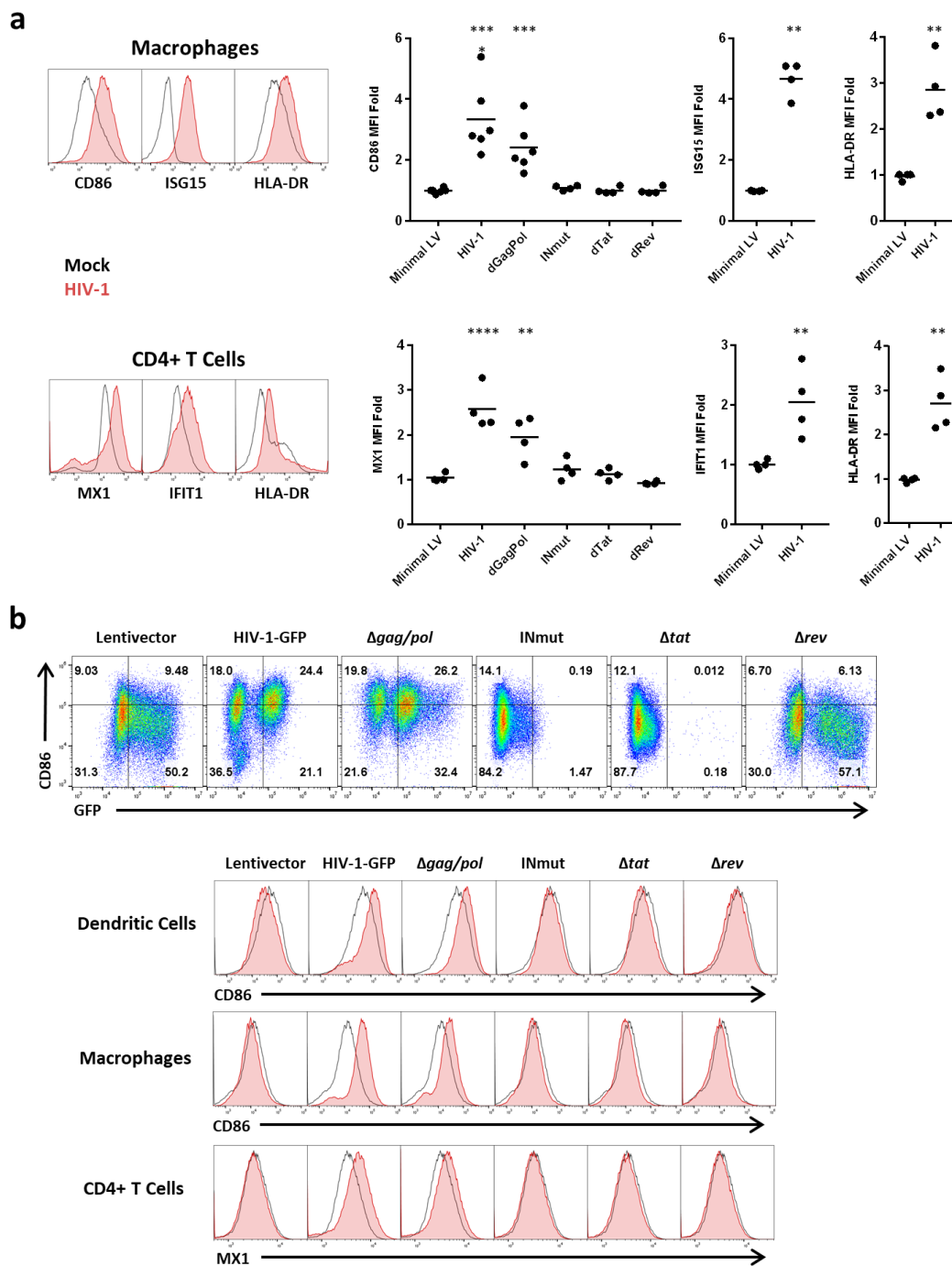
d
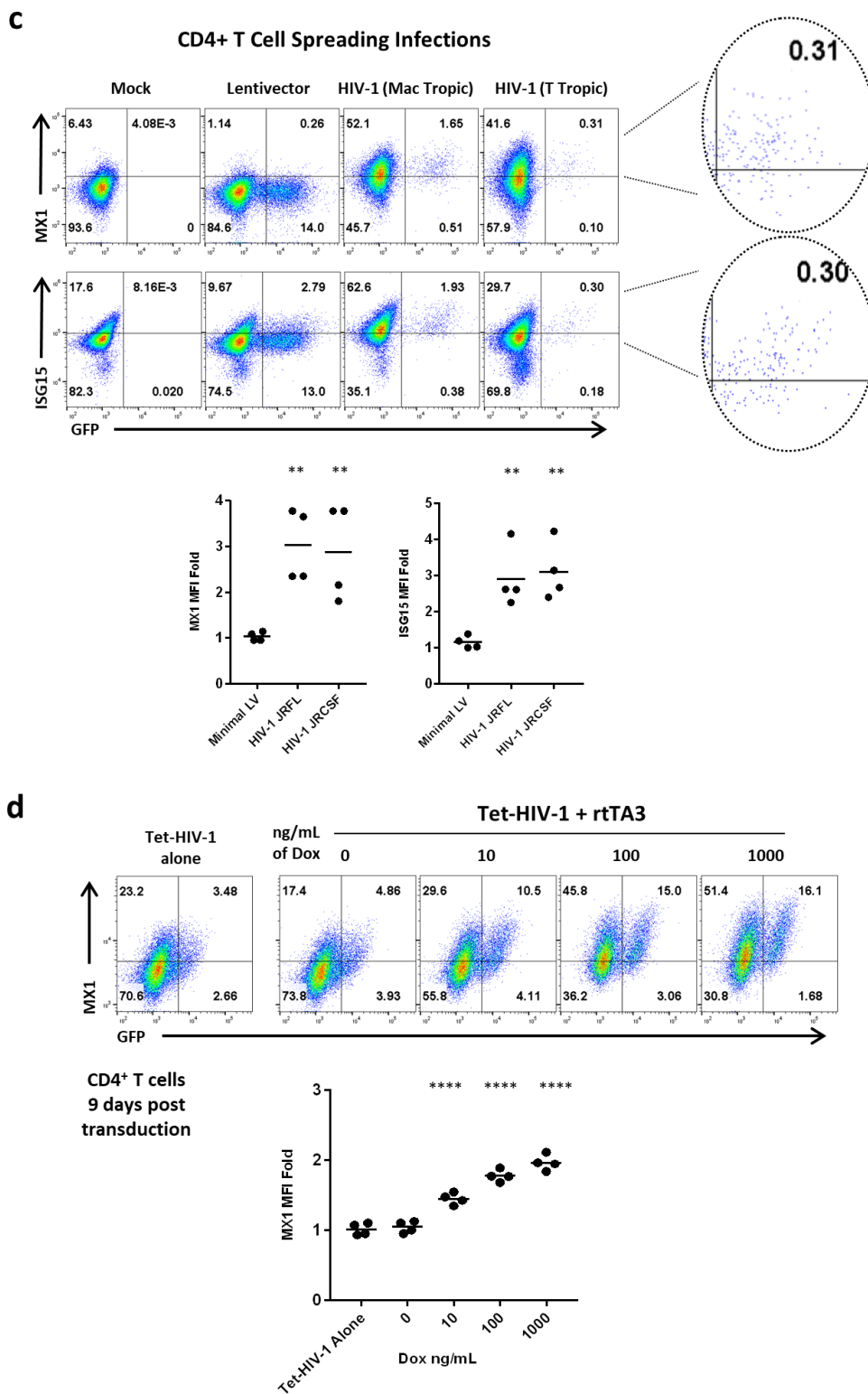
Figure 2.4 Innate immune activation in macrophages and CD4+ T cells by HIV-1 proviral transcription

a, Macrophages and CD4$^+$ T cells were transduced with HIV-1-GFP and assayed 3 days later for the indicated activation markers. **b**, DCs, macrophages, and CD4$^+$ T cells were challenged with HIV-1-GFP or the indicated mutants. When an essential viral component was disrupted within HIV-1-GFP, the factor in question was provided in trans during assembly in transfected HEK293 cells, as appropriate (see Methods). The upper panel shows flow cytometry of the DCs for GFP and CD86. The histograms show CD86 for DCs and macrophages or MX1 for CD4$^+$ T cells. **c**, 12 day spreading infections on CD4+ T cells with either macrophage-tropic or T cell-tropic, replication-competent HIV-1. **d**, CD4$^+$ T cells were stimulated for 3 days with PHA and IL2, and transduced with Tet-HIV-1 and rtTA3. Cells were then cultured without stimulation for 9 days. Doxycycline was then added at the indicated concentrations. Cells were assayed for GFP and MX1 3 days later. Shown are blood donor data representative of n=6 (a), n=4 (b, c, d).

To determine significance, the MFI of individual flow cytometry samples was calculated as fold-change of variable versus negative control for the indicated marker. Combined flow cytometry MFI fold change data for all donor replicates is represented by the associated dot plots. Significance was tested in all flow cytometry data by one-way ANOVA with Dunnett's post-test comparing test

against minimal lentivector negative (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001,

**** = p ≤ 0.0001).

2.6 Implications for research and antiviral therapy

The HIV-1 LTR generates a single primary transcript that gives rise to over 100 alternatively spliced RNAs (Ocwieja et al., 2012). The full-length, unspliced, intron-bearing transcript acts as viral genomic RNA in the virion and as mRNA for essential *gag*- and *pol*-encoded proteins. Expression of the unspliced transcript requires specialized viral and cellular machinery, HIV-1 Rev and CRM1(Fornerod et al., 1997), in order to escape from the spliceosome. Results here indicate that unspliced or partially spliced HIV-1 RNA is detected by human cells as a danger signal, as has been reported for inefficiently spliced mRNAs from transposable elements in distantly related eukaryotes (Dumesic et al., 2013). Transposable elements are mutagenic to the host genome and it stands to reason that molecular features such as transcripts bearing multiple, inefficient splice signals characteristic of retrotransposons, would activate innate immune signaling pathways.

HIV-1 genomic RNA contains extensive secondary and higher order structures that could be detected by innate immune sensors. Our knockdown of IRF3 and inhibition of TBK1, both required for signal transduction of the RNA sensors RIG-I, MDA5, and TLR3, did not impede HIV-1 maturation of DCs (Figure 2.2c). Furthermore, we suppressed an extensive list of innate signaling pathways and sensors including knockdowns of IRF's 1, 5, 7, and 9, STAT's 1 and 2, as well as pharmacologic inhibition of CypA, PKR, c-Raf, IkBa, NF-kB, MEK1+2, p38, JNK,

Caspase 1, pan-Caspases, ASK1, eIF2a, IKKe, TAK1, or NLRP3 (Table 2.2). None of these perturbations had any effect on limiting innate immune activation by HIV-1, suggesting requirement for an alternative detection mechanism. Such mechanisms might include uncharacterized RNA sensors, direct detection of stalled splicing machinery, or overload of the CRM1 nuclear export pathway itself.

The replication competent HIV-1 reservoir in memory CD4^{+} T cells has a 44 wk half-life and thus patients must take antiviral medication for life (Crooks et al., 2015). Long-lived, replication competent HIV-1 reservoirs in other cell types have not been clearly demonstrated, but these may also contribute to the HIV-1 reservoir (Kandathil et al., 2016). The common genetic determinants in HIV-1 for maturation of CD4^{+} T cells, macrophages, and DCs suggests that HIV-1 is detected by a mechanism that is conserved across cell types, and that this mechanism would be active in any cell type that possesses a transcriptionally active provirus. Data here show that proviruses need not be replication competent to contribute to inflammation. Rather, HIV-1 transcription and export of unspliced RNA, regardless of replication competence, is sufficient to induce immune activation. Consistent with our findings, T cell activation correlates directly with the level of cell-associated HIV-1 RNA in patients receiving antiretroviral therapy (El-Diwany et al., 2017). Furthermore, our data suggests that new drugs that block HIV-1 transcription, Tat-mediated transcriptional elongation, or Rev-mediated preservation of unspliced transcripts(Mousseau et

al., 2015), would limit inflammation, and offer an important addition to the current

anti-HIV-1 drug armamentarium.

Table 1 Plasmids used in this study

Plasmid Name	Purpose	Notes
pBS NL4-3 *env*^{FS} eGFP	Single-cycle, full-length HIV-1	"HIV-1-GFP" in the manuscript. HIV-1 NL4-3 in pBluescript, flanking host sequences deleted, frameshift in *env*, eGFP in place of *nef(He et al., 1997)*
pUC57mini NL4-3 Δ*env* eGFP	HIV-1 clade B molecular clone	Molecular clone of NL4-3 with deletion of 79 nucleotides following the Env signal peptide and eGFP in place of *nef*
pUC57mini AD17 Δ*env* eGFP	HIV-1 clade B molecular clone	Molecular clone of transmitted/founder virus HIV-1-AD17(Parrish et al., 2013) with deletion of 79 nucleotides following the Env signal peptide and eGFP in place of *nef*
pUC57mini Z331M-TF Δ*env* eGFP	HIV-1 clade C molecular clone	Molecular clone of transmitted/founder virus HIV-1-Z331M-TF(Deymier et al., 2015) with deletion of 79 nucleotides following the Env signal peptide and eGFP in place of *nef*
pUC57mini ZM249M Δ*env* eGFP	HIV-1 clade C molecular clone	Molecular clone of transmitted/founder virus HIV-1-ZM249M(Salazar-Gonzalez et al., 2009) with deletion of 79 nucleotides following the Env signal peptide and eGFP in place of *nef*
pROD9 Δ*env* eGFP	HIV-2 $_{ROD}$ molecular clone	Molecular clone of HIV-2-ROD. Contains frameshift in *env* and eGFP in place of Nef
pMD2.G	VSV G	Pseudotype HIV-1 vectors with VSV G
psPAX2	HIV-1 *gag-pol*	"3-part" lentivector or for complementation of assembly-incompetent HIV-1 vectors
SIV3+	SIV$_{MAC251}$ *gag-pol/vpx*	Production of SIV VLPs containing Vpx protein
pALPS(Pertel et al., 2011)	Minimal lentivector	Includes *cis*-acting elements required for reverse transcription and integration, psi RNA packaging element, RRE, cPPT, PPT, mutation in the 3'LTR U3 that eliminates LTR-based transcription, and SFFV promoter to express genes of interest.
pALPS eGFP	eGFP lentivector	Encodes eGFP

pALPS *gag*	*gag* lentivector	Encodes codon optimized NL4-3 *gag*
pALPS *env*	*env* lentivector	Encodes codon optimized JR-CSF *env*
pALPS *tat*	*tat* lentivector	Encodes codon optimized NL4-3 *tat*
pALPS *rev*	*rev* lentivector	Encodes codon optimized NL4-3 *rev*
pALPS *vif*	*vif* lentivector	Encodes codon optimized NL4-3 *vif*
pALPS *vpr*	*vpr lentivector*	Encodes codon optimized NL4-3 *vpr*
pALPS *vpu*	*vpu lentivector*	Encodes codon optimized NL4-3 *vpu*
pALPS *tat*-P2A-*rev*	*tat* and *rev* *lentivector*	Lentivector expressing codon optimized NL4-3 *tat* and *rev* linked by P2A peptide coding sequence (GSGATNFSLLKQAGDVEENPGP)
pBS NL4-3 *env*^{FS} eGFP RT-D185K/D186L(De Iaco and Luban, 2011)	RT mutant	pBS NL4-3 *env*^{FS} eGFP with mutation that disrupts RT catalytic activity
pBS NL4-3 *env*^{FS} eGFP IN-D116A(De Iaco and Luban, 2011)	IN mutant	pBS NL4-3 *env*^{FS} eGFP with mutation that disrupts IN catalytic activity
pBS NL4-3 *env*^{FS} eGFP PR-D25A(De Iaco and Luban, 2011)	PR mutant	pBS NL4-3 *env*^{FS} eGFP with mutation that disrupts Protease catalytic activity
pALPS puro miR30-L1221(Pertel et al., 2011)	Luciferase knockdown	negative control for knockdowns target site: CTTGTCGATGAGAGCGTTTGT
pALPS puro miR30-IRF1(Pertel et al., 2011)	IRF1 knockdown	Target site TTGCTCTTAGCATCTCGGCTG
pALPS puro miR30-IRF3(Pertel et al., 2011)	IRF3 knockdown	Target site ATCAGATCTACAATGAAGGGC
pALPS puro miR30-IRF5(Pertel et al., 2011)	IRF5 knockdown	Target site TATTTCCCTGTCTCCTTGGCC

pALPS puro miR30-IRF7(Pertel et al., 2011)	IRF7 knockdown	Target site ATAAGGAAGCACTCGATGTCG
pALPS puro miR30-IRF9(Pertel et al., 2011)	IRF9 knockdown	Target site AATTATCACAAAGAGGACAGG
pALPS puro miR30-STAT1(Pertel et al., 2011)	STAT1 knockdown	Target site ATATCCAGTTCCTTTAGGGCC
pALPS puro miR30-STAT2(Pertel et al., 2011)	STAT2 knockdown	Target site TTTAAGTTCCACAGACTTGGA
pALPS puro miR30-TAK1(Pertel et al., 2011)	TAK1 knockdown	Target site AGCGCCCTTCAATGGAGGAAAT
pALPS_3'LTR GFP@gag start SFFV-	U3+ lentivector GFP at *gag* start SFFV promoter	Repaired U3 allows LTR-based transcription by the provirus with GFP as a marker for expression. WPRE was deleted.
pALPS_3'LTR GFP@*gag* start SFFV *tat*	U3+ lentivector GFP at *gag* start SFFV-*tat*	LTR drives GFP and internal SFFV promoter drives codon optimized *tat*
pALPS_3'LTR GFP@*gag* start SFFV *rev*	U3+ lentivector GFP at *gag* start SFFV-*rev*	LTR drives GFP and internal SFFV promoter drives codon optimized *rev*
pALPS_3'LTR GFP@*gag* start SFFV *tat*-P2A-*rev*	U3+ lentivector with SFFV-*tatP2Arev*	LTR drives GFP and internal SFFV promoter drives codon optimized *tat* and *rev*.
pNL4-3 *env*^{FS} eGFP *gag*^{2xFS}	No Gag synthesis	1st frameshift is CG nucleotide insertion in MA at nt 832. 2nd is a CTAG addition in CA at nt 1508.
pNL4-3 *env*^{FS} eGFP NoStarts	No Gag synthesis	All ATGs from the start of *gag* to NC mutated to ATC except the first which was mutated to ACG
pBS NL4-3 *env*^{FS} eGFP Δ*gag/pol*	*gag-pol* deletion	Deletion from the start of *gag* until 229 bases before the cPPT
pNL4-3 *env*^{FS}	*vif*/*vpr* deletion	Deletion from NL4-3 nt 5582-6199

eGFP Δ*vif/vpr*		encompassing Vif and Vpr coding sequence
pNL4-3 *env*^{FS} eGFP Δ*vpu/env*	*vpu*/*env* deletion	Deletion from NL4-3 nt 6054-7489 encompassing all of *vpu* and *env* until before the RRE
pBS NL4-3 *env*^{FS} eGFP Δ*nef*/U3	*nef*/U3 deletion	Deletion from NL4-3 nt 8911-9022 and 9088-9377. This deletes *nef* and U3 LTR sequences
pBS NL4-3 *env*^{FS} eGFP 5'CMV Δ*tat*	HIV-1 with inactivating mutations in *tat*	*tat* ATG->ACG (silent in *vpr* reading frame), nt 78 mutated T->G to change Tyr to stop codon, nt 116 mutated T->C to disrupt Met, 5'LTR replaced with CMV-R-U5 from pALPS for *tat*-independent transcription in HEK293E cells.
pBS NL4-3 *env*^{FS} eGFP Δ*rev*	HIV-1 with inactivating mutations in *rev*	All mutations in *rev* are silent with respect to the *tat* reading frame. Start ATG->ACG and nts 68-71 were mutated AGC->TCA to change tyrosine to a stop.
pBS NL4-3 *env*^{FS} eGFP 5'CMV Δ*tat* ΔTARx2_d2TetOp(Das and Berkhout, 2016)	Tet-inducible, *tat*-independent HIV-1	2xTet Operator inserted between NFkB and Sp1 sites in U3 of HIV-1 Δ*tat* with 5' CMV-R-U5. 5' and 3' TAR elements were mutated to: 5'-GGTCTCTCTGGTTAGACCAGA<u>AAG</u>GAGC<u>A</u> T<u>T</u>GGAGCTCTCTGGCTAACTAGGGAACCC-3'
pALPS rtTA3_V14(Das and Berkhout, 2016)	rtTA3 lentivector	Codon optimized rtTA3 used *in trans* with Tet inducible HIV-1
pSC101 NL4-3 *env*^{FS} Δ*rev* ΔRRE RTEm26CTE(Smul evitch et al., 2006)	Rev (CRM1) independent HIV-1	HIV-1 ΔRev was cloned into pSC101 and modified to include an RTEm26CTE element in order to utilize the NXF1 RNA export pathway. The RRE was also mutated.

Table 2 Drugs and maturation effects

Drug	Action	Source	Working concentration	HIV-1 DC maturation
Doxycycline	rtTA3 activator	Sigma (D9891)	10-1000 ng/mL	-
cGAMP	STING activator	Invivogen (tlrl-nacga23)	25 µg/mL	-
PHA-P	T cell mitogen	Sigma (L1668)	5 µg/mL	-
2'deoxyguanosine monohydrate	For nucleoside assisted transductions	Sigma (D0901)	2 mM	-
2' deoxythymidine	For nucleoside assisted transductions	Sigma (T1895)	2 mM	-
2'deoxyadenosine monohydrate	For nucleoside assisted transductions	Sigma (D8668)	2 mM	-
2'deoxycytidine hydrochloride	For nucleoside assisted transductions	Sigma (D0776)	2 mM	-
Sendai Virus (SeV) Cantell strain	Challenge virus	Charles River Labs (VR-907)	200 HA units/mL	-
Nevirapine	Reverse transcriptase inhibitor	NIH AIDS reagent program (4666)	5 µM	Inhibits
Raltegravir	Integrase inhibitor	NIH AIDS reagent program (11680)	10 µM	Inhibits
Leptomycin	CRM1 inhibitor	Invivogen (tlrl-lep)	25 nM	Inhibits
Cyclosporin A	Cyclophilin A inhibitor	Sigma (30024)	5 µM	No effect
GW-5075	c-Raf	Sigma (G6416)	1, 5, 25 µM	No effect
BAY11-7082	IkB-a Inhibitor	Invivogen (tlrl-b82)	1, 2.5, 10 µM	No effect
U0126	MEK1 and MEK2 Inhibitor	Invivogen (tlrl-u0126)	10, 25, 50 µM	No effect
SB203580	p38 MAP Kinase Inhibitor	Invivogen (tlrl-sb20)	1, 2.5, 10 µM	No effect

MCC950	NLRP3-inflammasome inhibitor	Invivogen (inh-mcc)	1, 2.5, 10 µM	No effect
SP600125	JNK Inhibitor	Invivogen (tlrl-sp60)	10, 25, 100 µM	No effect
Z-VAD-FMK	Pan-Caspase Inhibitor	Invivogen (tlrl-vad)	1, 5, 20 µM	No effect
NQDI-1	ASK1 inhibitor	Sigma (SML0185)	1, 10, 100 µM	No effect
ISRIB	eIF2a phosphorylation inhibitor	Sigma (SML0843)	1, 10, 100 µM	No effect
VX-765	Caspase 1 inhibitor	Invivogen (inh-vx765i)	1, 10, 100 µM	No effect
Dexamethasone	NF-kB and MAPK inhibitor	Invivogen (tlrl-dex)	10, 100, 1000 nM	No effect
Chloroquine	inhibitor of endosomal acidification	Invivogen (tlrl-chq)	1, 10, 100 µM	No effect
Amlexanox	TBK1/IKKε inhibitor	Invivogen (inh-amx)	1, 10, 100 ug/mL	No effect
BX795	TBK1/IKKε inhibitor	Invivogen (tlrl-bx7)	0.5, 1, 2 µM	No effect
NG25 trihydrochloride	TAK1 & LYN, MAP4K2 and Abl inhibitor	Sigma (SML1332)	50, 100, 500, 1000 nM	No effect
5Z-7-Oxozeaenol	TAK1 & MAP4K2 inhibitor	Sigma (O9890)	50, 100, 500, 1000 nM	No effect
C16	PKR inhibitor	Sigma (I9785)	1 µM	No effect
2AP	PKR inhibitor	Invivogen (tlrl-apr)	5 µM	No effect

2.7 Materials and Methods

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Plasmids. The plasmids used here were either previously described or generated using standard cloning methods(Pertel et al., 2011). The full list of plasmids used here, along with their purpose and characteristics, is provided in Supplementary Table 1. All plasmid DNAs with complete nucleotide sequence files are available at www.addgene.com.

Cell culture. Cells were cultured at 37°C in 5% CO_2 humidified incubators and monitored for mycoplasma contamination using the Lonza Mycoplasma Detection kit by Lonza (LT07-318). HEK293 cells (ATCC) were used for viral production and were maintained in DMEM supplemented with 10% FBS, 20 mM L-glutamine (ThermoFisher), 25 mM HEPES pH 7.2 (SigmaAldrich), 1 mM sodium pyruvate (ThermoFisher), and 1x MEM non-essential amino acids (ThermoFisher). Cytokine conditioned media was produced from HEK293 cells stably transduced with pAIP-hGMCSF-co (Addgene #74168), pAIP-hIL4-co

(Addgene #74169), or pAIP-hIL2 (Addgene #90513), as previously

described(Pertel et al., 2011).

Leukopaks were obtained from anonymous, healthy, blood bank donors (New

York Biologics). As per NIH guidelines

(http://grants.nih.gov/grants/policy/hs/faqs_aps_definitions.htm), experiments

with these cells were declared non-human subjects research by the UMMS IRB.

PBMCs were isolated from leukopaks by gradient centrifugation on Histopaque-

1077 (Sigma-Aldrich). CD14+ mononuclear cells were isolated via positive

selection using anti-CD14 antibody microbeads (Miltenyi). Enrichment for CD14+

cells was routinely >98%.

To generate DCs or macrophages, CD14+ cells were plated at a density of 1 to 2

x 10^6 cells/ml in RPMI-1640 supplemented with 5% heat inactivated human AB+

serum (Omega Scientific, Tarzana, CA), 20 mM L-glutamine, 25 mM HEPES pH

7.2, 1 mM sodium pyruvate, and 1x MEM non-essential amino acids (RPMI-HS

complete) in the presence of cytokines that promote differentiation. DCs were

generated by culturing monocytes for 6 days in the presence of 1:100 cytokine-

conditioned media containing human GM-CSF and human IL-4. DC preparations

were consistently >99% DC-SIGN^high, CD11c^high, and CD14^low by flow cytometry.

Macrophages were generated by culturing for 7 days with GM-CSF conditioned

media in the absence of IL-4, and were routinely >99% CD11b. CD4+ T cells were isolated from PBMCs that had been depleted of CD14+ cells, as above, using anti-CD4 microbeads (Miltenyi), and were >99% CD4+. CD4+ T cells were then cultured in RPMI-1640 supplemented with 10% heat inactivated FBS, 20 mM L-glutamine, 25 mM HEPES pH 7.2, 1 mM sodium pyruvate, 1x MEM non-essential amino acids (RPMI-FBS complete), and 1:2000 IL-2 conditioned media. Cells from particular donors were excluded from experiments if percent enrichment deviated more than 5% from the numbers mentioned above, or if there was no increase in activation markers in response to control stimuli (LPS, Sendai virus, and wild-type HIV-1-GFP).

HIV-1 vector production. HEK293E cells were seeded at 75% confluency in 6-well plates and transfected with 6.25 uL Transit LT1 lipid reagent (Mirus) in 250 µL Opti-MEM (Gibco) with 2.25 µg total plasmid DNA. 2-part HIV-1 vectors based on HIV-1-GFP (He et al., 1997) and described in detail in Supplementary Table 1 were transfected at a 7:1 ratio in terms of µgs of HIV-1 plasmid DNA to pMD2.G VSV G expression plasmid DNA(Pertel et al., 2011). 3-part lentivectors were produced by transfection of the lentivector genome, psPAX2 GagPol vector, and pMD2.G, at a DNA ratio of 4:3:1. These also include 2- part HIV-1-GFP constructs that are mutated in such a way as to prevent GagPol, Tat, or Rev production. As these would be defective for viral production, psPAX2 was

included in the transfections at the same 4:3:1 ratio. VPX-bearing SIV-VLPs were produced by transfection at a 7:1 plasmid ratio of SIV3+ to pMD2.G(Pertel et al., 2011). 12 hrs after transfection, media was changed to the specific media for the cells that were to be transduced. Viral supernatant was harvested 2 days later, filtered through a 0.45 µm filter, and stored at 4°C.

Virions in the transfection supernatant were quantified by a PCR-based assay for reverse transcriptase activity(Pertel et al., 2011). 5 µl transfection supernatant were lysed in 5 µL 0.25% Triton X-100, 50 mM KCl, 100 mM Tris-HCl pH 7.4, and 0.4 U/µl RNase inhibitor (RiboLock, ThermoFisher). Viral lysate was then diluted 1:100 in a buffer of 5 mM $(NH_4)_2SO_4$, 20 mM KCl, and 20 mM Tris–HCl pH 8.3. 10 µL was then added to a single-step, RT PCR assay with 35 nM MS2 RNA (IDT) as template, 500 nM of each primer (5'-TCCTGCTCAACTTCCTGTCGAG-3' and 5'-CACAGGTCAAACCTCCTAGGAATG-3'), and hot-start Taq (Promega) in a buffer of 20 mM Tris-Cl pH 8.3, 5 mM $(NH_4)_2SO_4$, 20 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml BSA, 1/20,000 SYBR Green I (Invitrogen), and 200 µM dNTPs. The RT-PCR reaction was carried out in a Biorad CFX96 cycler with the following parameters: 42°C 20 min, 95°C 2 min, and 40 cycles [95°C for 5 s, 60°C 5 s, 72°C for 15 s and acquisition at 80°C for 5 s]. 2 part vectors typically yielded 10^7 RT units/µL, and 3 part vector transfections yielded 10^6 RT units/µL.

Transductions. 10^6 DCs/mL, or 5 x 10^5 macrophages/ml, were plated into RPMI-HS complete with Vpx$^+$ SIV-VLP transfection supernatant added at a dilution of 1:6. After 2 hrs, 10^8 RT units of viral vector was added. In some cases, drugs were added to the culture media as specified in Supplementary Table 2. In most cases, transduced DC were harvested for analysis 3 days following challenge. For gene knockdown or for expression of factors *in trans*, 2 x 10^6 CD14$^+$ monocytes/mL were transduced directly following magnetic bead isolation with 1:6 volume of SIV-VLPs and 1:6 volume of vector. When drug selection was required, 4 µg/mL puromycin was added 3 days after monocyte transduction and cells were selected for 3 days. SIV-VLPs were re-administered in all cases with HIV-1 or lentivector challenge. For DCs in Tet-HIV-1 experiments, fresh monocytes were SIV-VLP treated and co-transduced with rtTA3 and Tet-HIV-1. DCs were harvested 6 days later and treated with indicated doxycycline concentrations.

For deoxynucleoside-assisted transductions, DCs were plated at 10^6 DCs/mL and treated with 2mM of combined deoxynucleosides for 2 hrs before transduction with HIV-1. Deoxynucleosides were purchased from Sigma-Aldrich (2′deoxyguanosine monohydrate, cat# D0901; thymidine, cat# T1895; 2′deoxyadenosine monohydrate, cat# D8668; 2′deoxycytidine hydrochloride, cat#

D0776). A 100 mM stock solution was prepared by dissolving each of the four

nucleotides at 100 mM in RPMI 1640 by heating the medium at 80°C for 15 min.

CD4$^+$ T cells were stimulated in RPMI-FBS complete with 1:2000 hIL-2

conditioned media and 5 µg/mL PHA-P. After 3 days, T cells were replated at 10^6

cells/mL in RPMI-FBS complete with hIL-2. Cells were transduced with 10^8 RT

units of viral vector per 10^6 cells and assayed 3 days later. T cells were co-

transduced with rtTA3 and Tet-HIV-1 every day for 3 days after PHA stimulation.

Cells were then replated in RPMI-FBS complete with hIL-2. Transduced T cells

were cultured for 9 days with fresh media added at day 5. After 9 days,

doxycycline was added at the indicated concentrations and assayed 3 days later.

Non-HIV-1 Challenge Viruses. Sendai Virus Cantell Strain was purchased from

Charles River Laboratories. Infections were performed with 200 HA units/ml on

DCs for 3 days before assay by flow cytometry.

Spreading Infections. DCs were plated at 10^6 DCs/mL, in RPMI-HS complete

media, with or without Vpx$^+$ SIV-VLP transfection supernatant added at a dilution

of 1:6. After 2 hrs, 10^8 RT units of HEK-293 transfection supernatant of either

NL4-3-GFP-JRFL or NL4-3-GFP-JRCSF was added. Each is a construct of

pNL4-3 in which *env* was replaced from the end of the signal peptide (therefore retaining the NL4-3 signal peptide and Vpu sequence) to the stop codon with either macrophage-tropic JR-FL *env* (GenBank: U63632.1) or T cell-tropic JR-CSF *env* (GenBank: M38429.1). Every 3 days (for a total of 12 days) samples were harvested for detection of viral RT activity in supernatant and flow cytometry assessment.

CD4$^+$ T cells were stimulated in RPMI-FBS complete with 1:2000 hIL-2 conditioned media and 5 µg/mL PHA-P. After 3 days, T cells were replated at 10^6 cells/mL in RPMI-FBS complete with hIL-2 and transduced with 10^7 RT units of NL4-3-GFP with JRFL (mac tropic) or JRCSF (T tropic) env. Cells were harvested every 3 days (for a total of 12 days) and assayed for infectivity and activation via flow cytometry.

Cytokine analysis. Supernatants from DCs were collected 3 days following transduction with HIV-1-GFP or minimal lentivector. Supernatant was spun at 500 x g for 5 mins and filtered through a 0.45 µm filter. Multiplex soluble protein analysis was carried out by Eve Technologies (Calgary, AB, Canada).

qRT-PCR. Total RNA was isolated from 5 x 10^5 DCs using RNeasy Plus Mini (Qiagen) with Turbo DNase (ThermoFisher) treatment between washes. First-strand synthesis used Superscript III Vilo Master mix (Invitrogen) with random hexamers. qPCR was performed in 20 μL using 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 1 μL cDNA, and 1 μL TaqMan Gene Expression Assays (ThermoFisher) specified in Supplementary Table 3. Amplification was on a CFX96 Real Time Thermal Cycler (Bio-Rad) using the following program: 95°C for 10 min [45 cycles of 95°C for 15 s and 60°C for 60 s]. Housekeeping gene OAZ1 was used as control (Pertel et al., 2011).

Flow cytometry. 10^5 cells were surface stained in FACS buffer (PBS, 2% FBS, 0.1% Sodium Azide), using the antibodies in Supplementary Table 4. Cells were then fixed in a 1:4 dilution of BD Fixation Buffer and assayed on a BD C6 Accuri. BD Biosciences Fixation and Permeabilization buffers were utilized for intracellular staining. Data was analyzed in FlowJo.

Sampling. All individual experiments were performed with biological duplicates, using cells isolated from two different blood donors. Flow cytometry plots in the figures show representative data taken from experiments performed with cells from the number of donors indicated in the figure legends.

Statistical Analysis. Experimental n values and information regarding specific statistical tests can be found in the figure legends. The mean fluorescence intensity for all live cells analyzed under a given condition was calculated as fold-change to negative control/mock. The exception to this methodology was in Figures 1g and 4c where the percent infected cells was too low to use MFI for the bulk population; in these cases MFI was determined for the subset of cells within the GFP+ gate. Significance of flow cytometry data was determined via one-way ANOVA. A Dunnett's post-test for multiple comparisons was applied, where MFI fold change was compared to either mock treatment or positive treatment depending on the experimental question. qRT-PCR and luminex data was analyzed via two-way Anova, with Dunnett's post-test comparing all samples to mock. All ANOVAs were performed using PRISM 7.02 software (GraphPad Software, La Jolla, CA).

Data availability. The plasmids described in Supplementary Table 1, along with their complete nucleotide sequences, are available at www.addgene.com.

CHAPTER III

Conclusion

3.1 Overview and concepts

The effect of HIV-1 infection on different cell types, especially antigen presenting cells such as dendritic cells and macrophages, has been a matter of significant research and debate. DCs are tasked with maintenance of self-tolerance and the surveillance of pathogenic attack. Upon pathogen detection, DCs undergo maturation and present cognate peptides to antigen specific T cells. Competent antiviral responses require that the antigen presenting cells communicate the nature of the threat with specific coreceptor and cytokine signals. In contrast, even when viral replication is well controlled under antiretroviral treatment, T cell responses to HIV-1 infection are well characterized by decreased effector and cytotoxic activity. (Boasso and Shearer, 2008; Day et al., 2006; Khaitan and Unutmaz, 2011; Trautmann et al., 2006). Although chronic systemic inflammation has been suggested to contribute to immune exhaustion, the cause of T cell dysfunction in HIV-1 infection is largely unknown. One hypothesis proposes that the deficient immune response observed in HIV-1 infection is due, at least in part, to the deficient stimulation of HIV-1 specific T cells by DCs that do not communicate an antiviral response.

DCs have been have been shown to resist innate activation following challenge with HIV-1 mainly due to their resistance to infection. Limited CD4 expression inhibits Env mediated viral entry (Chauveau et al., 2017; Granelli-Piperno et al., 2004, 2006; Piguet and Steinman, 2007). Instead, interactions with c-type lectins

like DC-SIGN direct entry into endosomal compartments that drive processing

and presentation of antigen in the absence of innate stimulation and DC

maturation. Notwithstanding the limits to viral entry, the host cytoplasmic

nuclease SAMHD1 provides a block to reverse transcription that further prohibits

productive infection of DCs and other non-dividing cells (Bloch et al., 2014;

Lahouassa et al., 2012; White et al., 2013). Without recognition of the virus, DCs

may communicate a tolerogenic response in HIV-1 antigen specific T cells with

limited antiviral activity (Ahmed et al., 2015; Brown et al., 2010; Day et al., 2006;

Khaitan and Unutmaz, 2011; Moris et al., 2006; Smed-Sörensen and Loré, 2011;

Smed-Sörensen et al., 2004).

3.2 Competing mechanisms

The disagreement and multitude of competing observations and mechanisms

over how antigen presenting cells and T cells can react to HIV-1 infection is both

intriguing and discouraging. Dendritic cells are essential to initiate a potent and

effective antiviral immune response. An understanding of whether and how DCs

detect and communicate HIV-1 infection is crucial to the goal of developing a

protective, prophylactic vaccine. Which makes the lack of consensus all the

more thwarting. Early studies did not report innate detection of HIV-1 infection in

DCs regardless of experimental facilitation via VSV-G pseudotyping or Vpx

addition (Beignon et al., 2005; Fonteneau et al., 2004; Granelli-Piperno et al.,

2004; Smed-Sörensen et al., 2005). However, more recent studies have shown

strong evidence of sensing of HIV-1 infection both with and without artificial

assistance. However, while many conflicting explanations have been offered

over many years, a collectively clearer answer may be possible.

3.3 Detection of HIV-1 cDNA

The recently discovered cytoplasmic DNA sensor, cGAS, has become a

prominent candidate for HIV-1 sensing. Viral cDNA, produced following reverse

transcription, may be open to detection before it enters the nucleus and

undergoes genomic integration. Indeed, studies have reported cGAS mediated

innate activation of both DCs and macrophages.   In most cases, Vpx addition

through the use of SIV viral like particles was used to promote reverse

transcription thereby leading to the production, and assumed accumulation, of

cytoplasmic HIV-1 cDNA. In several cases, this was sufficient by itself to induce

a type 1 interferon response in macrophages and DCs (Chen et al., 2016; Gao et

al., 2013; Wu et al., 2013; Yoh et al., 2015). In other reports, HIV-1 has been

shown to circumvent cGAS detection in DCs and macrophages regardless of Vpx

addition. This restriction to detection of viral cDNA was alleviated with mutations

to the viral capsid to change the affinity for CypA and CPSF6 (Lahaye et al.,

2013; Rasaiyaah et al., 2013). Protected by the viral capsid, reverse

transcription takes place ostensibly protected from cytoplasmic sensors like cGAS until nuclear import and integration.

Detection of HIV-1 cDNA would logically be a process independent of viral integration. It stands to reason that nuclear import and chromosomal integration of viral cDNA would negate its capacity to be detected by cytoplasmic DNA sensors. In seeming conflict with this rationale, CD4+ T cells were reported to induce type 1 interferon following HIV-1 infection in a manner dependent on both cGAS sensing of viral cDNA and proviral integration and expression (Vermeire et al., 2016). Disruption of the cGAS signaling axis abrogated innate activation leading the authors to suggest an elaborate model of HIV-1 detection involving cGAS detection determinants both before and after integration. A mechanism for this detection is unknown and was not described in cells other than T cells (Vermeire et al., 2016).

While these observations appear incongruous, a recent report may provide a degree of corroboration. In partial agreement with the aforementioned study, Vpx assisted HIV-1 infection of DCs was shown to induce maturation involving steps both before and after viral integration (Johnson et al., 2018). Immediately following HIV-1 challenge, a low level interferon response was detected that was unaffected by inhibition of integration. This low level response was found to be mediated by the cGAS signaling pathway. However, robust DC maturation, as evident by a much stronger type 1 interferon response and upregulation of

costimulatory CD86, was only found following integration and expression. This secondary amplification of the antiviral response required both the initial cGAS "priming" response and subsequent activation of NF-κB. While it is unknown by what mechanism HIV-1 transcription is sensed or how NF-κB is recruited, the concept of a multiple sensing mechanisms with determinants both before and after integration may offer an explanation to seemingly conflicting observations.

3.4 Detection of HIV-1 RNA processing

Naked HIV-1 RNA sequences and structures have been shown to be substrates for cytoplasmic RNA receptors like RIG-I (Berg et al., 2012; Solis et al., 2011). The proposed mechanism in these studies argue for the detection of incoming HIV-1 genomic RNA prior to reverse transcription and subsequent RNAse H degradation. However, these studies mainly utilized transfections of genomic HIV-1 RNA or HIV-1 RNA motifs as opposed to genuine HIV-1 challenge. In addition to the questionable relevance of transfected RNAs, this methodology would be unable to distinguish whether *de novo* HIV-1 RNAs, as opposed to incoming virion associated genomic RNA, could be detected. In contrast, one study has described an integration dependent mechanism in which newly transcribed HIV-1 RNA is sensed by the host helicase DDX3 (Gringhuis et al., 2016). Transfection studies in DCs implicated short, abortive HIV-1 RNA products to be determinants for interferon production through the direct binding of

DDX3 and signaling through MAVS. However, it is unknown whether abortive transcription products constitute the determinant for detection in primary viral infection.

Interestingly, DDX3 has also been shown to be a contributing factor for the Rev-CRM1 mediated export of intron containing HIV-1 RNAs (Mahboobi et al., 2015; Valiente-Echeverría et al., 2015; Yedavalli and Jeang, 2011; Yedavalli et al., 2004). In addition, DDX3 acts to promote HIV-1 translation initiation by binding directly to the TAR stem loop structure as a substitute for eIF4E mRNA 5' cap binding (Lai et al., 2013; Liu et al., 2011; Soto-Rifo et al., 2013). As such, it is apparent that DDX3 binds to *de novo* transcribed genomic HIV-1 RNA following integration to both promote its export and its translation.

In two simultaneous studies, DCs, macrophages, and CD4+ T cells were all shown to induce an antiviral response upon HIV-1 challenge (Akiyama et al., 2017; McCauley et al., 2017). Similarly to the preceding studies, this innate activation was dependent on integration and expression of the provirus. However, the authors found no specific viral sequence determinants nor a connection or reliance on cGAS-STING-IRF3 signaling. Innate activation was shown to be entirely dependent on Rev mediated export of unspliced and partially spliced HIV-1 RNA through the CRM1 RNA export pathway. Furthermore, transduction of the virus could be separated from RNA transcription for at least 9 days suggesting that RNA export detection is independent of any

steps prior to integration as well as the process of integration itself. The authors

conclude that, as long as Rev-CRM1 export of the RNA is still maintained, a

provirus need not be intact or replication competent to induce innate activation

(Akiyama et al., 2017; McCauley et al., 2017). The outstanding question is how

HIV-1 unspliced RNA export itself is detected; whether it is by escape of

unspliced RNA, the overt use of the CRM1 pathways itself, binding of cellular

signaling determinants such as RIG-I or DDX3, or some combination thereof.

3.5 Putting the pieces together

Disagreement exists over whether cGAS is responsible for post-integration

signaling or if it is limited to detection of viral DNA before integration. HIV-1

infection of CD4+ T cells has been shown to induce type 1 interferon in a

mechanism dependent on cGAS signaling and on proviral production (Vermeire

et al., 2016). Considering reverse transcribed HIV-1 cDNA would theoretically

only be available to detection prior to nuclear import and chromosomal insertion,

it is difficult to conjecture a mechanism by which cGAS detects viral DNA

following integration. The authors suggest that, rather than incoming viral cDNA,

cGAS might detect released mitochondrial DNA as due to stress from viral

production or viral cDNA from other simultaneous infection events. However, this

mechanism has not been identified nor has it been observed in other cell types.

Other studies have found that HIV-1 integration and subsequent expression are necessary and sufficient to activate DCs without apparent cGAS signaling. Detection of *de novo* transcribed HIV-1 RNA in DCs is a process that inherently occurs subsequently to viral integration. An important exception is that of limited transcription from transient, unintegrated HIV-1 DNA circles (Brussel and Sonigo, 2004; Kelly et al., 2008; Sloan et al., 2010; Thierry et al., 2016; Wu and Marsh, 2003). However, this seems unlikely, as experimental inhibition of integration does not inhibit accumulation of unintegrated DNA, and hence, it's transcription (Munir et al., 2013).

In partial agreement with the aforementioned, post integration sensing of HIV-1 RNA has been shown in two accompanying studies to occur in DCs, macrophages, and CD4+ T cells (Akiyama et al., 2017; McCauley et al., 2017). Innate activation required both proviral integration and expression and was exclusive of the cGAS-STING-IRF3 signaling axis. It was further found that induction of type 1 interferon was contingent on Rev-CRM1 RNA export of intron containing HIV-1 RNA. In addition, this response was separable from HIV-1 transduction by at least nine days suggesting a plenary independence of pre-integration sensing events (McCauley et al., 2017). While it is unresolved to what adaptor or mechanism links HIV-1 incompletely spliced RNA export to induction of type 1 interferon, it remains plausible evidence of post integration, cGAS independent sensing.

As alluded to prior, cellular detection of pre-integrated HIV-1 cDNA by cGAS or of post integration transcription may not be mutually exclusive mechanisms. A recent effort has proposed that both may occur either independently or as multiple steps in a connected mechanism (Johnson et al., 2018). In this study, microarray and ATAC-seq analysis on HIV-1 infection of Vpx treated DCs clearly demonstrate two stages of innate activation. The first manifests as a low level interferon response immediately following infection, unaffected by inhibition of integration. In contrast, a far more potent interferon and maturation response was observed not until two days following HIV-1 challenge. Similarly to the aforementioned studies, this robust secondary response was found to require both integration and transcription of the provirus. Knockdowns in DCs and CRISPR knockouts in THP1 derived DC-like cells suggested that the early, limited interferon response was mediated by cGAS-STING-IRF3 signaling. In addition, this secondary amplification was shown to require both the initial cGAS dependent response and the subsequent activation of NF-κB by an unknown mechanism. These observations led the authors to offer a model in which pre-integration cGAS sensing of HIV-1 cDNA induces a "priming" response that is further amplified by NF-κB activation following HIV-1 transcription.

It would seem likely that this strong, successive interferon induction seen in HIV-1 infected DCs is the same response described in Akiyama et al. and McCauley et al. to sense incompletely spliced HIV-1 RNA export. Both proposed mechanisms are dependent on integration and subsequent transcription of the

provirus. Each was characterized by a potent type 1 interferon response delayed

significantly following transduction with peak response two days post HIV-1

challenge. Nonetheless, knockdowns and drug inhibitors of cGAS-STING-IRF3

signaling failed to abolish interferon induction and DC maturation (Akiyama et al.,

2017; McCauley et al., 2017). This is in contrast to the two stage sensing

hypothesis by Johnson et al., in which cGAS sensing was proposed to "prime" a

pro-antiviral state that is later amplified by viral replication. Interestingly, careful

analysis of my work in the preceding chapter (as well as McCauley et al.) does

indicate that inhibition of integration by integrase mutation or raltegravir did not

fully abolish ISG induction. Indeed, integration deficient samples yielded 100 fold

higher CXCL10 RNA over background (McCauley et al., 2017). However, as

inhibition of integration resulted in 1000 fold less CXCL10 RNA expression and

abrogation of surface CD86 as compared to unhindered HIV-1 infection, this

observation was not further investigated. Nonetheless, it is plausible that the

significantly reduced ISG induction observed upon integration inhibition may

reflect early, cGAS mediated innate activation.

3.6 Outstanding questions and speculations

The theory that best connects and explains the various observations presented is

that of a two stage response to HIV-1 infection of dendritic cells. The initial

response is that of a low level type 1 interferon induction that occurs prior to

integration and is dependent on HIV-1 cDNA detection by cGAS and subsequent signaling through STING and IRF3. The second response is characterized by a far larger induction of interferon and ISGs and is dependent on viral integration, transcription, and Rev-CRM1, HIV-1 RNA export. While this serves to identify the causative factor necessary for innate activation, it still leaves several unanswered questions.

It is also not known whether other dendritic cell subtypes react similarly to monocyte derived dendritic cells and macrophages. Monocyte differentiated cells are, by far, the most common experimental model for dendritic cell and macrophage biology. This is mainly due to the fact that dendritic cells are extremely heterogeneous and exist in fleeting numbers *in vivo*. Isolation and differentiation of CD14+ monocytes limits the variability between donors and greatly increases the total cell yield. As a result, data on the infection of proper dendritic cells both *in vivo* and *ex vivo* is limited (Ahmed et al., 2015; Granelli-Piperno et al., 2006). Interestingly, it has been found that *ex vivo* isolated myeloid and plasmacytoid dendritic cells are even more resistant to HIV-1 transduction than monocyte derived cells (Bloch et al., 2014). It was found that the levels of cellular SAMHD1 were much higher in *ex vivo* myeloid dendritic cells and that Vpx was insufficient to alleviate the block to reverse transcription. However, the term myeloid dendritic cell encompasses multiple cell subtypes more classically referred to as common dendritic cells. Different myeloid dendritic cell subtypes have widely varying functions, interactions, and tissue

locations. As such, the development of effective immunotherapies will necessitate a better understanding of how individual dendritic cell subtypes react to HIV-1 challenge.

As mentioned before, the delayed but robust interferon and maturation response seen in HIV-1 infected DCs may or may not be dependent on early cGAS signaling. In more molecular terms, is the export of unspliced HIV-1 RNA via CRM1 sufficient to induce dendritic cell maturation or is a prior stimulus needed to induce a state of heightened sensitivity? In some cases, suppression of cGAS signaling appears to prevent downstream, integration dependent innate activation (Johnson et al., 2018; Vermeire et al., 2016). In other cases, cGAS sensing appears to be dispensable for a potent immune response (Akiyama et al., 2017; Gringhuis et al., 2016; McCauley et al., 2017; Nasr et al., 2017). It has been suggested that, if a priming response is required, another stimulus may be able to functionally substitute for cGAS detection (Johnson et al., 2018). Furthermore, addition of a secondary stimulus following integration deficient HIV-1 infection was shown to dramatically increase the resultant activation state to a degree similar to that of unhindered infection.

Technical limitations limit experimental design: how does one isolate post-integration sensing events from pre-integration ones? This question was partly addressed through our use of a doxycycline inducible, Tat independent HIV-1 construct (McCauley et al., 2017). By transducing monocytes with Tet-HIV-1

immediately following isolation, the process of reverse transcription and integration occurs during the differentiation of the dendritic cells without immediate transcription. With this technique, viral challenge was separated from transcription initiation by 9 days in dendritic cells and CD4+ T cells with innate activation directly correlating with doxycycline concentration (McCauley et al., 2017). This would suggest that the detection of HIV-1 RNA export is sufficient to induce activation sans prior stimulation from cGAS or otherwise. It is important to note that a prior stimulation may induce permanent epigenetic and transcriptional changes separable by no length of time. However, this would seem unlikely considering that the cells are transduced as monocytes and must undergo large scale epigenetic and transcriptional changes just to differentiate.

The most significant unknown is the mechanism linking CRM1 export of unspliced HIV-1 RNA to innate signaling and interferon activation. Continued genetic experimentation on the HIV-1 genome will likely determine whether or not the sequence of the Env intron is necessary for innate detection. It is possible that the overt or improper use of the CRM1 export pathway sufficient is sufficient to induce activation without direct sequence specificity. Indeed, CRM1 RNA export is a highly specific pathway necessitating the use of secondary proteins to directly bind to RNA and bridge transport (Delaleau and Borden, 2015; Okamura et al., 2015). In HIV-1, this role is fulfilled by the Rev protein. However, CRM1 is primarily a protein transporter for complexes containing canonical nuclear export signals and commonly exports highly diverse cargos. As such, it is difficult to

imagine a mechanism by which use of the CRM1 pathway itself would activate immune signaling.

A more likely scenario is one where HIV-1 RNA acts as the direct determinant for the innate sensing. In this scenario, the genomic HIV-1 RNA, inclusive of the Env intron, is the substrate for direct or indirect innate detection. It is possible that interactions occur directly with a known signaling adapter or recognition receptor; however, this reasoning would not serve to explain the necessity for CRM1 export. Another possibility is that sequence specific interactions with HIV-1 RNA co-export proteins not normally found outside the nucleus. Perhaps these are proteins that are regularly removed from RNAs during NXF1/TAP mRNA export but are retained when exported via CRM1. Once in the cytoplasm, these mislocalized proteins might be recognized as a danger signal leading to interferon activation.

The HIV-1 RNA is itself known to associate with a diverse array of proteins (Knoener et al., 2017; Kula et al., 2011; Marchand et al., 2011; Milev et al., 2012; Naji et al., 2012). Moreover, intron containing HIV-1 RNA is known to associate with many unique proteins not found with its accompanying spliced RNAs (Kula et al., 2011; Naji et al., 2012). These include RNA helicases, splicing regulators, HNRNPs, and DNA damage associated proteins among many others. Of note, Rev exported HIV-1 RNA binds multiple RNA helicases of the DEAD/H box protein family including DDX1, DDX3, DDX5, DDX7, DHX9, DDX17, DDX21, and

DDX24. Furthermore, DDX1 (Edgcomb et al., 2012; Fang et al., 2004), DDX3

(Yedavalli et al., 2004), DDX5 (Zhou et al., 2013), DHX9 (Bolinger et al., 2010)

DDX17 (Lorgeoux et al., 2013; Sithole et al., 2018), and DDX24 (Ma et al., 2008)

have all been shown to have widely varying effects on HIV-1 replication; some

positive and some negative. Many of these helicases also have known functions

in various innate processes such as direct detection of nucleic acids, adapters for

signaling, and regulation of viral replication (Cheng et al., 2018; Fullam and

Schröder, 2013; Ma et al., 2013; Rahman et al., 2017; Sekiba et al., 2018; Shan

et al., 2012; Valiente-Echeverría et al., 2015; Xu et al., 2010; Zhang et al., 2011).

In fact, DDX3 has been implicated in the direct sensing of transcribed HIV-1 RNA

in an integration dependent manner (Gringhuis et al., 2016). Although the

authors implicate abortive, Tat independent transcription, as opposed to CMR1

exported RNA, as the determinants for activation, it still serves to illustrate the

potential for DDX mediated detection. As such, it is not difficult to imagine that

an RNA helicase could act as a direct recognition receptor for HIV-1 RNA. This

hypothetical binding factor would then escape the nucleus by co-transporting with

the unspliced HIV-1 genome via CRM1 and induce innate activation once

recognized in the cytoplasm.

3.7 Experimental considerations

While the focus of this text has been on innate detection of HIV-1 in dendritic cells, the research cited and discussed was performed in a variety of cell types including whole PBMCs, CD4+ T cells, and macrophages, as well as numerous cell lines. It serves to reason that any number of observations made may not be relevant outside the cited system. It very well may be that different cell types react differently to HIV-1 infection. Indeed, plasmacytoid DCs have been shown, without experimental assistance, to readily sense HIV-1 through detection of incoming viral RNA via TLR7 (Beignon et al., 2005; Fonteneau et al., 2004; Haupt et al., 2008; Lepelley et al., 2011; Smed-Sörensen et al., 2005). However, the lack of TLR7 (and downstream IRF7) expression in myeloid, or common, dendritic cells prevents this route of innate activation (Bloch et al., 2014; Izaguirre et al., 2003; Jarrossay et al., 2001; Wacleche et al., 2018). In addition, even when observations are made in the same or similar cell types, eg. DCs and macrophages, differences in cell culturing, viral production, and assay conditions can drastically change the experimental outcome.

Difficulties determining the dependence for cGAS detection of HIV-1 infection in DCs may also stem from differences in reagent and assay conditions. In most studies, HIV-1 virus is produced via transfection of 293 cells and is harvested days later as virally enriched supernatant. In addition to virus, this media may also contain stimulating byproducts of cellular metabolism as well as leftover

transfection reagents and DNA. HIV-1 preparations themselves are amalgamations of functional viral particles mixed with other products contained within cell treated supernatants. Even purportedly purified viral particles are either precipitated out of solution with other exosomes or centrifuged on a sucrose gradient that would not exclude similarly dense vesicles.

Dendritic cells are a highly heterogeneous cell type with numerous subtypes with classifications dependent on *in vivo* localization, marker expression, and T cell presentation capacity (Kaisho, 2012; Liu, 2001; Palucka and Banchereau, 2013; Piguet and Blauvelt, 2002; Piguet and Steinman, 2007; Schreibelt et al., 2010; See et al., 2017; Villani et al., 2017; Wacleche et al., 2018). Furthermore, the majority of dendritic cell reports utilize monocyte derived dendritic cells (mdDC) as these can be produced reliably and in larger quantities than DCs isolated *ex vivo*. These are a cell type differentiated from monocytes *in vitro* by culturing in the presence of GM-CSF and IL4. Originally considered to be a culturing oddity with questionable disease relevance, mdDCs have been shown to be a true DC subset that differentiate *in vivo* during inflammation (León and Ardavín, 2008; León et al., 2007; Nakano et al., 2009; Wacleche et al., 2018). The specific culturing conditions can dramatically alter the phenotype of DCs and macrophages differentiated from monocytes *in vitro* (Qu et al., 2014; Safi et al., 2016; Wacleche et al., 2018). For example, during differentiation of macrophages from monocytes, the use of fetal bovine serum induces a transition to a G1 state as compared to a G0 state from when human serum is used.

Moreover, this G1 state induces the phosphorylation of SAMHD1 thereby reducing its capacity to restrict HIV-1 reverse transcription (Mlcochova et al., 2017; Moldenhauer et al., 2003). This greatly increases the permissivity to HIV-1 infection without the use of Vpx protein to degrade SAMHD1, which, as discussed, may or may not be a limiting factor in cGAS detection of reverse transcribed HIV-1 cDNA.

Dendritic cells are immune cells possessing a complex collection of sensors specific to a diverse array of host and pathogenic molecules. As such, they are highly sensitive to differences in experimental conditions and will alter their behavior in unknown ways. It is entirely possible that differences in culturing conditions can cause monocytes to differentiate along subtly disparate DC lineages that react opposingly to HIV-1 infection. Indeed, unpublished observations indicate that mdDCs differentiated in the presence of fetal bovine serum express a different surface marker profile than those in human serum. FBS derived mdDCs express a profile of CD1a+, CD1c++, CD11c+, CD14-, DC-SIGN+, as well as HLA-DR++, CD80+, CD83++, and CD86+ upon stimulation. Human serum derived mdDCs express a similar repertoire with minor differences including: CD1a+/-, CD1c++, CD11c+, CD14+, DC-SIGN+ and, upon stimulation, express HLA-DR+, CD80+, CD83+, and CD86++. Interestingly, recent studies of single cell RNA sequencing on *ex vivo* isolated monocytes and DCs report similar expression profiles in CD1c+ DC subsets (See et al., 2017; Villani et al., 2017). Villani et al describes two, closely related, but distinct, DC subsets in the

classical CD1c+ DC lineage. Comparison of expression profiles would suggest that FBS derived mdDCs maintain similarity to "CD1c_A" DCs whereas human serum derived DCs appear closer to "CD1c_B" DCs. Both DC subsets were shown to be potent stimulators of naive T cell expansion and to react similarly to LPS, poly(I:C), and R848 (Villani et al., 2017).

It is entirely possible that different DC subsets, regardless of similarity, may react opposingly to HIV-1 infection and could account for disparities observed in separate studies. Johnson et al. described a "priming" response potentiated by cGAS detection of pre-integrated HIV-1 cDNA that led to a chromatin state permissive to downstream, post integration DC maturation. Perhaps the necessity for prior epigenetic priming is dissimilar between different DC subtypes or other cell types like macrophages and T cells. A better understanding of the functional differences of *in vivo* DC lineages will be essential to determining exactly how HIV-1 interacts with the innate immune system on a functional level. Furthermore, advances in genetic and expression manipulation will allow for the elucidation of the dependence of particular sensors and signaling pathways to the HIV-1 response. From there, it may be possible to manipulate the response to induce a protective and permanent anti-HIV-1 immune response.

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