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HIV-1 cell-to-cell transfer and dissemination in myeloid cells

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HIV-1 cell-to-cell transfer and dissemination in myeloid cells

Par Maorong XIE

Thèse de doctorat de **Infectiologie et Microbiologie**

Dirigée par **Dr. Serge BENICHOU**

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Abstract

Dendritic cells (DCs), macrophages as well as osteoclasts (OCs) are emerging as target cells of HIV-1 involved in i) sexual transmission, ii) virus dissemination in different tissues, and iii) establishment of persistent virus reservoirs. While the mechanisms of virus cell-to-cell transmission toward these myeloid cells remain poorly understood, we recently reported a novel very efficient mechanism for virus transfer and dissemination in macrophages through a two-step cell fusion process, leading to the formation of long-lived and highly HIV-1-productive multinucleated giant cells (MGCs). In the work presented here, we show that HIV-1 uses similar cell-fusion mechanisms for virus transfer from infected T lymphocytes to macrophages, OCs and immature DCs (iDCs), and subsequent dissemination in these target cells. The establishment of contacts with infected T cells leads to heterotypic cell fusion for the fast and massive transfer of viral material in macrophage, OC and iDC targets. This process subsequently triggers homotypic fusion with non-infected neighboring macrophages (or OCs, or iDCs) for intercellular virus dissemination. Both cell fusion steps are mediated by viral envelope-receptor interactions, and are highly efficient for macrophage-tropic CCR5- and CXCR4-using viruses, dual-tropic R5X4 viruses, and to a lesser extent for non-macrophage-tropic R5 viruses, including transmitted/founder viruses. Interestingly, these cell-to-cell fusion processes result in the formation of infected MGCs able to produce fully infectious virus particles. Together, our results reveal original mechanisms for viral transfer and dissemination in HIV-1 myeloid cell targets and for the formation of the MGCs observed *in vivo* in lymphoid and non-lymphoid tissues of HIV-1-infected patients. Thus far, numerous studies on HIV-1 co-receptor usage and cellular tropism have been performed using cell-free virus particles only, however, our data strongly indicate that the relationship between co-receptor usage and cellular tropism should be revisited in the context of HIV-1 cell-to-cell transfer. They contribute to a better understanding of the cellular processes involved in virus transmission, dissemination, and formation of viral reservoir during HIV-1 infection.

Keywords: HIV-1 cell-to-cell transfer, cell fusion, CD4+ T cells, myeloid cells

Résumé

Les cellules dendritiques (DCs), les macrophages ainsi que les ostéoclastes (OCs) représentent des cellules cibles importantes du VIH-1 impliquées dans i) la transmission sexuelle, ii) la dissémination du virus dans différents tissus, et iii) la mise en place de réservoirs tissulaires persistants de virus. Alors que les mécanismes de transmission de cellule à cellule du virus vers ces cellules myéloïdes restaient mal compris, nous avons récemment révélé un nouveau mécanisme très efficace pour le transfert et la dissémination intercellulaire du VIH-1 dans les macrophages par le biais d'un processus de fusion cellulaire en deux étapes, conduisant à la formation de cellules géantes multinucléées (MGC) hautement productives de virus. Dans le travail présenté ici, nous montrons également que le VIH-1 utilise des mécanismes de fusion cellulaire similaires pour le transfert de virus de lymphocytes T infectés vers des OCs et des DCs immatures (iDC), puis leur dissémination dans ces cellules cibles. L'établissement de contacts avec des cellules T infectées conduit à la fusion hétérotypique des cellules T infectées avec les cibles macrophages, OCs et iDCs permettant le transfert rapide et massif de matériel viral. Ce processus déclenche ensuite une seconde étape de fusion homotypique avec des macrophages (ou OCs, ou iDCs) voisins non infectés pour la dissémination intercellulaire du virus dans ces cellules cibles myéloïdes. Les deux étapes de la fusion cellulaire sont médiées par des interactions enveloppe virale-récepteur et sont très efficaces pour les virus utilisant CCR5 et CXCR4 à tropisme pour les macrophages, les virus à double tropisme pouvant utiliser CCR5 ou CXCR4 et, dans une moindre mesure, pour les virus non-tropique pour les macrophages utilisant CCR5, comme les virus transmis/fondateurs. Il est intéressant de noter que ces processus de fusion de cellule à cellule aboutissent à la formation de MGCs infectés capables de produire des particules virales totalement infectieuses. L'ensemble de nos résultats révèlent des mécanismes originaux pour le transfert et la dissémination viraux dans les cellules cibles myéloïdes du VIH-1 permettant la formation des MGC observés *in vivo* dans les tissus lymphoïdes et non lymphoïdes de patients infectés par le VIH-1. Ils permettent également de revisiter les notions de tropisme cellulaires et d'utilisation des

souches virales qui avaient jusqu'à présent été étudiés uniquement dans le cadre d'infections par des virus libres. Ces résultats contribuent à une meilleure compréhension des processus cellulaires impliqués dans la transmission, la dissémination et la formation d'un réservoir viral lors de l'infection par le VIH-1.

Mots clés : Transfert de cellule à cellule du VIH-1, fusion cellulaire, cellules T CD4+, cellules myéloïdes

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Abbreviations

AGM: African green monkey
AIDS: Acquired immunodeficiency syndrome
ALCAM: Activated leukocyte cell adhesion molecule
APC: Antigen presenting cell
ART: Anti-retroviral therapy
APOBEC: Apolipoprotein BmRNA-editing catalytic polyprotein
AZT: Azidothymidine
BBB: Blood-brain barrier
BCCA3: Blood DC antigen 3
bNAbs: Broadly neutralizing antibodies
BST2: Bone marrow stromal antigen 2
CCR5: C-C Chemokine receptor 5
CD4: Cluster of differentiation 4
cDC1: Conventional DC1
cDC2: Conventional DC2
CDP: Common DC progenitor
CXCR4: C-X-C motif chemokine receptor 4
CXCR6: C-X-C motif chemokine receptor 6
CMP: Common myeloid progenitor
CNS: Central nervous system
CRFs: Circulating recombinant forms
DC: Dendritic cell
DAP12: DNAX activation protein of 12kb
DC-SIGN: Dendritic cell-specific intracellular adhesion molecule grabbing non-integrin
DC-STAMP: Dendrocyte expressed seven transmembrane protein
dNTP: Deoxynucleoside triphosphate
DNA: Deoxyribonucleoside acid
ECLs: Extracellular loops
ELISA: Enzyme-linked immunosorbent assay
EM: Electron microscopy
Env: Envelope glycoprotein
ERM: Ezrin/radixin/moesin
FBGCs: Foreign body giant cells
GM-CSF: Granulocyte macrophage colony-stimulation factor
GMP: Granulocyte and macrophage progenitor
GPCR: G-protein-coupled receptor
HAART: Highly active retroviral therapy
HIV: Human immunodeficiency virus
HIV-1: Human immunodeficiency virus type 1
HIV-2: Human immunodeficiency virus type 2

HSC: Haematopoietic stem cell
HTLV-I: Human T cell leukemia virus-type 1
iDCs: Immature DCs
ICAM-1: Intercellular adhesion molecule 1
ICAM-3: Intercellular adhesion molecule 3
IFITM: Interferon-induced transmembrane protein
IFN: Interferon
IFN- λ : Type III interferon
ILVs: Intraluminal vesicles
IS: Infectious synapse
JAM-A: Junctional adhesion molecule-A
JXG: Juvenile xanthogranuloma
LCs: Langerhans cells
LDL: Low-density lipoprotein
LGCs: Langerhans giant cells
LFA-1: Leukocyte function-associated antigen 1
LNs: Lymph nodes
LPS: Lipopolysaccharide
MA: Matrix protein
MDDCs: Monocyte-derived dendritic cells
MDP: Macrophage and DC progenitor
MECs: Mammary epithelial cells
MGCs: Multinucleated giant cells
MLV: Murine leukemia virus
MPS: Mononuclear phagocyte system
MRC1: Mannose receptor C type 1
MSM: Men who have sex with men
MV: Measles virus
MVBs: Multivesicular bodies
MVC: Maraviroc
NC: Nucleocapsid
OCs: Osteoclasts
PBLs: Peripheral blood lymphocytes
PDB: Paget's disease of bone
pDCs: Plasmacytoid DCs
PIC: Pre-integration complex
RANKL: Receptor activation of NF- κ B ligand
RSV: Respiratory syncytial virus
RT: Reverse transcriptase
RTIs: Reverse-transcriptase inhibitors
Siglec-1/CD169: Sialic acid-binding Ig-like lectin 1
SIV: Simian immunodeficiency virus
SLE: Systemic lupus erythematosus
SAMHD1: Sterile alpha motif and HD domain-containing protein- 1

SIRP α : Signal regulated protein alpha
SMM: Sooty mangabey monkeys
STAT6: Signal transducer and activator of transcription 6
TCLA: T-cell line-adapted
TGCs: Touton giant cells
TNTs: Tunnelling nanotubes
Tcm: Central memory CD4+ T cells
Tem: Effector memory CD4+ T cells
TGM2: Transglutaminase 2
Tn: Naïve CD4+ T cells
TNF: Tumor-necrosis factor
TRIM5 α : Tripartite motif-5 α
Tat: Trans-activator
TCR: T-cell receptor
VCC: Virus containing compartment
Vif: Viral infectivity factor
VN: Vitronectin
Vpr: Viral protein R
Vpu : Viral protein U
VS : Virological synapse
URFs: Unique recombinant forms

INTRODUCTION

Chapter 1. The human immunodeficiency virus

Since the beginning of the pandemic, the human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS), caused more than 75 million infections and approximately 32 million people have died from AIDS-related diseases. There are now approximately 37.9 million individuals living with HIV worldwide. In 2018, it has been estimated that around 1.7 million individuals were newly infected with HIV, and the heterosexual transmission remains the predominant mode of spreading of HIV-1 infection and accounts for approximately 85% of all HIV-1 infections (De Cock et al., 2012; Pape et al., 1985). HIV infection continues to be one of the major global public health issue, and AIDS is one of the leading causes of mortality and morbidity worldwide.

1.1 HIV subtype diversity

The two types of HIV (HIV-1 and HIV-2) probably arise from different cross-species transmission events from African monkeys (Keele et al., 2006) (Figure 1). The HIV-1 pandemic has been phylogenetically divided into several distinctive groups, including group M (for main), group O (for outlier), group N (for non-M/non-O) and group P, each of which has been shown to results from a separate cross-species transmission event (Aldrich and Hemelaar, 2012; Charneau et al., 1994; Plantier et al., 2009; Robertson et al., 2000; Simon et al., 1998). The HIV-1 group M (main) represents the predominant circulating HIV-1 strains found worldwide. This group has been then further divided into genetically distinct clades (or subtypes), including clades A, B, C, D, F, G, H, J, and K. Within some clades, high genetic variation leads to further classification into sub-subtypes (Robertson et al., 2000). For example, clade F has been subdivided into clade F1 and clade F2 (Lihana et al., 2012). In terms of viral diversity, HIV-1 clade C continues to be the major subtype of HIV-1 and accounts for approximately 50% of infections worldwide (Hemelaar et al., 2006; Thomson and Najera, 2005). Moreover, the recombination between distinct HIV-1 clades has generated numerous unique recombinant forms (URFs), some of which have disseminated in the population and developed into circulating recombinant forms

(CRFs). The HIV-1 group M and group N are closely related and have been shown to originate from independent transmission events of simian immunodeficiency virus (SIV) from chimpanzees of the subspecies *Pan troglodytes troglodytes* (SIVcpz) into humans in West-Central Africa (Gao et al., 1999; Keele et al., 2006). HIV-1 group N has been found in a small number of infected individuals in Cameroon. It has been estimated that HIV-1 group O caused more than ten thousands of infections in West-Central Africa (Bush and Tebit, 2015; Hemelaar, 2012). So far, HIV-1 group P has only been identified in two infected individuals originating from Cameroon (Plantier et al., 2009; Vallari et al., 2011). It has been indicated that HIV-1 group P is closely related to SIV from gorillas (SIVgor), and probably results from gorilla-to-human transmission (Plantier et al., 2009).

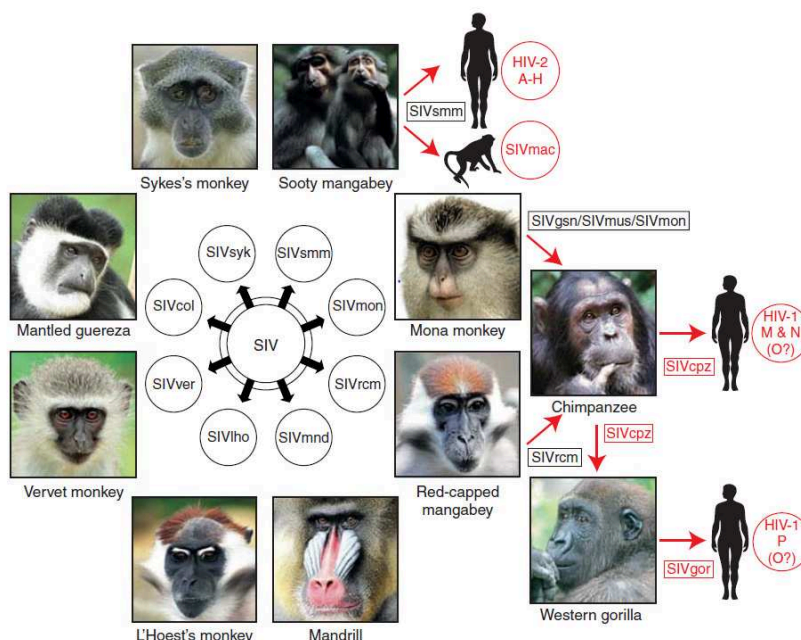


Figure 1: Origins of human AIDS viruses.

Old World monkeys are naturally infected simian immunodeficiency viruses (SIVs) with a suffix to indicate their primate species of origin (e.g., SIVagm from African green monkey). Several SIVs crossed the species barrier to apes and humans, generating the human AIDS viruses. Adapted from Sharp et al., 2011.

1.2 HIV transmission and the course of infection

Heterosexual transmission of HIV-1 is the predominant mode of spreading of HIV-1 infection and is responsible for approximately 85% of all HIV-1 infections worldwide (De Cock et al., 2012; Pape et al., 1985). Less frequent routes for HIV-1 transmission

include exposure of blood via transfusions, maternal-infant transmission, transmission via drug injection and men who have sex with men (MSM) (Beyrer et al., 2012).

To establish new infection in a naïve host, either cell-free or cell-associated HIV particles have to interact with epithelial cells and cross the mucosal barrier through several pathways, including transcytosis, productive infection, or by endocytosis and subsequent exocytosis (Hladik and McElrath, 2008). Interestingly, transcytosis of cell-associated viruses secreted from HIV-1 infected seminal leukocytes seems to be much more efficient than cell-free virions (Alfsen et al., 2005; Bomsel, 1997; Van Herrewege et al., 2007). It has been reported that HIV-1 could also productively infect cervical epithelial cells (Phillips et al., 1998; Trujillo et al., 2000), although this observation remains controversial (Dezzutti et al., 2001; Greenhead et al., 2000; Ray et al., 1998). Regardless of the mode, HIV-1 penetration through the cervicovaginal epithelium occurs within 30-60 minutes after exposure (Hu et al., 2000). Compelling evidences from non-human primate models and human tissue explant models indicate that vaginal transmission leads to infection of small numbers of CD4⁺ T cells, Langerhans cells, dendritic cells, and tissue macrophages located in the lamina propria (Haase, 2005, 2010; Hladik and McElrath, 2008; Pope and Haase, 2003). Langerhans cells are permissive for HIV-1 infection and are able to traffic to the submucosa. The submucosa of cervix and vagina contain dendritic cells, which are capable of capturing infectious viral particles for subsequent transmission to CD4⁺ T cells (Boggiano and Littman, 2007; Boggiano et al., 2007; Hladik et al., 2007). Most Transmitted/Founder (T/F) viruses can replicate efficiently in CD4⁺ T cells but poorly infect macrophages, raising the question about the role of tissue macrophages in HIV-1 transmission (Salazar-Gonzalez et al., 2009).

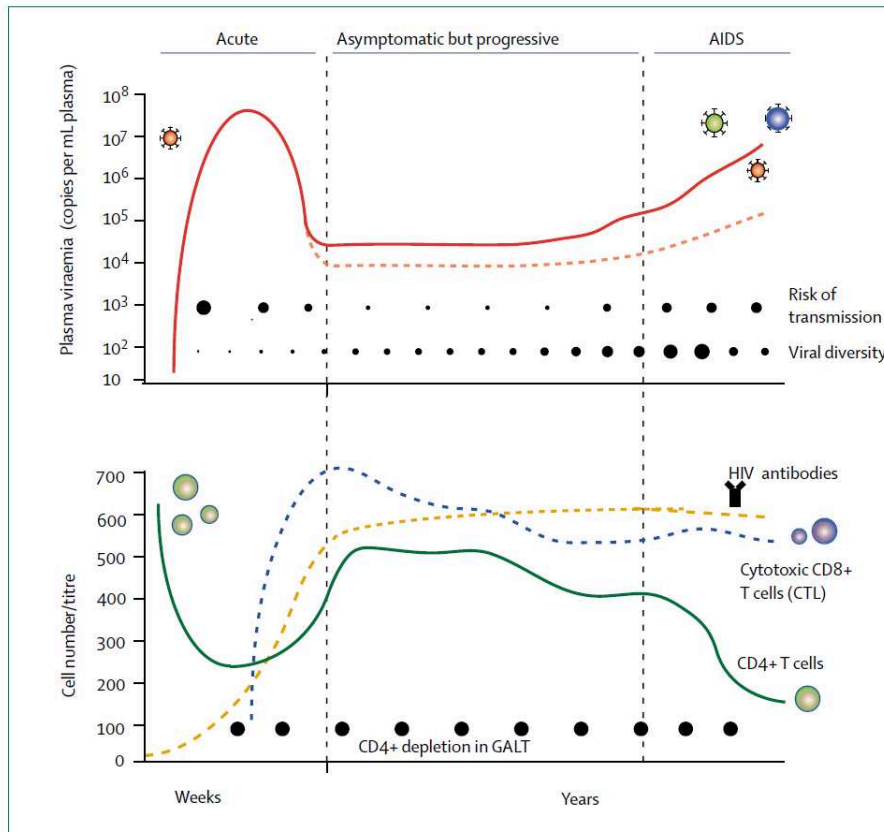


Figure 2: The course of HIV-1 infection.

Plasma viraemia (top), and the dynamic changes of T-lymphocyte compartments (bottom). Primary infection of HIV is characterised by the high plasma viraemia (red line, top), low CD4 cells (green line, bottom), as well as absence of HIV-1 specific antibodies (orange line, bottom). Viraemia drops when cytotoxic CD8+ T-lymphocytes develop (blue line, bottom), and viral-load set point is reached during the chronic infection. Viral diversity increases throughout the disease progression (closed circles, top). Adapted from Simon et al., 2006.

The initial viral replication is composed of few HIV-1 variants and occurs in the draining lymph nodes. With the migration of infected CD4+ T cells or cell-free viral particles into the bloodstream, HIV-1 replication is boosted in the spleen, gastrointestinal tract and bone marrow, leading to massive infection of susceptible cells.

Approximately 2 to 4 weeks following HIV-1 transmission, most of the HIV-1 infected patients would experience an acute HIV syndrome (Figure 2). This acute syndrome is characterized by high plasma viraemia and decrease of blood CD4+ T cells due to massive viral replication. As the anti-HIV-1 specific immune response developed, the plasma viraemia drops and reaches an individual viral-load set point. A progressive depletion of activated and memory CD4+ T cells in the gut-associated lymphoid tissues (GALT) could be identified early after infection and accompanied disease progression (Mehandru et al., 2004; Simon et al., 2006).

In the chronic phase of HIV-1 infection, viral replication continues in the lymph nodes, while the level of plasma viraemia differs from the peak of the acute phase by one or two orders of magnitude. This reduction of plasma viraemia is largely attributed to the development of HIV-1 specific antibodies and cytotoxic CD8⁺ T cells response.

HIV-1 replication is dynamic and viral diversity increases *in vivo* throughout the infection. The gradual destruction of both naïve and memory CD4 T⁺ cells is one of the hallmark of HIV-1 infection, with the number of blood CD4⁺ T cells below 200 cells/mm³, and AIDS developed in the last infection stage, when the loss of cytotoxic CD8⁺ T cells response is also observed whereas the plasma viral load increases rapidly (Simon et al., 2006; Swanstrom and Coffin, 2012). Then, severe immunodeficiency results in opportunistic infections such as tuberculosis, neurological diseases, pneumonia, and cancer, leading to the death of HIV-1infected patients (Deeks et al., 2015; Simon et al., 2006).

1.3 Genomic organization of HIV

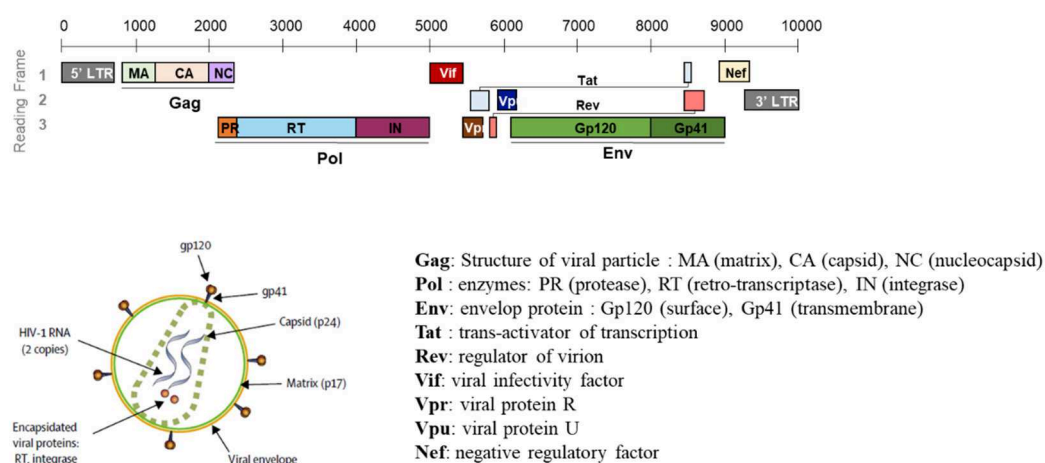


Figure 3: Genomic organization of HIV.

The HIV-1 genome encodes three structural genes (Gag, Pol and Env). In addition, HIV-1 genome also encodes for the regulatory and auxiliary proteins (Rev, Tat, Vif, Vpr, Vpu and Nef). Adapted from Simon et al., 2006.

HIV is an enveloped retrovirus that can integrate viral DNA into host genome and belongs to the lentivirus genus of Retroviridae family. The genome of HIV encodes three major genes that have been found in all retroviruses, env, pol and gag, encoding

for the viral envelope glycoprotein, the viral enzyme and the structural protein of viral core and matrix, respectively (Figure 3). These proteins are initially synthesized as polyprotein precursors and subsequently cleaved into mature, particle-associated proteins. The 55-kD Gag precursor is cleaved by HIV protease into the capsid (CA/p24) which encompasses the viral RNA and enzymes, nucleocapsid (NC/p7) and matrix (MA /p17) which resides at the core of virus and bound to viral RNA. Other products of Gag precursor include two spacer peptides SP1 and SP2, as well as p6 which is essential for viral budding. The envelope glycoprotein (Env) is produced as a polyprotein gp160, which can be cleaved by the cellular protease into surface gp120 and transmembrane gp41. The pol protein is initially produced as the gag-pol polyprotein due to the overlapping of the *pol* and *gag* open reading frames. The Pol protein could be cleaved by the HIV protease to give rise to the heterodimeric RT, the protease (PR), and integrase (IN) proteins. The HIV genome also contains nonstructural genes encoding for regulatory proteins (Rev and Tat) and auxiliary proteins (Vpr, Vpu, Vif and Nef). The Rev and Tat regulatory proteins are essential for the replication of HIV in all cells, which serve to induce the transcription of proviral DNA and also to transport viral RNA transcripts from nucleus to the cytoplasm, respectively. While the accessory proteins, including Vif, Vpu, Nef, Vpx and Vpr, were found to be necessary for HIV to replicate in some cell types.

1.4 The HIV life cycle and host restriction factors

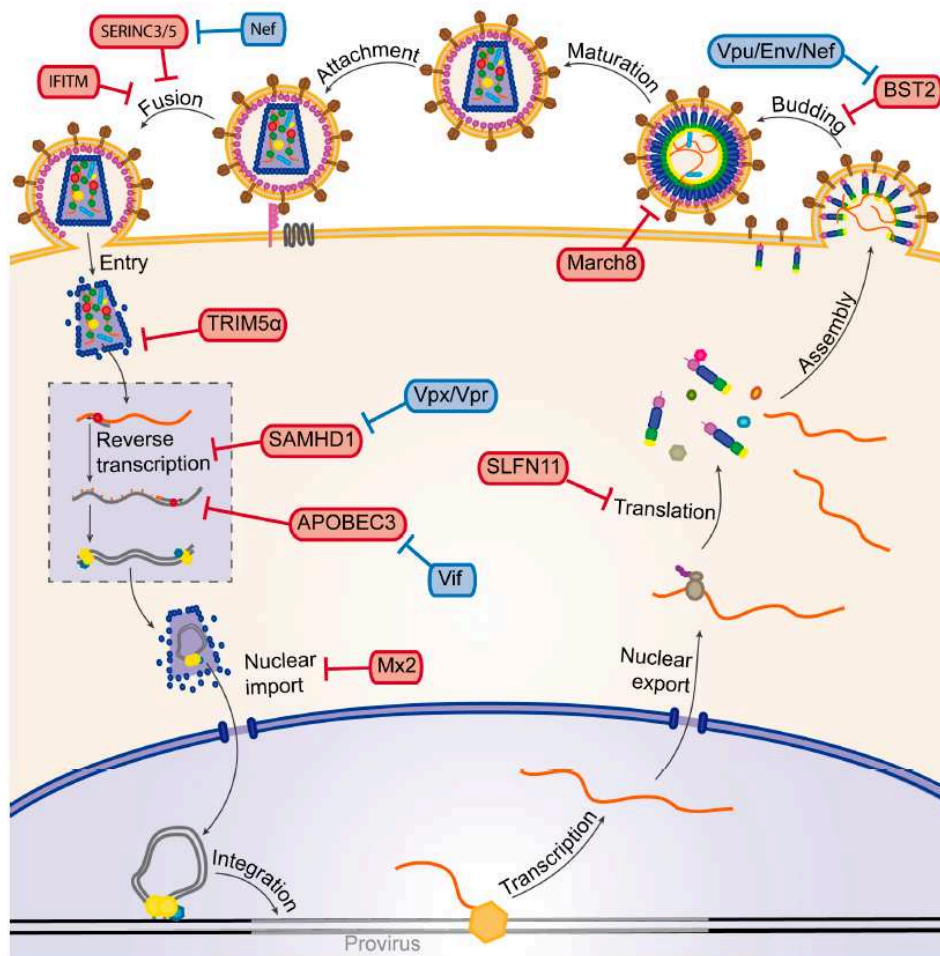


Figure 4: HIV life cycle and host restriction factors.

The IFITM proteins and the newly discovered SERINC proteins could block viral entry into the host cell by interfering viral fusion with target cells. Both IFITM proteins and SERINC proteins are efficiently incorporated into virions and impair the fusion process. Nef could prevent the incorporation of SERINC3 and SERINC5 into HIV virions by mediating their re-localization to late endosomes. After viral entry, APOBEC3 and SAMHD1 interfere with the reverse transcription (SAMHD1) or modify reverse-transcribed viral DNA (APOBEC3). Tetherin/BST2 could act late in the viral replication cycle to prevent the release of virions. Vif could counteract the APOBEC3-driven mutagenesis of viral genome, by preventing its packaging into viral particles. Vpx relieves SAMHD1-mediated block to viral reverse transcription. Vpu could prevent BST2/tetherin from holding on to the virions at the plasma membrane. SIVs and HIV-2 that lack Vpu could use Env and Nef, respectively, to counteract BST2/tetherin by sequestering it intracellularly. Adapted from Seissler et al., 2017.

1.4.1 Entry

HIV-1 infection of host cells by cell-free virus particles initiates with the binding of viral surface envelope glycoprotein gp120 (Env) to the cellular surface CD4 receptor, which triggers a conformation change in Env and subsequently exposes the binding sites for the co-receptor, either CCR5 or CXCR4. Following interaction of CD4-bound gp120 to the co-receptor, the fusion peptide of the transmembrane gp41 is exposed and then inserts into the host cell plasma membrane, leading to the formation of fusion pore that subsequently triggers the fusion between viral and cellular membranes, and released of the viral contents into the host cell cytoplasm (Clapham and McKnight, 2001; Wilen et al., 2012).

The SERINC proteins are multipass transmembrane proteins and play a putative role in the incorporation of serine into sphingolipids and phosphatidylserine (Inuzuka et al., 2005). Among the five SERINC proteins, SERINC3 and SERINC5 have been identified as restriction factors for HIV-1, while the anti-viral activity of SERINC5 is more potent (Rosa et al., 2015; Usami et al., 2015). SERINC3 and SERINC5 have been shown to incorporate into virions and impair viral fusion with target cells (Rosa et al., 2015; Usami et al., 2015). However, the auxiliary protein Nef could mediate the endocytosis of SERINC5 through interaction with clathrin adaptor complex AP2 (Craig et al., 1998; Trautz et al., 2016; Usami et al., 2015). Recent studies have revealed that the susceptibility of virions to SERINC5 is also dependent on the envelope glycoprotein since Ebola glycoprotein (GP), VSVG and envelope from certain HIV-1 isolates could render viral particles resistant to the restriction imposed by SERINC5 (Ahi et al., 2016; Beitari et al., 2017). Thus, it appears that HIV-1 has developed two distinct strategies to counteract the action of SERINC5-mediated by the auxiliary protein Nef or by the viral Env glycoprotein (Firrito et al., 2018; Seissler et al., 2017).

The interferon-induced transmembrane (IFITM) proteins are expressed at a basal level on multiple cell types and could be strongly induced by type I interferons. The human IFITM family consists of five members, including IFITM1, IFITM2, IFITM3, as well as IFITM5 (Diamond and Farzan, 2013). IFITM1, 2, and 3 are members of small interferon-stimulated genes (ISGs) and have been shown to restrict a large range of enveloped viruses by preventing the fusion of viral envelopes with host cell membranes (Shi et al., 2017; Zhao et al., 2018). IFITM5 (also known as BRIL) is an osteoblast-specific transmembrane protein, which has been shown to be involved in osteoblast mineralization *in vitro* (Guillen-Navarro et al., 2014; Hanagata, 2016). Moreover, a unique dominant mutation in the 5'UTR of *IFITM5* is associated with osteogenesis imperfect (OI) (Cho et al., 2012; Hanagata, 2016). The IFITM1–3 utilizes

several mechanisms to inhibit virus infection of cells. The IFITM1 is mainly expressed at the plasma membrane (Spence et al., 2019), whereas IFITM2 and IFITM3 localized to specific intracellular compartments, such as late endosomes and lysosomes. Therefore, IFITM1 could restricts enveloped viruses that enter host cells through the plasma membrane, whereas IFITM2 and IFITM3 restrict enveloped viruses that enter host cells through the acidic endosomes by interfering with the fusion of viral envelopes with the membrane of endosome membrane(Amini-Bavil-Olyaei et al., 2013; Ferreira et al., 2013; Smith et al., 2019). Furthermore, IFITM1–3 could act cooperatively and promote the shuttling of incoming virus particles to lysosomes for destruction (Spence et al., 2019). Interestingly, several studies have suggested that the IFITM proteins could decrease membrane fluidity to prevent fusion of virus particles with host cell membranes (Li et al., 2013; Lin et al., 2013). More recently, it has been shown that IFITM proteins are also involved in syncytin-mediated fusion of trophoblast cells and the formation of multinucleated syncytiotrophoblast (ST) layer (Buchrieser et al., 2019; Kellam and Weiss, 2019). In addition to the role of IFITM proteins in blocking viral entry through inhibition of membrane fusion, IFITM proteins provide additional protective mechanisms. IFITM proteins could incorporate into the membrane of nascent budding virions, the presence of IFITMs could reduce virion infectivity, probably through a mechanism which alters membrane fusion dynamics (Compton et al., 2014; Tartour et al., 2014; Tartour et al., 2017). Furthermore, IFITM proteins have been shown to inhibit the processing of HIV-1 envelope glycoprotein, thereby restricting viral infection (Yu et al., 2015).

1.4.2 Reverse transcription and integration

Once inside the cytoplasm, the viral RNA is then reverse transcribed into double-stranded DNA by the reverse transcriptase (RT) enzyme. The synthesis of viral DNA leads to the formation of a pre-integration complex (PIC) containing the newly made DNA, cellular proteins, as well as viral proteins, such as the enzyme IN, Vpr and matrix (MA). The PIC is then transported into the nucleus and integrated into the host genome. Integration generally occurs at the transcriptionally active sites of host genome, enabling efficient viral transcription.

The HIV genome initially enter the cytoplasm of host cells as part of the viral core, which is surrounded by the viral capsid. The viral capsid facilitates reverse transcription, shields the viral RNA from cytoplasmic sensors and also recruits host factors that required for nuclear import of the viral DNA(Ganser-Pornillos and Pornillos, 2019). TRIM5 α could block retroviral infection through recognition and inactivation of

the viral capsid that protects the incoming retroviral core (Ganser-Pornillos and Pornillos, 2019; Ohmine et al., 2010; Stremlau et al., 2004; Stremlau et al., 2006). The Myxovirus resistance 2 (Mx2/B) could also restrict HIV-1 by recognizing the viral capsid protein to prevent nuclear import and subsequent genome integration (Fribourgh et al., 2014; Goujon et al., 2013; Liu et al., 2013).

After virus entry, the RT enzyme have to generate viral DNA using the pool of dNTPs in the cytoplasm. The sterile alpha motif and HD-domain containing protein 1 (SAMHD1) is a triphosphohydrolase enzyme with deoxynucleoside triphosphate triphosphohydrolase (dNTPase) activity which maintains the intracellular level of dNTPs (Ballana and Este, 2015). SAMHD1 is highly expressed in resting T cells, as well as myeloid cells, including monocytes, macrophages, dendritic cells, where it localizes in both the nucleus and cytoplasm (Baldauf et al., 2012; Descours et al., 2012; Laguette et al., 2011). The SAM domain–and HD domain–containing protein 1 (SAMHD1) was identified as a restriction factor to block HIV-1 reverse transcription (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 can deplete dNTP pools, which prevents HIV-1 reverse transcription. In the case of SIV or HIV-2 infection, the auxiliary protein Vpx could be released from the viral particle and induce the degradation of SAMHD1 in the cytoplasm (Figure 4). The auxiliary protein Vpx could counteract SAMHD1-mediated restriction of HIV infection by directly binding to the C-terminal domain of SAMHD1 and inducing the degradation of SAMHD1 by the proteasome. Furthermore, SAMHD1 has been shown to restrict other retroviruses and several DNA viruses, including hepatitis B virus, vaccinia virus, herpes simplex virus type 1 (HSV-1), by limiting the intracellular deoxynucleoside triphosphate (dNTP) pools (Gramberg et al., 2013; Hollenbaugh et al., 2013). SAMHD1 harbors a cyclin binding motif which could interact with cyclin-CDK complexes and controls SAMHD1 protein expression, phosphorylation and stability, as well as dNTPase activity and HIV-1 restriction(St Gelais et al., 2016; St Gelais et al., 2018). Increased phosphorylation of SAMHD1 at threonine 592 (T592) due to high expression levels of the cyclin A-CDK complexes during the S-phase of cell cycle is associated with increased permissiveness of dividing cells to HIV-1 infection(Cribier et al., 2013; Pauls et al., 2014; St Gelais et al., 2014; White et al., 2013).

The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), a member of single-stranded DNA cytidine deaminase enzyme family, can potently block HIV-1 replication through cytidine deamination of viral genome, leading to abortive reverse transcription (Harris et al., 2002; Sheehy et al., 2002). The viral auxiliary Vif protein can counteract APOBEC3G and target it for proteasomal degradation (Desimmie et al., 2014; Mariani et al., 2003).

1.4.3 Transcription and nuclear export of viral RNA

The integrated provirus is subsequently transcribed into viral mRNA by the host RNA polymerase II. The viral mRNAs are spliced into different alternatively spliced RNA products, and the Rev protein is required for the nuclear transport of unspliced and partially spliced RNA transcripts containing the rev-responsive element (Blissenbach et al., 2010; Cochrane, 2014). The human schlafen 11 (SLFN11) could block viral protein synthesis in a codon-usage-dependent manner in HIV-1 infected cells (Li et al., 2012; Stabell et al., 2016).

1.4.4 Assembly, and budding and maturation

Translation of Gag-Pol, Gag and most of the auxiliary proteins takes place on the cellular ribosomes, while the translation of Vpu and Env occurs on the rough endoplasmic reticulum (ER). All viral proteins subsequently and a dimer of the full-length genomic viral RNA traffic to the plasma membrane for viral assembly. Viral assembly occurs at the lipid rafts, which are cholesterol and sphingolipid-rich microdomains of the plasma membrane where the Gag protein precursor initiates the assembly into immature virions (Benaroch et al., 2010; Carter and Ehrlich, 2008; Sundquist and Krausslich, 2012). The auxiliary protein Vpr is incorporated into the viral particle via interaction with the p6 C-terminal domain of Gag. The tetherin (also known as BST2) is an IFN-inducible antiviral protein which tether nascent virus to the plasma membrane. Tetherin can block virus release, activate the innate immune response and is counteracted by the HIV-1 auxiliary Vpu protein (Varthakavi et al., 2003). HIV-2 and most SIVs lack Vpu protein, but they can employ Env and Nef, respectively, to counteract the tetherin restriction factor by intracellular sequestering (Bour and Strebel, 1996; Jia et al., 2009; Lim et al., 2010; Zhang et al., 2009).

Membrane-associated RING-CH 8 (MARCH8) belongs to the MARCH family of RING-finger E3 ubiquitin ligases (Bartee et al., 2004; Goto et al., 2003). MARCH8 was initially identified as the cellular homolog of viral RING-CH E3 ligases, such as the K3 of murine γ -2 herpesvirus 68, the myxomavirus homolog M153R as well as the K3 and K5 of Kaposi's sarcoma-associated herpesvirus (KSHV)(Bartee et al., 2004; Goto et al., 2003). The viral RING-CH proteins predominantly down-regulate the cell surface expression of the major histocompatibility complex (MHC)-I molecules (Coscoy and Ganem, 2000; Ishido et al., 2000; Stevenson et al., 2000). Similarly, MARCH8 has been

shown to down-regulate numerous transmembrane proteins, including CD44, CD81, CD86, CD98, Bap31, MHC-II, TRAIL receptor 1 and IL-1 receptor accessory protein5(Bartee et al., 2010; Eyster et al., 2011; Lapaque et al., 2009; Ohmura-Hoshino et al., 2006; Tze et al., 2011; van de Kooij et al., 2013). MARCH8 is also able to down-regulate the cell surface expression of HIV-1 envelope glycoprotein and prevents its incorporation into virus particles, thereby reducing the infectivity of virions (Tada et al., 2015). More recently, it has been reported that MARCH1 and MARCH2 could also down-regulate viral envelope glycoproteins from the cell surface, leading to reduced incorporation of HIV-1 envelope glycoproteins into nascent virions (Zhang et al., 2018; Zhang et al., 2019).

Chapter 2. Mononuclear phagocyte system in HIV-1 infection

The mononuclear phagocyte system (MPS) has been defined as the cell lineages which comprise mainly dendritic cells (DCs), monocytes and macrophages (Gordon and Taylor, 2005; Guilleams et al., 2014; van Furth et al., 1972). Cells of the MPS are widely distributed in tissues throughout the body. In addition, osteoclasts (OCs) are specialized phagocytic cells of the bones, originating from macrophage fusion, and were also initially proposed as one of the members of MPS (van Furth et al., 1972). Monocytes, DCs and macrophages originate from a common myeloid progenitor termed myeloid blasts (Ly-6C⁺/CD31⁺) (Figure 5), and MPS cells of different maturation stages are able to differentiate into OCs (de Bruijn et al., 1994), but myeloid blasts were found to be more prone to differentiate into functional multinucleated osteoclasts in the presence of the RANK ligand (RANKL) (de Vries et al., 2009).

Mononuclear phagocytes can be found in different tissue compartments, including peripheral blood circulation, and play pivotal role in tissue homeostasis and exhibit multiple functions in immunity. Monocytes represent a subset of circulating blood cells which constitute around 10% of peripheral leukocytes in humans, and can further differentiate into tissue macrophages and DCs. Monocytes as well as DCs and macrophages play crucial role during pathogen invasion and inflammation, and DCs and macrophages are also referred as antigen presenting cells (APCs) which mediates adaptive host immune response by antigen processing and presentation.

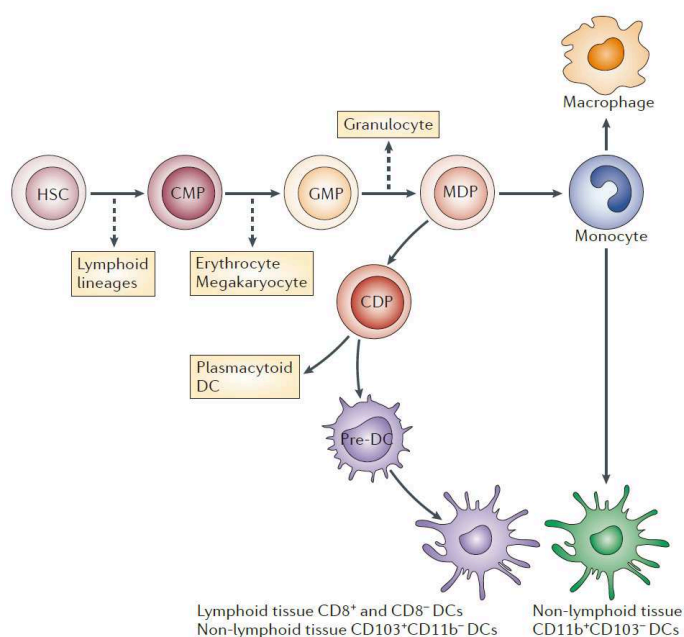


Figure 5: Lineage of mononuclear phagocytes.

Commitment to differentiation into the monocyte, dendritic cell (DC) or macrophage takes place at the stage of the macrophage and DC progenitor (MDP). MDPs could give rise to monocytes or common DC progenitors (CDPs). Monocytes can give rise to macrophages or some CD11b⁺CD103⁻ DCs. CDPs could give rise to both CD8⁻ and CD8⁺ DCs in lymphoid tissues as well as CD103⁺CD11b⁻ DCs in the peripheral non-lymphoid tissues. HSC, haematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte and macrophage progenitor. Adapted from Chow et al., 2011.

Therefore, cells of MPS play important roles in both innate and adaptive immune responses. In addition, they are also important host cell targets for HIV permissive for HIV-1 infection and represent natural host cell targets of HIV-1 since they all express the CD4 receptor and CCR5 and CXCR4 co-receptors for virus entry. The role of the mononuclear phagocyte system in HIV infection have been described as early as 1990's (Roy and Wainberg, 1988). *In vivo*, MPS cells could be involved in viral control, but they also contribute to virus dissemination and cell-to-cell transmission (Connor and Ho, 1994; Martin and Bandres, 1999; Orenstein et al., 1997; van't Wout et al., 1994).

2.1 Monocyte subsets and HIV-1 infection

Monocytes are crucial members of the MPS (Guilliams et al., 2014), and are mobile mononuclear phagocytes originating from bone marrow myeloid precursors, which circulate in blood for a few days and then migrate into tissues where they differentiate into some tissue macrophages and DCs (Auffray et al., 2009). (Bain et al., 2014; Ginhoux and Jung, 2014). Monocytes play an essential role in tissue development and homeostasis, as well as host defense against pathogens (Serbina et al., 2008) and are implicated in a wide range of inflammatory diseases (Libby et al., 2008).

Monocytes are heterogeneous and can be classified into different subsets based on the expression of the lipopolysaccharide (LPS) receptor CD14 and the Fc γ III receptor CD16 (Passlick et al., 1989): these subsets include classical monocytes (CD14⁺⁺/CD16⁻), intermediate monocytes (CD14⁺⁺/CD16⁺) and non-classical monocytes (CD14⁺/CD16⁺⁺) (Figure 6). The classical monocytes express high level of CD14 but no CD16, and make up 80-90% of monocytes. The intermediate monocytes show high level of CD14 expression and low CD16 expression, and the non-classical monocytes express a high level of CD16 and low CD14 (Wong et al., 2012; Ziegler-Heitbrock et al., 2010). The three monocyte subsets have distinct phenotypes

and functions, and different abilities to produce cytokines. The classical monocytes are highly phagocytic and can migrate rapidly to the sites of infection, where they recognize and phagocytose pathogens, produce reactive oxygen species (ROS) and secrete pro-inflammatory cytokines (Jakubzick et al., 2017; Serbina et al., 2008). The non-classical monocytes exhibit patrolling behaviors on the endothelium and may have important role in anti-viral responses (Auffray et al., 2007; Cros et al., 2010). Finally, the intermediate monocytes are involved in antigen processing and presentation, T cell stimulation and activation, as well as angiogenesis (Sampath et al., 2018; Wong et al., 2011; Wong et al., 2012).

Monocytes can undergo transition from classical monocytes to non-classical monocytes in human, and intermediate monocytes has been proposed as a monocyte subset in transition from classical to non-classical monocytes. Recent findings suggest that monocytes follows a linear differentiation pattern, a small population of classical monocytes differentiate into circulating intermediate monocytes, whereas the majority of circulating intermediate monocytes eventually mature into non-classical monocytes (Patel et al., 2017; Tak et al., 2017). CD16⁺ monocytes has been shown to expand under inflammatory conditions and during viral infection (Fingerle et al., 1993; Nockher and Scherberich, 1998; Thieblemont et al., 1995).

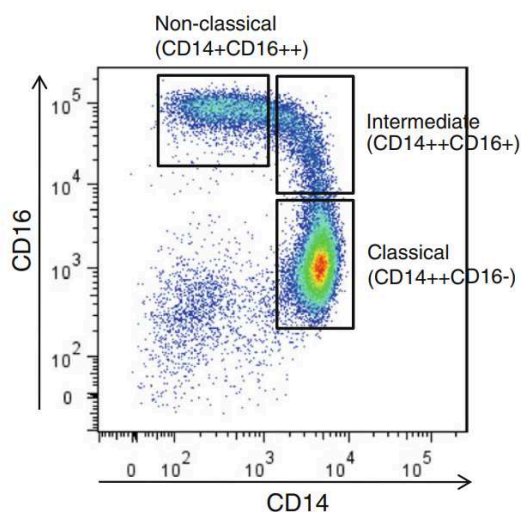


Figure 6: Gating strategy of monocyte subsets.

Flow cytometry dot plot shows the gating of classical, intermediate and nonclassical monocyte subsets. The classical monocytes express high levels of CD14 but no CD16, intermediate monocytes express high levels of CD14 and low CD16, while the non-classical monocytes express low CD14 but high CD16. Adapted from Wong et al., 2012.

Compared to CD4⁺ T cells, monocytes are much more resistant to HIV-1 productive infection owing to the high level expression of host cell restriction factors. Monocytes, as well as macrophages and DCs, express differential restriction factors

including SAMHD1, viperin and APOBEC variants which inhibit HIV-1 replication at different stages of the viral life cycle (Berger et al., 2011a; Berger et al., 2011b; Nasr et al., 2012; Nguyen et al., 2014). Monocytes are relatively refractory to HIV-1 productive infection *in vitro* (Sonza et al., 1996; Triques and Stevenson, 2004). However, CD14⁺CD16⁺ monocytes likely play important roles in inflammatory responses during HIV-1 infection, since they are significantly expanded in the peripheral blood and make up to 20-60% of the monocytes in HIV-1 infected patients even under effective anti-retroviral therapy (ART) (Kim et al., 2010; Pulliam et al., 1997; Williams et al., 2015). Similarly, it has been shown that a significant expansion of intermediate monocytes and increased levels of soluble CD14 in the peripheral blood occur during HIV-2 infection (Cavaleiro et al., 2013). Importantly, the levels of HIV-1 co-receptor CCR5 expression is highest on the intermediate monocytes (Ancuta et al., 2003), and the infection and expansion of CD16⁺ monocytes can be inhibited following treatment intensification with the MVC CCR5 antagonist (Ndhlovu et al., 2014). Moreover, monocyte differentiation could enhance their permissiveness to HIV-1 infection (Ancuta et al., 2006; Buckner et al., 2011; Triques and Stevenson, 2004). Indeed, it has been initially shown that CCR5 expression increases during monocyte differentiation into macrophages and directly mediates macrophages susceptibility to HIV-1 infection (Naif et al., 1998; Tuttle et al., 1998). Of note, CD16⁺ monocytes are considered to have a more mature phenotype compatible with macrophages, which may partly explain for their susceptibility to HIV-1 infection (Ellery et al., 2007; Jaworowski et al., 2007). Thus, monocyte maturation and differentiation may differently regulate the susceptibility of monocytes to HIV infection (Campbell et al., 2014; Wacleche et al., 2018). Furthermore, the CD14⁺CD16⁺ monocytes are more permissive to HIV infection *in vivo* and can be found in the CNS of HIV-1-infected individuals (Buckner et al., 2011; Burdo et al., 2010; Ellery et al., 2007; Fischer-Smith et al., 2001). It has been proposed that CD14⁺CD16⁺ monocytes may indeed facilitate HIV-1 entry into CNS, contributing to the development of HIV-associated neurocognitive disorders (Williams et al., 2015; Williams et al., 2014), since HIV-1 infected CD14⁺CD16⁺ monocytes express increased levels of the JAM-A and ALCAM cell-adhesion molecules on cell surface and preferentially transmigrate through the blood-brain barrier (Veenstra et al., 2017). The activated leukocyte cell adhesion molecule (ALCAM) and junctional adhesion molecule-A (JAM-A) are cell adhesion molecule expressed on blood-brain barrier (BBB) endothelial cells and also leukocytes, and are implicated in the processes of cell migration and adhesion (Lecuyer et al., 2017; Sugano et al., 2008).

2.2 Dendritic cells and HIV-1 infection

As the most powerful professional antigen-presenting cells, DCs have been considered to be the ‘sentinels’ of the immune system. They also orchestrate the immune response by bridging innate and adaptive immune systems. Immature DCs (iDCs) are distributed in peripheral tissues, where they recognize invading pathogens and process antigens. The sensing of microbial components such as lipopolysaccharide triggers the maturation of DCs for trafficking to the draining lymph nodes where they present foreign antigens to naive CD4+ T cells and initiate adaptive immune responses.

Human DCs in blood have been classified into two main groups: plasmacytoid DCs (pDCs) and ‘myeloid’ or ‘classical’ DCs. Myeloid or classical DCs were further subdivided into two subsets, including CD141+ DCs (cDC1) and CD1c+ DCs (cDC2) (Haniffa et al., 2012; McIlroy et al., 2001; Schlitzer et al., 2013; Segura et al., 2012; Summers et al., 2001; Yu et al., 2013a; Ziegler-Heitbrock et al., 2010).

2.2.1 Plasmacytoid DCs (pDCs)

The pDCs represent only 0.2–0.5% of the circulating blood leukocytes and are responsible for large quantities of type I interferon (IFN) production in response to viral infection (Fitzgerald-Bocarsly et al., 2008). The pDCs can respond to a variety of enveloped viruses, including HIV-1 and HCV, with production of type I interferon (IFN) as well as type III interferon (IFN- λ), which signal through the type III IFN receptor (IFNLR) consisting of subunits IL-10R β and IFNLR1 (Fitzgerald-Bocarsly et al., 2008; Mesev et al., 2019). Furthermore, pDCs can also produce several pro-inflammatory cytokines, such as TNF- α and IL-6, as well as a variety of chemokines, including CCL4, CCL5 and CXCL10, in response to viral stimulation (Megjugorac et al., 2004; Penna et al., 2002a; Penna et al., 2002b).

The pDCs have been shown to be permissive to HIV-1 infection and are able to produce IFN robustly in response to HIV-1 infection (An et al., 2007; Fong et al., 2002; Smed-Sorensen et al., 2005; Yonezawa et al., 2003). In addition, the deficient in IFN- α production is associated with progressive depletion of pDCs in blood of HIV-1-infected individuals (Howell et al., 1994; Lopez et al., 1983). The mechanism for significantly loss of circulating pDCs and their dysfunction in HIV-1 infected individuals remain poorly understood. It has been suggested HIV-1 infection leads to redistribution of pDCs to lymphoid organs, thus accounting for the depletion of

circulating pDC in the blood; indeed, the significant loss of pDCs in the peripheral blood of systemic lupus erythematosus (SLE) patients is due to redistribution of circulating pDCs to lesions in the skin (Baccala et al., 2007). The redistribution of pDCs to lymphoid organs has been observed in acute SIV infection of nonhuman primates (Brown et al., 2009; Malleret et al., 2008; Wijewardana et al., 2009). Similarly, it has been reported that pDCs and cDCs accumulated in the lymph nodes (LNs) of untreated, asymptomatic HIV-1-infected patients when pDCs were modestly depleted in the peripheral blood (Dillon et al., 2008; Lehmann et al., 2010). Furthermore, pDCs have also been shown to accumulate in the lymphoid organs, such as spleen and LNs, of chronically HIV-1-infected subjects (Foussat et al., 2001; Nascimbeni et al., 2009). Another possibility that could explain the loss of blood pDCs in HIV-1-infected patients is due to infection-induced cell death (Lehmann et al., 2010; Smed-Sorensen et al., 2005). Interestingly, HIV-1 infection of pDCs results in the formation of multinucleated giant cells *in vitro* and *in vivo* in tonsils from HIV-1 infected patients (Fong et al., 2002; Schmidt et al., 2004). Upon HIV-1 infection, IFN secretion by pDCs could promote maturation of pDCs and cDCs and also inhibit HIV-1 replication in CD4⁺ T cells (Li et al., 2014; Miller and Bhardwaj, 2013; Royle et al., 2014). However, pDCs has been shown to efficiently transmit viral particles to CD4 T⁺ cells, which may exacerbate T cell depletion (Evans et al., 2011; Fong et al., 2002). Thus, pDCs may play different roles in HIV-1 infection, and the redistribution and loss of pDCs at late stage of HIV-1 infection may contribute to immunodeficiency.

2.2.2 Conventional DC1 (cDC1)

The human cDC1 corresponds to myeloid CD141⁺ DCs, or blood DC antigen3 (BDCA3⁺), which constitute approximately 0.03% of human PBMCs (Macri et al., 2018). The myeloid CD141⁺ DCs can also be found in human LN, tonsil and spleen, as well as non-lymphoid tissues, including skin, lung, liver and gut (Bamboate et al., 2009; Haniffa et al., 2012; Jongbloed et al., 2010; Poulin et al., 2012; Poulin et al., 2010). Human myeloid CD141⁺ DCs are considered to be human cross-presenting DCs, which exhibit enhanced ability to take up necrotic or dead cells through CLEC9A (Bachem et al., 2010; Sancho et al., 2009), sense viral nucleic acids and cross-present foreign antigens to CD8⁺ T cells (Croizat et al., 2010; Haniffa et al., 2012; Jongbloed et al., 2010; Poulin et al., 2010). Blood and skin CD141⁺ DCs are capable of secreting CXCL10 and TNF- α upon stimulation (Haniffa et al., 2012; Jongbloed et al., 2010). CD141⁺ DCs are the major producers of IFN- λ in response to viral infection, such as HCV (Yoshio et al., 2013).

2.2.3 Conventional DC2 (cDC2)

The myeloid CD1c⁺ DCs corresponds to conventional DC2 (cDC2) and represent approximately 1% of human PBMCs. The CD1c⁺ DCs are also present in lymphoid tissues, including lymph nodes, spleen, tonsils, liver, skin, lungs, kidneys and gut (Mittag et al., 2011; Persson et al., 2013; Segura et al., 2012; Watchmaker et al., 2014). Human blood cDC2 are also able to cross-present foreign antigens and are potent stimulators of naïve CD4⁺ T cells (O'Keeffe et al., 2015). Skin CD1c⁺ DCs could secrete IL-8, IL-10, IL-23, and TNF- α upon stimulation (Haniffa et al., 2012; Morelli et al., 2005).

Both human CD141⁺ cDCs and CD1c⁺ cDCs are present in blood and peripheral tissues. Each subset in the blood is quite similar to its tissue counterpart but seems less differentiated (Haniffa et al., 2012; Schlitzer et al., 2013; Segura et al., 2012). It has been suggested that less differentiated blood cDCs migrate through the blood to replenish the cDCs pool in peripheral tissues (Collin et al., 2011; Haniffa et al., 2013; Segura et al., 2012).

Some studies have shown that cDCs can support some level of HIV-1 infection *in vitro* (Cameron et al., 2007; Martin-Gayo et al., 2015; Smed-Sorensen et al., 2005). However, the cytoplasmic protein SAM domain and HD domain-containing protein 1 (SAMHD1) is one of the major restriction factors that limits HIV-1 infection in cDCs. SAMHD1 is highly expressed in cDCs and can block viral replication through depletion of endogenous intracellular pools of dNTPs, but also directly through degradation of the viral RNA (Lahouassa et al., 2012; Ryoo et al., 2014). Furthermore, interaction of DCs with CD4⁺ T cells leads to a significant down-regulation of SAMHD1 expression in DCs (Su et al., 2014), which may renders primary cDCs more permissive to HIV-1 infection (Hertoghs et al., 2015; Martin-Gayo et al., 2015). However, there are paradoxical findings regarding the permissiveness of cDCs to HIV-1 replication. Primary cDCs from elite controllers (ECs) patients are more permissive to HIV-1 replication, while cDCs from HIV-1 infected individuals with progressive infection are much more resistant to HIV-1 infection. It has been suggested that SAMHD1-dependent restriction of viral replication in primary cDCs might benefits HIV-1 more than the host, since restriction of HIV-1 replication by SAMHD1 may allow viral evasion from the innate immune response and could also impair the ability of cDCs to prime HIV-1-specific T cells responses (Martin-Gayo et al., 2015).

Recent studies indicate that primary CD141⁺ cDCs and CD1c⁺ cDCs may differ in the susceptibility to HIV-1 replication. In this regard, CD141⁺ cDCs possess an innate resistance to HIV-1 infection due to the constitutive expression of a vesicle-trafficking protein Rab15 which prevents fusion of viral particles in CD141⁺ cDCs, while CD1c⁺ cDCs are more susceptible to HIV-1 infection (Silvin et al., 2017). Similar to pDCs, circulating cDCs are capable of capturing viral particles and transmit to CD4⁺ T cells, a process called *trans*-infection (Groot et al., 2006). Consistent with the role of cDCs in facilitating HIV-1 trans-infection in the lymphoid tissues, it has been reported that depletion of endogenous cDCs from the tissue suspension cultures significantly decreased the efficiency of HIV-1 infection of memory T cells (Reyes-Rodriguez et al., 2016).

2.2.4 Langerhans cells (LCs)

Langerhans cells (LCs) are the major antigen presenting cells (APCs) in the epidermis of human skin. LCs have been proposed as classical sentinels which are frontier of contact with invading pathogens. However, the exact classification of LCs is always controversial since their discovery in 1868. It has been heavily debated whether LCs could be classified as macrophages or dendritic cells. Functionally speaking LCs are more like DCs, since they pick up foreign antigens as efficiently as DCs, and can migrate out of the epidermis to lymph nodes to present foreign antigens to naïve T cells. However, it has been evidenced that human LCs are capable of self-renewing and persistence in epidermis of human skin, and can be repopulated from bone marrow precursors after hematopoietic stem cell transplantation or during inflammation (Collin et al., 2006; Czernielewski and Demarchez, 1987; Kanitakis et al., 2011; Kanitakis et al., 2004). Thus, ontogenically speaking, they are more similar to tissue resident macrophages, both LCs and macrophages arise from yolk sac-derived myeloid precursors and are capable of self-renewing and persistence in tissues (Gomez Perdiguero et al., 2015; Guilliams et al., 2016; Malissen et al., 2014; Mass et al., 2016; Schulz et al., 2012). LCs have been characterized by the expression of CD1a and C-type lectin Langerin (CD207), which functions as receptor for many invading microbial pathogens (Cunningham et al., 2010). It has been reported that LCs are potent stimulators of CD4⁺ T cell response and can induce their polarization to helper type 2 (Th2) T cells (Klechevsky et al., 2008). Furthermore, it has been shown that LCs are able to present non-peptide antigens to T cells in a Langerin-dependent and CD1a-restricted pattern (Hunger et al., 2004).

To establish successful infection in a naive host, HIV-1 has to penetrate the mucosal barrier to gain access to its potential target cells, such as Langerhans cells, CD4⁺ T cells, DCs and macrophages. LCs have numerous dendrites that could extend through intercellular spaces of mucosa, and these dendrites could even reach up to the outer surface of the epithelium, where HIV-1 could bind directly to them (Hladik and McElrath, 2008; Miller et al., 1992; Nishibu et al., 2006). The expression of CD4, CCR5 and C-type lectin receptors on LCs in foreskin and endocervical mucosa has been thought to be the basis of preferential sexual transmission for CCR5-using viruses. Early studies have shown that the LCs are permissive to HIV-1 infection both *in vivo* and *ex vivo*, indicating a critical role of LCs in HIV-1 transmission (Berger et al., 1992; Cimarelli et al., 1994; Dezutter-Dambuyant, 1995; Henry et al., 1994; Kawamura et al., 2003; Kawamura et al., 2008; Peressin et al., 2011). Indeed, epidermal LCs could be preferentially infected by CCR5-using viruses but not CXCR4-using viruses *ex vivo*, and could produce high amount of infectious viral particles when co-cultured with autologous CD4⁺ T cells (Kawamura et al., 2000; Ogawa et al., 2013). Monocyte-derived LCs express CD4 as well as CCR5 and are also readily permissive to infection by R5 HIV-1 (Kawamura et al., 2001). Furthermore, it has been evidenced that the majority of initially infected cells were Langerhans cells in Rhesus macaques following exposure to intra-vaginal SIV (Hu et al., 2000). Similarly, it has also been shown that epidermal LCs are initial targets for HIV-1 using *ex vivo* human foreskin explants model (Ganor et al., 2010; Zhou et al., 2011).

LCs may act as “Trojan horse” which carrying infectious viral particles for HIV-1 invasion of the mucosa barrier, and then migrate through epidermis and transmit HIV-1 to CD4⁺ T cells in the draining lymph nodes for efficient virus dissemination (Cunningham et al., 2008; Kawamura et al., 2000).

2.2.5 *In vitro* monocyte-derived DCs (MDDCs)

Most of our knowledge on human DC functions has been obtained using monocyte-derived dendritic cells (MDDCs) differentiated from blood monocytes *in vitro* using GM-CSF and IL-4. MDDCs can undergo further maturation in the presence of TNF- α or LPS, or the cytokine cocktail containing IL-1 β , TNF- α , IL-6 and PEG2 (Sallusto and Lanzavecchia, 1994). Despite there are some overlaps in functions and phenotypes between MDDCs and CD1c⁺ DCs, the MDDCs are genetically distinct from CD141⁺ DCs and CD1c⁺ DCs and arise from different bone marrow precursors

(Haniffa et al., 2012; Lee et al., 2015; Robbins et al., 2008). Although the physiological relevance of MDDCs is currently unclear, it has been suggested that these MDDCs are closely related to DCs found *in vivo* in inflamed tissues, namely inflammatory DCs (Palucka et al., 1998; Sallusto and Lanzavecchia, 1994; Segura and Amigorena, 2013; Segura et al., 2013; Xu et al., 2007). MDDCs has been broadly used in clinic, especially as vaccines to induce immune response against tumors in cancer patients (Radford et al., 2014).

The early observations of HIV-1 infection of DCs were constrained by the limited availability of DCs, while culturing monocytes with GM-CSF and IL-4 provide an easy way to generate large quantity of MDDCs that closely resembles DCs found *in vivo*. Many studies using this *in vitro* model can recapitulate the results obtained using primary DCs isolated from blood, namely MDDCs are permissive to HIV-1 infection (Turville et al., 2004), and can transfer viral particles to CD4⁺ T cells independent of HIV-1 infection, they can also transfer virus to CD4⁺ T cells when they are productively infected (Blauvelt et al., 1997; Turville et al., 2004). MDDCs express high levels of the C-type lectin DC-SIGN which can efficiently bind to viral envelope and play import role in HIV-DC interaction (Geijtenbeek et al., 2000). Although SAMHD1 has been reported to be a key restriction factor that limits HIV-1 replication in MDDCs and can also inhibit HIV-1 cell-to-cell transmission from HIV-1 infected CD4⁺ T cells to MDDCs (Hrecka et al., 2011; Puigdomenech et al., 2013), recent studies demonstrated that the MDDCs do support productively HIV-1 infection to a certain degree, despite high levels expression of SAMHD1 (Hertoghs et al., 2015).

In general, HIV-1 replication is relatively inefficient in immature DCs, and mature DCs are even more resistant to HIV-1 productive infection (Izquierdo-Useros et al., 2010; Steinman et al., 2003; Wu and KewalRamani, 2006), partially due to the high expression of cellular restriction factors, such as SAMHD1, TRIM5 α , APOBEC3A (Berger et al., 2011b; Laguette et al., 2011; Ribeiro et al., 2016; Stremlau et al., 2004). It has been well proposed that DCs play a critical role early in HIV-1 primo-infection since they are one of the first cell types which take up HIV-1 virus particles for transmission to CD4⁺ T cells (Boggiano and Littman, 2007; Boggiano et al., 2007; Hladik et al., 2007; Wu and KewalRamani, 2006). It has been reported that DC-SIGN⁺, CD1a-sorted, HIV-1 exposed cDCs could survive and release fully infectious viral particles for up to 45 days, and are still capable of transmitting viral particles to CD4⁺ T cells (Popov et al., 2005). Thus, HIV-1 infection of DCs could be essential for long-term survival of the virus and may prolong the infection process, DCs could capture viral particles and migrate to lymph nodes and became trapped in the lymph nodes, viral particles in the VCCs of DCs can preserve for a long period and are still infectious and can be transmitted to CD4⁺ T cells (Popov et al., 2005).

DCs were found to preferentially support productive infection by CCR5-using

virus rather than by CXCR4-using virus (Granelli-Piperno et al., 1998; Pena-Cruz et al., 2018). However, different DC subpopulations may differ in their permissiveness to HIV-1 infection (Silvin et al., 2017). Even if our knowledge regarding the intrinsic ability of cDCs and pDCs to sense HIV-1 infection and stimulate innate immune response has greatly improved during the last decade, to which degree individual DC subsets could influence the effective immune response to HIV-1 infection and contribute to viral control remains open questions and require further investigations. The development of humanized animal models that could recapitulate human DCs biology could be critical to characterize the complexity of HIV-DC interactions *in vivo*.

2.3 HIV-1 infection and macrophage polarization

Macrophages are heterogeneous mononuclear phagocytes, strategically distributed throughout tissues where they display great functional diversity and play key role in tissue homeostasis. Within a specific tissue, macrophages can differentiate into specialized cells which perform distinct function from macrophages from other tissues. For example, in a specialized tissue microenvironment, macrophages could differentiate into osteoclasts (in bone), alveolar macrophages (in lung), microglial cells (in brain), Kupffer cells (in liver) or histiocytes (within interstitial connective tissues). Historically, it has been suggested that these tissue resident macrophages arise from circulating adult blood monocytes produced in the bone marrow (van Furth et al., 1972). However, it has then become evident that most tissue-resident macrophages in fact develop during embryogenesis, and they arise from hematopoietic precursors in the yolk sac and maintain themselves in tissues through adulthood by longevity and self-renewal. (Epelman et al., 2014; Ginhoux et al., 2010; Ginhoux and Guilliams, 2016; Perdiguero and Geissmann, 2016). During inflammation, tissue-infiltrating monocytes could also contribute to tissue macrophage compartments. However, these monocyte-derived macrophages display limited life spans in general. Of note, both embryonic-derived macrophages and monocyte-derived macrophages can undergo proliferation when given the certain stimulus.

Macrophages play key roles in the immune response as both modulator and effector cells, since their activation is crucial for the immune system. In response to microenvironmental stimuli, macrophages can undergo functional polarization for initiation and orchestration of immune responses. Similar to Th1 and Th2 polarization of T cells (Kapsenberg, 2003; Neurath et al., 2002), macrophages can undergo either classical M1 polarization when stimulated by LPS and IFN- γ , or alternative M2

polarization when stimulated with anti-inflammatory cytokines such as IL-10, IL-4 or IL-13 (Figure 7). The two groups of macrophages display distinct phenotypic and functional characteristics. However, depending on the anti-inflammatory stimuli, M2 macrophages can be further classified into M2a macrophages (stimulated by IL-4 and IL-13), M2b macrophages (stimulated by a combination of immune complexes and TLR or IL-1R agonists) and M2c macrophages (stimulated by IL-10) (Mantovani et al., 2004). M1 macrophages are characterized by high production of cytokines IL-23, IL-12, reactive oxygen species (ROS) and high antigen presentation feature. M2 macrophages are more heterogeneous than M1 macrophages, and participate in a wide range of immune activities, including phagocytosis of pathogens, tissue repair, suppressing inflammation and parasite elimination (Gordon, 2003; Mantovani et al., 2004; Martinez et al., 2009). M1 macrophages are able to produce several inflammatory cytokines such as IL-1, IL-6 and TNF- α , while M2 macrophages are characterized by expression of specific cell surface markers including mannose receptor (MRC1), transglutaminase 2 (TGM2), DC-SIGN, CD163 and CD23 (Gordon, 2003; Mantovani et al., 2004; Martinez et al., 2009) (Figure 8).

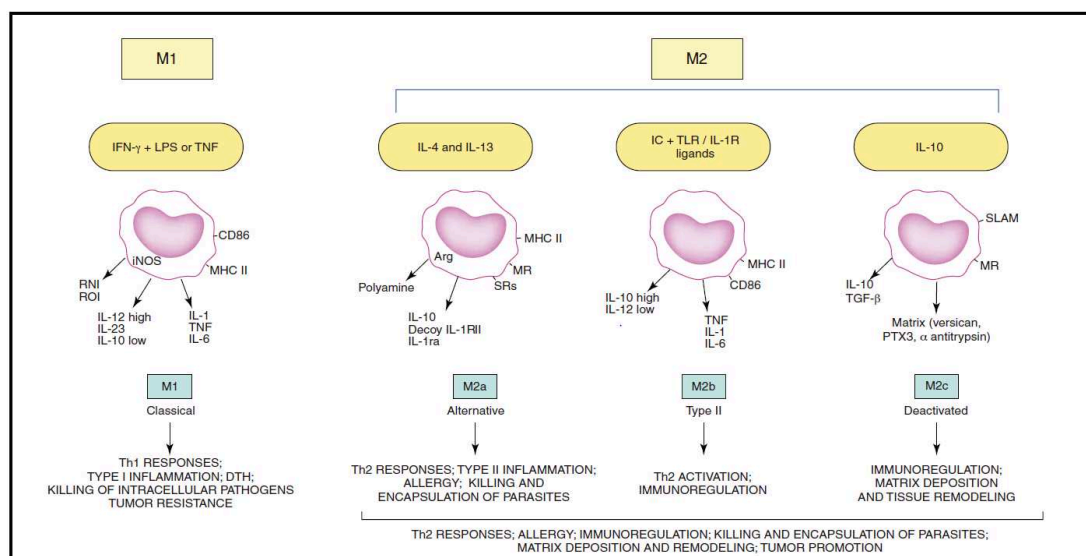


Figure 7: Macrophage polarization.

Mantovani and colleagues have proposed an M1-M2 macrophage model, in which M1 polarization included interferon-gamma (IFN- γ) plus lipopolysaccharide (LPS) or tumor necrosis factor (TNF), while M2 stimulation has been subdivided to interleukin-4 (IL-4) (M2a), immune complex + Toll-like receptor (TLR) ligands (M2b), as well as IL-10 and glucocorticoids (M2c) (Mantovani et al., 2004). Adapted from Martinez et al., 2014.

M1	M2
B7 (CD80)	M130 (CD163)
B7.2 (CD86)	CD206 (MRC1, mannose receptor)
CCR7 (MCP-3)	FcεRII (CD23)
CCL22 (MDC1) CD64	CD36
CXCL10 (IP-10) SOCS1	IL-1 Ra SOCS1
TLR-2	Nucleotide receptors (GPR86, GPR105, P2Y8, P2Y11, P2Y12)
TLR-4	C-type lectin-like receptor dectin-1
FcγRIII (CD16)	DC-SIGN (CD209)
FcγRII (CD32)	DCIR (CLECSF6)
LAM-1 (CD62)	CLACSF13
IL-1 R1	FIZZ1, ST2 (mouse)
IL-7R (CD127)	Phagocyte receptors (SR-A, M60)
IL-2R (α chain)	CXCR4, fusin (CD184)
IL-15R (α chain)	TRAIL
IL-17R (CTLA8) (CDw217)	

Figure 8: Markers of M1 and M2 macrophages.

Markers of M1 and M2 phenotypes, adapted from Kim et al., 2009. Markers in bold are those most commonly used.

HIV-1 could hide and thrive inside macrophages to evade immune surveillance, since HIV-1-infected macrophages are resistant to viral cytopathogenic effect and can survive for several months *in vitro* (Orenstein et al., 1988; Salahuddin et al., 1986; Sattentau and Stevenson, 2016; Sharova et al., 2005; Swingler et al., 2007). Furthermore, HIV-1 productively infected macrophages continuously produce high amounts of infectious viral particles over long periods of time by assembling and accumulating virus particles both in tetraspanin-enriched virus-containing compartments (VCCs) and at the plasma membrane (Benaroch et al., 2010; Carter and Ehrlich, 2008; Deneka et al., 2007; Mlcochova et al., 2013; Orenstein et al., 1988). Thus, macrophages may contribute to the formation long term tissue reservoirs during HIV-1 infection (Carter and Ehrlich, 2008; Cohen et al., 2011; Goodenow et al., 2003; Herbein and Varin, 2010).

Monocyte-derived macrophages (MDMs) are commonly used *in vitro* to investigate the phenotypes and functions of macrophages. A wealth of studies have investigated the effects of bacterial products and cytokines on the susceptibility of MDMs to HIV-1 infection (Alfano et al., 2008; Brown et al., 2008; Herbein and Khan, 2008; Kedzierska and Crowe, 2001). M-CSF has been found to strongly induce the expression of CD4 and co-receptor CCR5 and CXCR4 on MDMs, while GM-CSF leads to a marked reduction of CXCR4 expression on MDMs (Lee et al., 1999). However, there is only a few studies having investigated the consequences of macrophages polarization on viral infection (Brown et al., 2008; Cassol et al., 2009; Chan et al., 2008;

Chan et al., 2009). *In vitro*, unpolarised M0 MDMs are freshly differentiated macrophages from monocytes using M-CSF and are relatively permissive to HIV-1 infection, whereas M1 or M2 macrophages are relatively more resistant to HIV-1 infection (Alfano et al., 2013; Cassol et al., 2010; Lugo-Villarino et al., 2011). HIV-1 replication typically drive macrophages toward a pro-inflammatory M1-like phenotype (Brown et al., 2008; Porcheray et al., 2006), and HIV-1 macrophages infection results in an increased secretion of M1-associated pro-inflammatory chemokines, including CCL3, CCL4 and CCL5, and significant down-regulation of M2-associated specific markers, such as the mannose receptor CD206, CD163, IL-10 and CCL18 (Brown et al., 2008; Porcheray et al., 2006) (Figure 9). Compared with unpolarized MDMs, short-term exposure of MDMs to M1 (TNF- α plus IFN- γ) or M2a (IL-4) stimuli prior to HIV-1 infection led to a significant decreased capacity to support HIV-1 infection (Cassol et al., 2009). TNF- α plus IFN- γ -induced M1 polarization results in a significant reduction of the CD4 and CXCR4 expression on macrophages. In addition, IFN- γ stimulation of MDMs has been found to induce an increased expression of several restriction factors for HIV-1 infection, including cyclophilin A, APOBEC3G, TRIM22, TRIM5 α (tripartite motif 5- α) and Bst-2/theterin (Cobos Jimenez et al., 2012). Thus, the M1-dependent restriction of HIV-1 replication is associated with a more profound reduced CD4 expression and a greater inhibition of virus replication compared with M2 macrophages. M1 restriction of HIV-1 infection is also associated with a significant suppression of HIV-1 DNA synthesis, indicating that HIV-1 infection is inhibited at an early pre-integration step of the virus life cycle (Cassol et al., 2009).

M2a polarization of MDMs with IL-4 leads to inhibition of HIV-1 replication. However, since there is no detectable impairment of the viral DNA synthesis in M2a-polarized MDMs, this indicates that HIV-1 inhibition then occurs at a post-transcriptional/post-translational step of the virus life cycle in M2a macrophages (Cassol et al., 2009). Compared with unpolarised macrophages, M2 macrophages have been shown to express high levels of C-type lectins, such as DC-SIGN (Gordon, 2003; Gordon and Martinez, 2010), whereas this lectin is strongly down-regulated on M1 macrophages. Interestingly, M2a macrophages can efficiently transmit both R5 and X4 viruses to CD4⁺ T lymphocytes in a DC-SIGN-dependent manner *in vitro* (Cassol et al., 2013). Collectively, all these studies indicate that macrophage polarization can regulate and modulate macrophage susceptibility to HIV-1 infection and play an important role in HIV-1 transmission and dissemination (Figure 10).




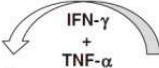

	 M1-MDM	 Unpolarized MDM	 M2a-MDM
	 IFN- γ + TNF- α		 IL-4, IL-13
R5 HIV-1 replication	+/-	++++	++
HIV-1 attachment and entry receptors	DC-SIGN (-) CD4 (+/-) CCR5 (++)	DC-SIGN (+) CD4 (+++) CCR5 (++)	DC-SIGN (+++) CD4 (+) CCR5 (++)
Cytokine and chemokine secretion	IL-6, IL-12, TNF- α , CCL2, CCL3, CCL4, CCL5 CXCL8, CXCL9, CXCL10, CXCL11	low to undetectable	IL-1ra, IL-10, CCL13, CCL14, CCL20, CCL22, CCL23
HIV-1 life cycle interference	Entry, pre-integration (post-integration)	n.a.	Post-integration
HIV-1 restriction factor expression	APOBEC3A (APOBEC3G, SAMHD1, TRIM22)	APOBEC3G, TRIM22	Undefined

Figure 9: MDM polarization and HIV-1 infection *in vitro*.

M1 polarization results in a significant reduction of the CD4 and CXCR4 expression on MDMs. M1 polarization induce an increased expression of several restriction factors for HIV-1 infection, including cyclophilin A, APOBEC3G, TRIM22, TRIM5 α . M2 macrophages express high levels of C-type lectins, such as DC-SIGN. Adapted from Alfano et al., 2013.

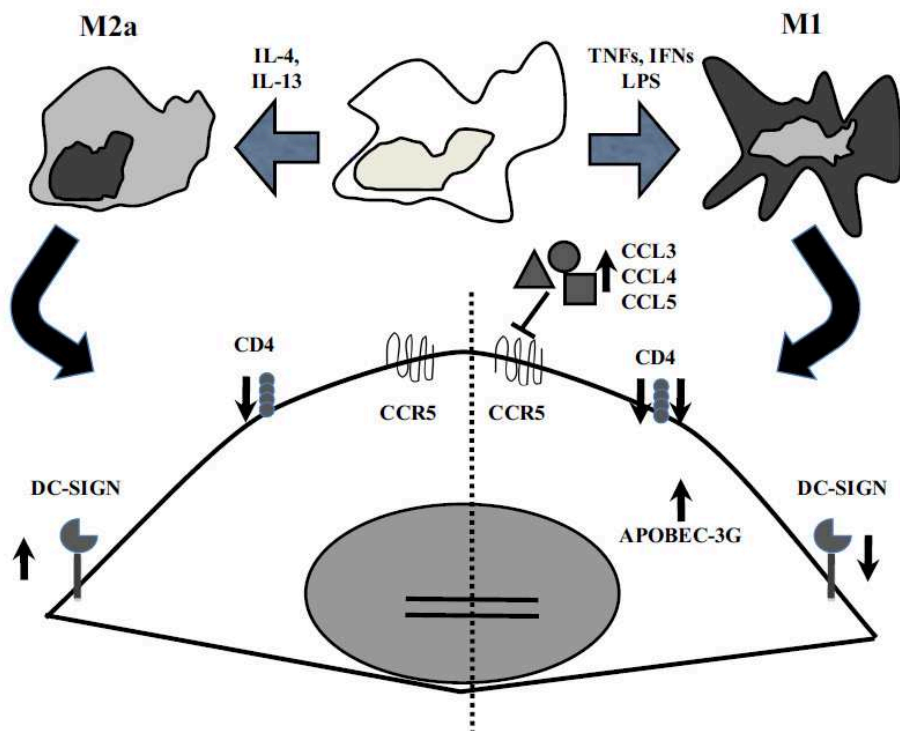


Figure 10: HIV-1 infection and macrophage polarization.

M1- and M2a-polarized MDM have both shown decreased levels of CCR5-dependent HIV-1 infection compared to unpolarized MDMs. This ultimate effect is likely due to differential and multi-factorial effects triggered by the macrophage polarization, including upregulation of APOBEC-3G, CCR5-binding chemokines, as well as profound down-regulation of CD4 in M1-MDM but not in M2-MDM (Cassol et al., 2009). A distinct regulation of DC-SIGN expression in M1-MDM versus M2-MDM may differentially contribute to spreading of captured HIV-1 to CD4+ T cells and other target cells. Adapted from Cassol et al., 2010.

2.4 Osteoclasts and HIV-1 infection

Osteoclasts (OCs) are multinucleated giant cells involved in bone resorption via proteolytic degradation and acid decalcification, leading to maintenance of bone matrix homeostasis and ensuring continuous remodelling of the bones. They are specialized phagocytic cells of the bone, originating from macrophage fusion, and were initially proposed as one of the members of mononuclear phagocyte system (van Furth et al., 1972). Bone-resorbing OCs arise by fusion of haematopoietic stem cells-derived monocytic precursors in the presence of RANKL and M-CSF (Ash et al., 1980; de Bruijn et al., 1994; Gomez Perdiguero et al., 2015; Lacey et al., 1998; Shalhoub et al., 2000), whereas myeloid blasts were found to be more prone to differentiate into functional multinucleated osteoclasts (de Vries et al., 2009). Monocyte subpopulations can first differentiate into multinucleated OCs and may contribute to the functional properties of OCs. It has been suggested that classical monocytes (CD14⁺⁺/CD16⁻) are the major source of OCs, while intermediate monocytes (CD14⁺⁺CD16⁺) could form multinucleated OCs with high resorption potential under inflammatory conditions and the non-classical monocytes (CD14⁺CD16⁺⁺) could differentiate into non-resorbing OC-like giant cells (Champion et al., 2018; Sprangers et al., 2016). Immature DCs are also capable of differentiating into functional multinucleated OCs in the presence of M-CSF and RANKL *in vitro* or through interactions with CD4⁺ T cells *in vivo* (Alnaeeli et al., 2006; Miyamoto et al., 2001; Speziani et al., 2007; Wakkach et al., 2008). Finally, differentiated macrophages still have the potential to fuse together and form multinucleated OCs upon stimulation with RANK ligand and M-CSF (Udagawa et al., 1990). However, recent studies revealed OC precursors that are independent from the haematopoietic stem cell (HSC) lineage, and may originate from the embryonic erythro-myeloid progenitor (EMP) lineage (Jacome-Galarza et al., 2019). It has been shown that fetal bone-resorbing OCs arise from EMPs in the ossification centers, and are essential for tooth eruption and normal bone development (Gomez Perdiguero et al.,

2015; Jacome-Galarza et al., 2019; McGrath et al., 2015). However, the iterative fusion of circulating HSC-derived blood leukocytes in long-lived multinucleated OCs in bones contribute to the postnatal maintenance of bone-resorbing OCs and bone remodelling (Jacome-Galarza et al., 2019).

Some viruses can directly infect bone cells and influence bone homeostasis. For example, measles virus infection of bone-resorbing OCs has been involved in the pathophysiology of the Paget's disease, and persistent expression of the measles virus nucleocapsid (MVNP) gene in OCs can induce Paget's disease-like bone lesions *in vivo* in transgenic mice (Kurihara et al., 2000; Kurihara et al., 2006). Multinucleated OCs in Paget's disease have shown to have significantly increased number of nuclei per cell, markedly increased in cell size, and display enhanced bone resorption capacity (Kurihara et al., 2000).

OCs has been shown to express CD4 and CCR5 and CXCR4 coreceptors, and support productive infection by HIV-1 (Gohda et al., 2015). HIV-1 infection of OCs results in enhanced bone resorption capacity, and may contribute to bone loss and osteoporosis observed in HIV-1-infected individuals (Raynaud-Messina et al., 2018). Interestingly, activation of CCR5 has been shown to positively regulate OC function, indicating that HIV-1 may directly stimulate bone resorption capacity of OCs (Lee et al., 2017). Moreover, it has also been reported that HIV-1 infection of macrophages induces the formation of multinucleated giant cells (MGCs), and further stimulation with RANKL significantly enhanced HIV-1-induced MGCs formation (Takeda et al., 2015). These HIV-1-induced MGCs express tartrate-resistant acid phosphatase (TRAP), a specific marker of OCs, suggesting a morphologically and functionally similarity to OCs (Takeda et al., 2015).

Chapter 3: Co-receptor usage and HIV-1 tropism

The cell tropism of a certain virus is defined by the cell types which are able to support productive infection by that virus. To fulfil a complete replication cycle, viruses are largely dependent on host cellular pathways, including not only the cell surface receptors for virus entry but also intracellular cofactors required for virus replication. All viruses infect target cells depending on the presence of receptors expressed on the host cell surface. In the case of HIV-1, the major receptor was discovered based on the observation that HIV-1 infection leads to significant loss of CD4⁺ T helper cells in patients and that specific antibody targeting the CD4 surface protein could efficiently block HIV-1 infection (Dalglish et al., 1984; Fahey et al., 1990; Klatzmann et al., 1984).

3.1 HIV-1 entry into host cells

Virus entry of cell-free HIV-1 particles into a specific host cell is mediated by the sequential interactions of the viral surface protein, known as the viral Envelope (Env) protein, the CD4 receptor and a seven transmembrane chemokine co-receptor CCR5 or CXCR4. Both CCR5 and CXCR4 are members of seven-transmembrane G-protein-coupled receptor (GPCR) superfamily, and are structurally formed of a cytoplasmic C-terminal tail, three intracellular loops, three extracellular loops (ECLs) and an extracellular N-terminal segment. The viral envelope glycoprotein (Env) spikes the exterior of virus particles and comprise both the outer surface glycoprotein gp120 (SU) and the transmembrane glycoprotein gp41 (TM). The surface gp120 SU and the transmembrane gp41 TM are non-covalently associated and form a trimer of 3 molecules of gp120 and 3 molecules of gp41 on each spike of viral particles. Binding of the viral gp120 to the cell surface receptor CD4 triggers a conformational change in gp120 and subsequently expose the binding sites for the co-receptor, either CCR5 or CXCR4. The third variable loop region (V3) of gp120 is a key determinant of co-receptor binding (Cardozo et al., 2007; Hoffman and Doms, 1999), and the overall positive charged residues of the V3 loop significantly affects co-receptor binding (Hartley et al., 2005). Following interaction of CD4-bound gp120 to the co-receptor, the fusion peptide of the transmembrane gp41 is exposed and then inserts into the host cell plasma membrane, leading to the formation of fusion pore that subsequently triggers the fusion between viral and cellular membranes, and release of the viral contents into the host cell cytoplasm (Clapham and McKnight, 2001; Wilen et al., 2012) (Figure 11).

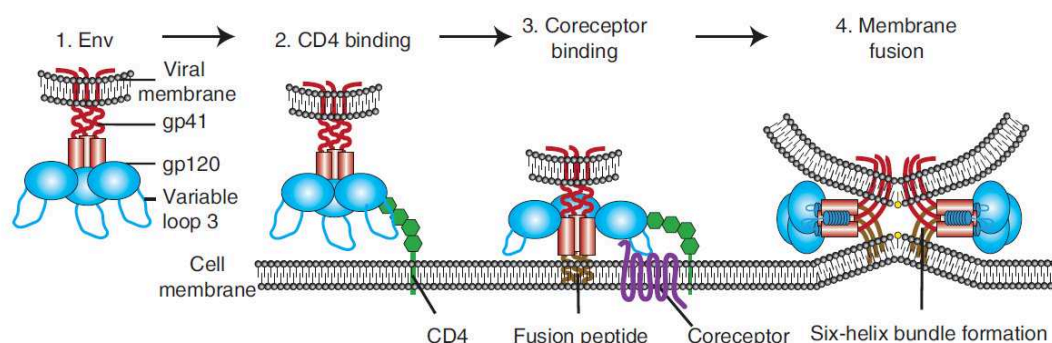


Figure 11: Overview of HIV entry.

To deliver the viral content into host cells, HIV Env, expressed on the surface of virions and comprised of gp120 and gp41 subunits (1), first attaches to the target cell, binding CD4 receptor (2). This induces conformational changes in viral Env, allowing coreceptor binding, which is partially mediated by the V3 loop of Env (3). This could initiate the membrane fusion process as the fusion

peptide of gp41 inserts into the plasma membrane of the target cell, followed by the six-helix bundle formation and complete membrane fusion (4). Adapted from Wilen et al., 2012.

3.2 HIV-1 co-receptor usage and cellular tropism

The co-receptor usage of HIV-1 has been defined as the ability of cell-free viruses to use CCR5 and/or CXCR4 for virus entry into target cells. Based on the co-receptor usage, HIV-1 isolates were initially classified as R5 viruses which use only CCR5 co-receptor for virus entry, X4 viruses using only CXCR4 co-receptor, and R5X4 viruses that are able to use both CCR5 and CXCR4 co-receptors for virus entry (Berger et al., 1998). Consequently, the terms R5, X4 and R5X4 virus became surrogates for functional characterization of cell-free HIV-1 cellular tropism as M-tropic (macrophage-tropic), T-tropic (lympho-tropic), and Dual-tropic, respectively. Although many M-tropic viruses utilize CCR5 as co-receptor for HIV-1 entry, not all R5 viruses are M-tropic. In fact, most of R5 viruses that are replication-competent in primary CD4⁺ T cells cannot replicate in macrophages, thus they are considered as R5 T-tropic (or non-M-tropic) viruses (Sterjovski et al., 2010). In addition, a subset of primary M-tropic viruses can utilize only CXCR4 for HIV-1 entry into macrophages (Simmons et al., 1998; Yi et al., 1998). Although co-receptor usage is closely related to HIV-1 cellular tropism, the level of CD4 expression on target cells is also associated with viral tropism. It has been indeed shown that CD4⁺ T cells and monocytes-derived macrophages (MDMs) differentiated using M-CSF express similar level of CD4 molecules on the cell surface (Lee et al., 1999), but the CD4 density (CD4/ μm^2) on MDMs is significantly lower than that of CD4⁺ T cells due to their larger cell size (Joseph et al., 2014). Since macrophages express low density of CD4 molecules compared to primary CD4⁺ T cells, M-tropic viruses are adapted to use low level of CD4 for virus entry in macrophages. Thus, the ability of HIV-1 primary isolates to utilize distinct density of CD4 receptor and CCR5 or CXCR4 as co-receptor is a more accurate predictor of macrophage tropism than co-receptor usage per se. Indeed, the determinants of HIV-1 cellular tropism are more complicated than the co-receptor specificity of HIV-1.

Actually, HIV-1 co-receptor usage is closely related to, but distinct from, cellular tropism. The distinction between HIV-1 tropism and co-receptor usage is illustrated by the functional studies of R5X4 dual-tropic viruses which utilize both CCR5 and CXCR4 for cell-free HIV-1 entry into target cells. Prototypic R5X4 viral strains such as DH12 and 89.6 infect macrophages efficiently through both CCR5 and CXCR4, but

they are largely restricted to CXCR4-mediated entry into primary CD4⁺ T cells (Loftin et al., 2011; Yi et al., 2005). However, analysis of a panel of R5X4 primary isolates revealed that they can vary greatly in the ability to utilize either co-receptor for efficient infection of CD4⁺ T cells (Loftin et al., 2010; Toma et al., 2010). Three different R5X4 HIV-1 subsets have been revealed that have preferential CCR5 usage, preferential CXCR4 usage, or have equivalent usage of either co-receptor. R5X4 HIV-1 primary isolates with preferential CCR5 usage have an increased ability to exploit limiting levels of CCR5 on primary CD4⁺ lymphocytes (Loftin et al., 2010). These variations in co-receptor preference of R5X4 HIV-1 isolates are in part governed by the V3 loop of the viral gp120 (Loftin et al., 2010), and are suggested to alternatively enhance the tropism of these R5X4 isolates for macrophages in the brain and tropism of for CXCR4-expressing CD4⁺ T cells in the blood and lymphoid tissues. In fact, the co-receptor preference of R5X4 HIV-1 isolates for primary CD4⁺ T cells and macrophages varies dramatically depending on the viral strain and primary cell type (Cashin et al., 2011; Gray et al., 2006; Gray et al., 2009; Loftin et al., 2011; Loftin et al., 2010; Yi et al., 1998; Yi et al., 2005). These studies demonstrated that the viral determinants for macrophage tropism of HIV-1 isolates are significantly more complex than the co-receptor specificity. Thus, the co-receptor usage and cellular tropism of HIV-1 are closely related but are distinct features of the virus.

There are in fact three classes of HIV-1 variants: (1) X4 T cell-tropic viruses, (2) R5 T cell-tropic viruses, and (3) M-tropic viruses. The X4 T cell-tropic viruses exclusively use CXCR4 for virus entry and are able to replicate efficiently in CD4⁺ T cell lines and naïve CD4⁺ T cells, and R5 T cell-tropic viruses use CCR5 and can replicate efficiently in memory CD4⁺ T cells. Most T cell lines and naïve CD4⁺ T cells typically express only CXCR4 and lack CCR5 expression, while memory CD4⁺ T cells are more likely to express both CCR5 and CXCR4 (Bleul et al., 1997; Lee et al., 1999; Ostrowski et al., 1999; Zamarchi et al., 2002). The M-tropic viruses can replicate efficiently in macrophages and possibly other cell types that express low levels of CD4 receptor. Specifically, many M-tropic viruses are actually able to utilize only CCR5 as co-receptor (R5 M-tropic viruses) or only CXCR4 as co-receptor (X4 M-tropic viruses), or even both CCR5 and CXCR4 (R5X4 M-tropic viruses). M-tropic viruses do remain the ability to infect primary T cells (Council and Joseph, 2018; Goodenow and Collman, 2006; Gorry et al., 2014; Joseph and Swanstrom, 2018), and the differential expression of CD4 and co-receptor on cell surface can impact the ability of HIV-1 variants to infect target cells. Monocyte-derived macrophages (MDMs) exhibit significant donor variability in the susceptibility to HIV-1 infection (Joseph et al., 2014; Naif et al., 1998; Sonza et al., 1995), with MDMs from some donors being more permissive to both R5 T cell-tropic viruses and M-tropic viruses (Colin et al., 2018; Lee et al., 1999). The

mechanisms underlying the donor variability of MDMs are currently unknown, and it is unclear whether tissue macrophages *in vivo* exhibit similar donor variability in the susceptibility to HIV-1 infection. It has been shown that expression of CD4 and CCR5 on M-CSF-differentiated MDMs is significantly higher than GM-CSF-differentiated MDMs, while CXCR4 is virtually eliminated on GM-CSF-differentiated MDMs (Lee et al., 1999).

3.3 Alternative co-receptors for HIV entry

It is widely accepted that CCR5 and CXCR4 are the major co-receptors for HIV-1 entry since all HIV-1 strains use at least one or both of these co-receptors for viral entry. However, co-receptors other than CXCR4 and CCR5 could also mediate HIV-1 infection of target cells. It has been shown that some HIV-1 strains can utilize alternative chemokine co-receptors for viral entry, including CCR1, CCR2b, CCR3, CCR4, CCR6, CCR8, CXCR3, CX3CR1, CXCR6 (BONZO/Str133), CXCR7 (RDC1), FPRL1 GPR1, GPR15 (BOB), CMKLR1 (ChemR23) and APJ (Combadiere et al., 1998; Doms and Trono, 2000; Gorry et al., 2007; Ho Tsong Fang et al., 2008; Moore et al., 2004; Pollakis and Paxton, 2012; Samson et al., 1998; Shimizu et al., 2000; Shimizu et al., 2008; Simmons et al., 2000). The different expression patterns of alternate co-receptors on host cell types may support HIV-1 infection and serve as viral reservoirs *in vivo*. For example, CCR3 is broadly expressed by microglia cells in the brain, and HIV-1 infection of microglia can be inhibited by an anti-CCR3 antibody and the CCR3 ligand eotaxin implicating the role of CCR3 in HIV-1 neuro-tropism (He et al., 1997). Recently, it has been shown that CCR4⁺/CCR6⁻ and CCR4⁻/CCR6⁺ T cell subsets are highly permissive for HIV-1 infection (Gosselin et al., 2010; Monteiro et al., 2011). CCR8 has been shown to function as co-receptor for many HIV-1 and HIV-2 primary isolates (Calado et al., 2010; Jinno et al., 1998) and can be frequently used as co-receptor (in addition to CCR5, CXCR4 or both) by these primary isolates. Recently, it has been shown that CCR8 *rs2853699*, CXCL10 *rs56061981* and CXCR6 *rs2234355* polymorphisms are associated with susceptibility to HIV-1 infection in subjects of European descent (Valverde-Villegas et al., 2017). Some studies indicate that CCR3, CCR8 and CXCR6 polymorphisms are associated with AIDS progression in Caucasian subjects (An et al., 2011; Duggal et al., 2003; Limou et al., 2010). Interestingly, novel CCR5 mutations have been identified in sooty mangabeys and are believed to promote the utilization of alternative co-receptors such as GPR15, GPR1 and CXCR6 for the non-pathogenic SIV infection *in vivo* (Riddick et al., 2010). In sooty

mangabeys, CXCR6 expression has been found to be enriched on effector memory CD4⁺ T cells (Tem), but infrequent on central memory CD4⁺ T cells (Tcm) or naïve CD4⁺ T cells (Tn). The expression of low levels of CXCR6 and highly restricted CCR5 expression render central memory CD4⁺ T cells (Tcm) of sooty mangabey relatively resistant to SIV infection (Wetzel et al., 2018). A better preservation of CD4⁺ T cell homeostasis may explain that naturally SIV-infected sooty mangabeys generally do not progress to AIDS in spite of high-level virus replication (Paiardini et al., 2011). HIV-2 is more closely related to SIV from sooty mangabeys (SIVsm)(Lemey et al., 2003). Similarly, the lower expression of CXCR6 in central memory CD4⁺ T cells has been found to be associated with the low pathogenicity of HIV-2 infection (Samri et al., 2019). Together, these studies indicate that the alternative co-receptors of HIV may play important role in viral pathogenesis.

3.4 Co-receptor usage switching

Heterosexual transmission is the predominant mode of spreading of HIV-1 infection (De Cock et al., 2012; Pape et al., 1985), and approximately 80% of heterosexual transmission are caused by a single HIV-1 variant, namely transmitted/founder virus (T/F virus) as evidenced by the analyses of virus complexity in blood during the acute stage of HIV-1 infection (Abrahams et al., 2009; Derdeyn et al., 2004; Haaland et al., 2009; Keele et al., 2008). Most T/F viruses primarily utilize CCR5 for virus entry, are exclusively T-tropic, and replicate efficiently in primary CD4⁺ T cells but not in monocyte-derived macrophages (Ochsenbauer et al., 2012; Ping et al., 2013). Indeed, R5 HIV-1 isolates account for the majority of primary HIV-1 infections regardless of transmission routes. Shortly after transmission, HIV-1 expands within the newly infected individuals followed by an asymptomatic period, and then virus populations diversify and diverge from the T/F virus (Herbeck et al., 2006; Shankarappa et al., 1999). During evolution of HIV-1 populations within the infected individuals, co-receptor switching from CCR5 to CXCR4 occurs in 50% of clade B HIV-1-infected patients (Connor et al., 1997; Schuitemaker et al., 1992), are less common in clade A and C HIV-1-infected individuals (Connell et al., 2008; Esbjornsson et al., 2010; Johnston et al., 2003; Ping et al., 1999), while infection with subtype D HIV-1 maybe associated with higher frequency of coreceptor switching (Huang et al., 2007). The appearance of X4 viruses is correlated with an accelerated CD4⁺ T cell loss and more rapid progression to AIDS, while it is still not clear whether the emergence of CXCR4-using variants cause or indicate more rapid disease

progression (Blaak et al., 2000; Brumme et al., 2005; Hunt et al., 2006; Schuitemaker et al., 1992). Although co-receptor switching is common during HIV-1 infection, it is not a prerequisite for disease progression. A large number of untreated HIV-1-infected naïve individuals progress to AIDS without the emergence of CXCR4-using variants. AIDS also develops in HIV-1-infected individuals with only the presence of CCR5-using viruses, and disease progression has been linked to the appearance of R5 M-tropic viruses which efficiently infect macrophages (Gorry et al., 2005; Li et al., 1999; Tuttle et al., 2002).

Recent studies have shown that, compared to R5 primary isolates from chronic stages of HIV-1 infection, R5 viruses isolated from HIV-1 infected subjects with AIDS display enhanced macrophage tropism, increased affinity for CD4 and CCR5, and reduced sensitivity to CCR5 antagonists (Sterjovski et al., 2007; Wade et al., 2010). Furthermore, R5 Envs isolated from late stages of HIV-1 infection exhibit enhanced fusogenicity and more rapid kinetic of viral entry (Sterjovski et al., 2007). In fact, both X4 and M-tropic viruses evolve from their R5 progenitors and appear at the late stage of AIDS, and are associated with disease progression. Indeed, the M-tropic viruses appear only in late disease and were associated with HIV-associated dementia (Spudich and Gonzalez-Scarano, 2012). Interestingly, several groups showed that macrophages also express CXCR4 co-receptor, as well as CCR5, and support CCR5-independent infection by some X4 and R5X4 primary isolates (Ghaffari et al., 2005; Gorry et al., 2001; Simmons et al., 1998; Simmons et al., 1996; Tuttle et al., 2002; Yi et al., 1999; Yi et al., 1998). Given that co-receptor switching and emergence of CXCR4-using viruses occur at the late stage of infection in 50% of clade B HIV-1-infected individuals and correlated with rapid disease progression (Connor et al., 1997; Schuitemaker et al., 1992), the shift in co-receptor usage is sometimes accompanied by the loss of ability to infect primary macrophages. More often, they retain the property to replicate in macrophages, but whether these CXCR4-using variants also display enhanced macrophage tropism should be carefully determined (Simmons et al., 1998; van Rij et al., 2000). Interestingly, CXCR4 usage and enhanced macrophage tropism were found in viral variants isolated from a laboratory worker (LW) who developed AIDS several years after accidental infection with HIV-1 IIIB which is a prototype X4 T-tropic strain (Beaumont et al., 2001). Similarly, progeny viruses isolated from a neonatal rhesus macaque inoculated with simian-human immunodeficiency virus nonpathogenic strain SHIV-vpu+, which encodes Env of the prototype X4 T-tropic strain HIV-1 IIIB, still use CXCR4 for virus entry but display enhanced macrophage tropism (Hofmann-Lehmann et al., 2002). Similar mutations and enhanced macrophage tropism were also described after *in vivo* passage of chimeric viruses encoding HIV-1 IIIB *env* in pig-tailed and rhesus macaques (Cayabyab et al., 1999; Joag et al., 1997; Liu et al., 1999; Raghavan et al., 1997; Stephens et al., 1997). Thus, the prototype X4 T-tropic IIIB *env*

evolved similarly and acquire the ability to replicate efficiently in macrophages at late stage of infection in three different species, including pig-tailed macaques, rhesus macaques and *Homo sapiens*. Indeed, progeny viruses harboring M-tropic envelope glycoprotein emerge only late in infection, that may indicate the important role of macrophage infection *in vivo*. Perhaps a switch from non-M tropic to M-tropic is more related to disease progression regardless of CCR5 or CXCR4 usage, since both R5 and X4 viruses are able to evolve and efficiently infect macrophages.

3.5 The role of V3 loop in co-receptor usage and viral tropism

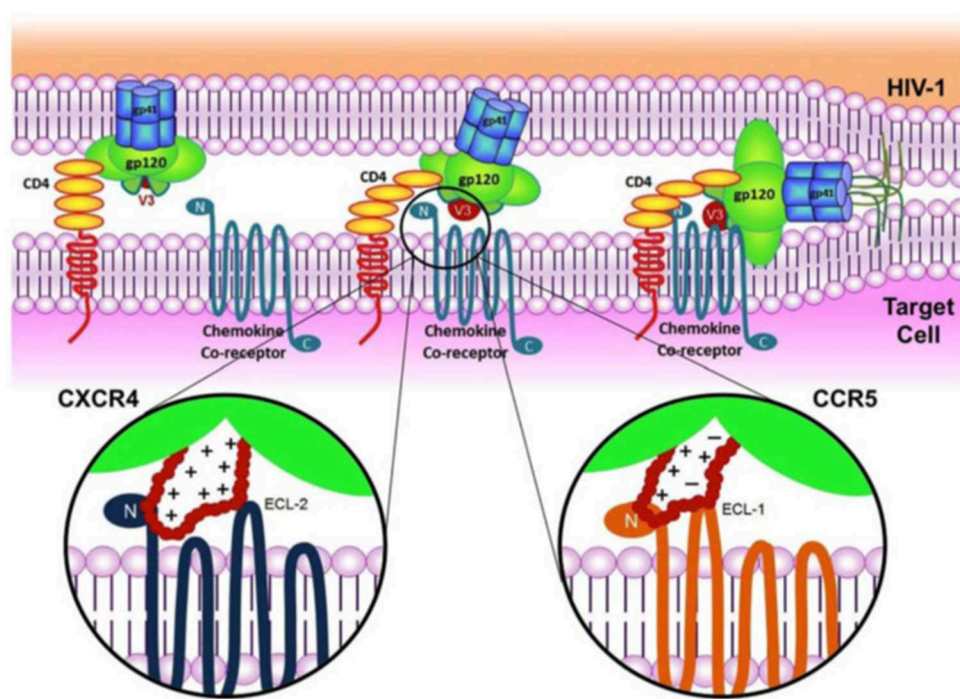


Figure 12: HIV-1 entry mechanism.

HIV-1 entry requires the binding of trimeric gp120 to the CD4 receptor on the target cell plasma membrane. This interaction conformational changes in viral Env that results in enhanced exposure of the V3 loop of viral Env, which is initially concealed by the V1/V2 region of Env. The V3 loop subsequently engages the coreceptor, either CXCR4 (left) or CCR5 (right). The overall charge of V3 loop largely determines the co-receptor usage, with CXCR4-using viruses having a greater net positive charge. In addition, in CXCR4-using viruses the V3 loop of gp120 interacts with the N-terminus and ECL-2 of co-receptor CXCR4, while the V3 loop of CCR5-using viruses interacts with the N-terminus and ECL-1 of the co-receptor CCR5. Finally, co-receptor binding initiates the membrane fusion process. Adapted from Aiamkitsumrit et al., 2014.

The V3 loop is a disulfide-bonded structure corresponding to the third variable region of the viral envelope gp120 glycoprotein containing approximately 35 amino

acid residues. It is highly variable, frequently glycosylated, and plays important role in HIV-1 co-receptor usage and cellular tropism. Early studies identified the V3 region of the viral envelope as the primary determinant of M-tropism vs. T-tropism (Chesebro et al., 1992; O'Brien et al., 1990). For example, T-cell line-adapted (TCLA) strains generally replicate poorly in primary macrophages, whereas the substitution of 20 amino acid residues from the V3 region of M-tropic strains confer a switch of T-tropic of TCLA strains to macrophage tropism (Hwang et al., 1991). It has also been shown that the V3 loop is a major determinant of CCR5 or CXCR4 usage (Carrillo and Ratner, 1996; Hung et al., 1999b). Though the V3 loop is not involved in CD4 receptor binding, the sequence and overall conformation of V3 loop play important roles in defining the extent to which the gp120-CD4 complex interacts with CCR5 and/or CXCR4 co-receptors (Wyatt et al., 1993) (Figure 12). Current models of viral Env binding to co-receptors suggest that the tip of the V3 loop interacts with the second ECL2 region, whereas the gp120 bridging sheet and V3 loop stem interact principally with the N-terminal segment of co-receptors (Brelot et al., 1999; Cormier and Dragic, 2002; Farzan et al., 1999; Huang et al., 2005). R5 HIV-1 isolates that preferentially binds to co-receptor CCR5 usually have low numbers of positively charged residues in the V3 loop, while CXCR4-using viruses typically have V3 regions with greater net numbers of positively charged amino acids, especially at position 11 or position 25 of the V3 loop (Briggs et al., 2000; Jensen et al., 2003; Milich et al., 1993; Resch et al., 2001). R5X4 viruses can use both CCR5 and CXCR4 for virus entry and have similar V3 charge pattern with X4 viruses. Thus, the determinants that control dual co-receptor usage are much more complicated and involve multiple discontinuous regions of the viral envelope glycoproteins (Cho et al., 1998; Hung et al., 1999a; Smyth et al., 1998). In general, co-receptor switching from CCR5 to CXCR4 is related to an increase of positively charged residues within the V3 loop of gp120 (Fouchier et al., 1992; Groenink et al., 1995; Shioda et al., 1994).

3.6 R5 M-tropic viruses exhibit higher affinity for CD4 receptor

As macrophages express less CD4 receptors on cell surface compared to CD4⁺ T cells (Lewin et al., 1996; Wang et al., 2002), most R5 HIV-1 primary isolates poorly infect macrophages (Gorry et al., 2001). However, R5 M-tropic viruses have adopted to use low level of CD4 receptor for virus entry in macrophages. The role of CD4 in defining HIV-1 entry phenotype in target cells has been clarified and assayed using the Affinofile cells which express a broad range of CD4 receptor and co-receptor CCR5

(Johnston et al., 2009). Further studies have confirmed that most blood-derived viruses are CCR5-using viruses that inefficiently infect cells expressing low CD4 density (Joseph et al., 2014; Parrish et al., 2012; Ping et al., 2013). It has been shown that CD4⁺ T cells and M-CSF-derived MDMs express similar level of CD4 molecules on the cell surface (Lee et al., 1999), but the CD4 densities (CD4/ μm^2) on MDMs are significantly lower than those of CD4⁺ T cells due to their larger cell size (Joseph et al., 2014). Thus, the true R5 M-tropic viruses use CD4 much more efficiently, with higher affinity of gp120, and are approximately 30-fold more efficient at infecting macrophages than R5 T-tropic viruses (Arrildt et al., 2015).

3.7 R5 M-tropic viruses exhibit reduced dependence on CCR5 expression

Since HIV-1 entry into host cells initiates when gp120 binds to CD4 molecule on cell surface, and then triggers the conformation change in viral envelope, the subsequent binding to CCR5 is critical for stabilizing the CD4-induced conformational changes and play essential role in HIV-1 entry (Shaik et al., 2019). Several studies have shown that enhanced gp120-CCR5 interactions contributes to macrophage tropism of blood-derived CCR5 using viruses (Cashin et al., 2011; Sterjovski et al., 2010), whereas reducing the affinity of HIV-1 Env for CCR5 attenuates CCR5-mediated M-tropism (Roche et al., 2011). Indeed, increasing expression of CCR5 levels on macrophages leads to partial or complete restoration of efficient macrophage infection by some non-M-tropic R5 viruses, depending on the viral strains. However, overexpression of CCR5 on macrophages has no effect on entry of R5 M-tropic viruses isolated from HIV-1 infected individuals with advanced disease, suggesting that a threshold of CCR5 level could be a major rate-limiting step in HIV-1 entry into macrophages (Gray et al., 2005). Similarly, overexpression of CXCR4 levels on macrophages largely rescues macrophage infection by some laboratory-adapted X4 T cell-tropic strains, but only modestly enhance macrophages infection by CXCR4-using primary isolates (Dimitrov et al., 1999; Tokunaga et al., 2001). These studies indicate that macrophage tropism maybe associated with the level of co-receptor expression on macrophages as well as the relative affinity of the viral gp120 envelope glycoprotein for the co-receptor, since it has been shown that macrophages express even lower density of CCR5 ($\sim 1.4 \text{ ABS}/\mu\text{m}^2$) than CD4 ($\sim 3.4 \text{ ABS}/\mu\text{m}^2$) (Joseph et al., 2014).

Maraviroc (MVC), a specific antagonist of CCR5, can bind to and modify conformation of the CCR5 extracellular loops (ECLs) and therefore blocks virus entry

of HIV-1. However, treatment of patients with MVC could result in virological rebound due to the emergence of MVC resistant viruses which can recognize MVC-bound conformation of CCR5 on the cell surface (Kuhmann et al., 2004; Pugach et al., 2007; Westby et al., 2007). Studies on *in vitro*-generated HIV-1 MVC-resistant strains show these viral strains exhibit increased CCR5 dependence, and that the altered gp120-CCR5 engagement by MVC-resistant variants dramatically attenuates macrophage tropism (Roche et al., 2011). Similar findings were also observed using MVC-resistant primary isolates from plasma of HIV-1 infected subjects who developed drug resistant during clinical trials of maraviroc (Flynn et al., 2013; Roche et al., 2013).

Interestingly, it has also been shown that certain HIV-1 M-tropic strains derived from the central nervous system (CNS) of patients exhibit reduced dependence on CD4/CCR5 and increased affinity for CCR5, suggesting that HIV-1 variants with enhanced CCR5 affinity may contribute to neurodegenerative diseases in HIV-1 infected patients (Gorry et al., 2002). However, the reduced dependence on CCR5 of brain-derived R5 M-tropic viruses is controversial. Some studies indicates that the macrophage tropism of R5 viruses predominantly correlates with sensitivity of viral envelopes to reagents that target gp120-CD4 interactions but not with reagents that block gp120-CCR5 interactions (Peters et al., 2008), suggesting that the macrophage tropism of R5 viruses is not related to the sensitivity to CCR5 antagonists (Martin-Garcia et al., 2006; Peters et al., 2004; Peters et al., 2008). Similarly, it has been shown that M-tropic viruses and R5 T cell-tropic viruses do not differ in their ability to utilize low levels of co-receptor CCR5 for virus entry, and have the ability to utilize alternative conformation of CCR5, i.e, the MVC-bound form of CCR5 (Joseph et al., 2014). An increased affinity of gp120 for the CD4 receptor may reduce the requirement of a high affinity for CCR5 (Platt et al., 1997). Brain-derived R5 envelopes tend to be more sensitive to CCR5 antagonists compared to non-M-tropic R5 envelopes isolated from lymph nodes (LNs) and blood (Peters et al., 2008).

3.8 Macrophages express CXCR4 and can support productively infection by X4 M-tropic viruses

All strains of primary HIV-1 isolates could replicate on primary CD4+ T cells, but vary dramatically in their ability to productively infect macrophages. Macrophages are important target for HIV-1 infection, but they express low density of CD4 molecules compared to primary CD4+ T cells, and M-tropic viruses are adapted to use low level of CD4 for viral entry in macrophages. In fact, primary macrophages express CXCR4,

as well as CCR5, and support entry of X4 viruses and R5X4 viruses (Simmons et al., 1998; Yi et al., 1999; Yi et al., 1998). It has been shown that the expression of CXCR4 on MDMs is differentially regulated by the chemokine stimulation used to differentiate from blood monocytes. M-CSF is the most potent inducer of CXCR4 expression, whereas GM-CSF strongly suppress CXCR4 expression on MDMs (Lee et al., 1999). Some primary X4 isolates and R5X4 HIV-1 strains are capable of infecting macrophages very efficiently via CXCR4 pathway (Aquino-De Jesus et al., 2000; Foda et al., 2001; Simmons et al., 1998). In fact, the progeny viral strains isolated from 40-50% of subtype B HIV-1-infected patients are CXCR4-using at the late stage of infection and are associated with rapid disease progression (Bjorndal et al., 1997; Connor et al., 1997). CXCR4-using primary strains can also be found in the CNS of HIV-1-infected individuals with AIDS, and uses CXCR4 exclusively for efficient infection of primary macrophages, indicating that CXCR4-using viruses may also participate in AIDS neuropathogenesis (Yi et al., 2003). X4 or R5X4 variants could emerge at late stages of infection, and these viruses may colonize naïve T-cell populations which express exclusively CXCR4 (Blaak et al., 2000; Ostrowski et al., 1999). Therefore, the broadened cellular tropism of X4 viruses and R5X4 viruses could contribute to more rapid disease progression in HIV-1-infected individuals who displayed co-receptor switch. When blood CD4⁺ T cells are significantly depleted at late stage of infection, tissue macrophages are likely to be the major cellular targets of CXCR4-using viruses during immunodeficiency. Interestingly, it has also been reported that CXCR4-using virus have adapted to using exclusively CXCR4 for macrophage infection in HIV-1-infected patient homozygous for CCR5-Δ32 (Naif et al., 2002). Several studies performed with SHIV-infected macaques indicated that tissue macrophages are responsible for the maintenance of high plasma viral loads when CD4⁺ T cells are depleted (Igarashi et al., 2001). The X4 T-tropic viral inoculum (SHIVDH12R) displayed enhanced macrophage tropism through adaptive alterations in viral envelope to facilitate efficient CXCR4-mediated infection of tissue macrophages (Igarashi et al., 2001). Similar mutations and enhanced macrophage tropism were also described after *in vivo* passage of chimeric viruses encoding HIV-1 IIIB Env in pig-tailed and rhesus macaques (Cayabyab et al., 1999; Joag et al., 1997; Liu et al., 1999; Raghavan et al., 1997; Stephens et al., 1997). Thus, the prototype X4 T-tropic IIIB *env* evolved similarly and acquire the ability to replicate efficiently in macrophages at late stage of infection in different species. In addition, further studies have shown that X4 M-tropic viruses are more likely to emerge in HIV-1-infected individuals with low CD4⁺T cell counts, indicating that tissue macrophages may be targeted by X4 viral strains and contribute to HIV-1 systemic infection at late stage of diseases in humans (Ghaffari et al., 2005).

3.9 Concluding remarks

In conclusion, analysis of the co-receptor utilization of the gp120 viral envelope glycoprotein has revealed three different types of HIV-1 variants: (1) X4 T cell-tropic viruses, (2) R5 T cell-tropic viruses, and (3) M-tropic viruses. R5 T cell-tropic viruses are the most common form of HIV-1 isolates which have adapted to enter into CD4⁺ memory T cells. X4 T cell-tropic viruses could emerge at the late stage of HIV-1 infection and are adapted to entry into naïve CD4⁺ T cells and T-cell lines. M-tropic viruses have adapted to efficiently infect macrophages with limiting levels of CD4 receptor and appear later in the course of infection.

Both CD4 and co-receptor expression levels can influence the efficiency of HIV-1 entry into target cells. When CD4 receptor is expressed at high levels on the cell surface, whereas low levels of co-receptor CCR5 or CXCR4 are sufficient to support HIV-1 entry into target cells. However, if CD4 receptor is present on cell surface at low levels, higher levels of co-receptor expression are needed to support virus entry (Kozak et al., 1997). Since macrophages have low CD4 densities on the cell surface, most blood derived R5 T-tropic viruses generally require high level of CD4 molecules for entry and thus inefficiently infect macrophages, while the true M-tropic viruses have evolved to utilize CD4 receptor much more efficiently to infect macrophages. Interestingly, it has been suggested that an increased affinity of gp120 for CD4 receptor may reduce the requirement of a high affinity for CCR5 (Platt et al., 1997). Thus, the relative affinity for CD4 receptor and co-receptor of the viral envelope could be the major rate limiting step in virus entry into target cells. The co-receptor usage and viral tropism of HIV-1 have important implications for HIV-1 pathogenesis and disease progression. For example, HIV-1 infected patients that have low levels of co-receptor CCR5 expression exhibit a significant delay in disease progression (Huang et al., 1996; Michael et al., 1997; Reynes et al., 2001). A 32-nucleotide deletion in the CCR5 coding sequence (CCR5- Δ 32) renders the transcript unstable and the variant protein cannot be detected in individuals homozygous for this deletion. Critically, CCR5- Δ 32 cells cannot be infected by CCR5-using viruses, and these individuals remained HIV-1-negative despite frequent exposure to HIV-1 (Liu et al., 1996; Samson et al., 1996). However, the cost of CCR5 loss-of-function mutation could associated with a significantly increase of mortality rate (Luban, 2019; Wei and Nielsen, 2019).

CCR5 is the major co-receptor for HIV-1 transmission, since most T/F viruses primarily utilize CCR5 for virus entry and are defined as exclusively T-tropic strains which replicate efficiently in primary CD4⁺ T cells but not in MDMs (Ochsenbauer et al., 2012; Ping et al., 2013). Although CD4-positive cells isolated from CCR5-negative

individuals are refractory to infection by CCR5-using viruses, they are readily permissive to CXCR4-using viruses (Liu et al., 1996; Rana et al., 1997; Samson et al., 1996; Zhang et al., 1997). However, it remains poorly understood why individuals homozygous for CCR5-Δ32 are only rarely infected by X4 viruses that utilize only CXCR4 for viral entry (Balotta et al., 1997; Biti et al., 1997; O'Brien et al., 1997; Theodorou et al., 1997). It appears that CXCR4-using viruses are rarely transmitted for unknown reasons (Joseph et al., 2015; Shaw and Hunter, 2012). Similarly, low levels of CCR5 expression is associated with low levels of SIV infection of central memory CD4⁺ T cells of sooty mangabey, and a better preservation of CD4⁺ T cell homeostasis may explain that naturally SIV-infected sooty mangabeys generally do not progress to AIDS in spite of high-level virus replication (Paiardini et al., 2011).

HIV strains can exhibit distinct viral and cell tropisms that have important influence on HIV pathogenesis and disease progression. While sooty mangabey monkeys (SMM) and African green monkeys (AGM) are natural hosts for SIV_{smm} and SIV_{agm}, respectively, SIV infection in these monkeys typically does not progress to AIDS despite high plasma viral load. SIVs generally utilize CD4 and CCR5 for virus entry into target cells *in vivo*, while other molecules such as CXCR6 and GPR15 also function as co-receptors for SIV *in vitro*. Recently, it has been shown that CXCR6 usage, in addition to CCR5, is a shared feature for SIV_{agm} and SIV_{smm} (Wetzel et al., 2018). The levels of CCR5 expression is extremely low on primary CD4⁺ T cells from natural hosts for SIV, including AGMs and SMMs (Pandrea et al., 2007). Low levels of CCR5 expression are associated with low levels of SIV infection in central memory CD4⁺ T cells of SM monkeys and it was postulated that the restricted CCR5 expression would protect critical target cells from SIV infection (Cartwright et al., 2014; Paiardini et al., 2011; Pandrea et al., 2007). However, it is still not clear that how could these low CCR5 expressing CD4⁺ T cells support high levels of viremia in natural hosts during SIV infection.

Recently, a CCR5 deletion common in sooty mangabeys has been identified and may promote the utilization of alternative co-receptors such as GPR15, GPR1 and CXCR6 for the nonpathogenic SIV infection *in vivo* (Riddick et al., 2010). SMMs are known to support productive SIV_{smm} infection *in vivo* through CCR5-independent pathways (Riddick et al., 2010), and the alternative co-receptor CXCR6 could mediate SIV_{smm} entry into SMM primary lymphocytes *ex vivo* (Elliott et al., 2015). In SMMs, CXCR6 expression has been found to be enriched on effector memory CD4⁺ T cells, but infrequent on central memory CD4⁺ T cells or naïve CD4⁺ T cells. The low levels of CXCR6 expression and highly restricted CCR5 expression renders central memory SMM CD4⁺ T cells relatively resistant to SIV_{smm} infection (Wetzel et al., 2018). In contrast, the effector memory CD4⁺ T cells of SMM express high levels of CXCR6

and may support productive SIVsmm infection, resulting in high plasma viral load. Therefore, the CXCR6 usage may direct SIV to target CXCR6 expressing cell types (e.g. effector memory T cells) that are easily replenished and whose loss during SIV infection is less likely to lead to AIDS development (Wetzel et al., 2018). Thus, CXCR6 utilization is a unique feature of non-pathogenic viruses.

HIV-2 is more closely related to SIVsm(Lemey et al., 2003), and compared to HIV-1 infection, HIV-2 infection is characterised by slower heterosexual transmission rates, longer asymptomatic stages, and slower declines in CD4 T cell counts and disease progression, and finally lower mortality rates (Alabi et al., 2003; Drylewicz et al., 2008; Hansmann et al., 2005; Holmgren et al., 2007; Kanki et al., 1994; Marlink et al., 1994; Poulsen et al., 1997; van Tienen et al., 2011). HIV-2 infection is thus postulated as a natural model of attenuated HIV infection, similar to the SMM model of attenuated SIV disease. Although HIV-2 can utilize the same co-receptors CCR5 and CXCR4 as HIV-1 (Choe et al., 1996; Doranz et al., 1996; McKnight et al., 1994), HIV-2 retains the ability to utilize CXCR6 as an alternative co-receptor (Blaak et al., 2005), whereas HIV-1 lost the ability to use CXCR6, and it was proposed that this could be a signature of pathogenic primate lentiviruses (Wetzel et al., 2018). In fact, CXCR6 is preferentially used by HIV-2 in aviremic infected individuals (Blaak et al., 2005). More recently, the lower expression of CXCR6 in central memory CD4⁺ T cells has been found to be associated with the low pathogenicity of HIV-2 (Samri et al., 2019). In HIV-2 infected individuals, it has been shown that the expression of CXCR6 on transitional memory CD4⁺ T cells is 94-fold higher than on central memory CD4⁺ T cells, and HIV-2 reservoirs exhibit a preferential distribution in these transitional memory CD4⁺ T cells (Samri et al., 2019). Therefore, the CXCR6 usage of HIV-2 may direct the virus to target transitional memory CD4⁺ T cells which highly express CXCR6, but not the central memory CD4⁺ T cells with limited CXCR6 expression. A better preservation of central memory CD4⁺ T cells may contribute to slower disease progression in HIV-2-infected patients. Unlike HIV-2, HIV-1 is closely related to SIV from chimpanzees (SIVcpz), and SIVcpz-infected chimpanzees (CPZ) generally develop AIDS. Similar to other pathogenic lentiviruses, HIV-1 has been shown to lose the ability to utilize CXCR6 as alternative co-receptor (Blaak et al., 2005; Wetzel et al., 2018), and this may contribute to faster disease progression than HIV-2 infection. Indeed, the transitional memory and central memory CD4⁺ T cells contribute to the major cellular reservoirs in HIV-1-infected patients (Chomont et al., 2009), while the preservation of central memory (Tcm) CD4⁺ T cells is a key determinant for long-term control of HIV-1 pathogenesis (de Masson et al., 2014). Together, these studies indicate that the alternative co-receptor usage for HIV may play important role in viral pathogenesis.

Chapter 4: Mechanisms for HIV-1 cell-to-cell transmission

To spread within an infected host, HIV-1 has evolved different strategies for insuring viral transmission and persistence. First, cell-free virus particles released from HIV-1-infected cells may infect neighbouring or distant cells. Second, virus particles or viral genetic material could be transferred directly from infected cells to neighbouring uninfected cells through cell-to-cell contacts. However, HIV-1 spread via cell-free virus particles is rather inefficient and rate limited by fluid uptake and virion attachment. Moreover, freely diffusing virions are vulnerable to antibody neutralization and antibody-mediated opsonization (Abela et al., 2012; Marechal et al., 2001; Sigal et al., 2011). In addition, cell-free virus particles must survive the innate and adaptive immune defenses to successfully establish infection in a new host. To circumvent these challenges, HIV-1 has adapted to benefit from cell-to-cell interactions for virus dissemination without appearing as cell-free virus particles, a mechanism described as HIV-1 cell-to-cell transmission. HIV-1 cell-to-cell transmission between CD4⁺ T cells mainly involves the formation of virological synapses (Alvarez et al., 2014; Chen et al., 2007; Hubner et al., 2009; Jolly et al., 2004; Jolly et al., 2007a; Rudnicka et al., 2009), but also through other membrane structures, such as nanotubes or filopodia, and has been estimated to be 100-1000 times more efficient than a cell-free virus infection process (Dimitrov et al., 1993; Sato et al., 1992). Interestingly, infectious viral particles seem to be not required for HIV-1 cell-to-cell transfer, Vif-mutated virus has been shown to be 1000 times less infectious than wild type virus in non-permissive cells, however it can spread by cell-to-cell transfer as efficiently as wild type virus (Strebel et al., 1987), although this observation remains controversial (Zotova et al., 2019). This mode of viral spread significantly enhance the efficiency of HIV-1 transmission several orders of magnitude compared with viral dissemination by cell-free particles (Carr et al., 1999; Chen et al., 2007; Dimitrov et al., 1993; Iwami et al., 2015; Maldarelli et al., 1995; Martin et al., 2010; Zhong et al., 2013a). In addition, since viral particles are concentrated at the sites of cell-to-cell contacts, HIV-1 cell-to-cell transmission could provide protection from neutralizing antibodies (Abela et al., 2012; Chen et al., 2007; Dufloo et al., 2018; Ganesh et al., 2004; Gombos et al., 2015; Gupta et al., 1989; Li et al., 2017; Malbec et al., 2013; Massanella et al., 2009; McCoy et al., 2014; Reh et al., 2015; Zhong et al., 2013a), and may overcome the inhibitory effects of several anti-viral restriction factors for HIV-1 such as TRIM5 α and tetherin/BST2 under certain circumstances (Casartelli et al., 2010; Coleman et al., 2011; Giese and Marsh, 2014; Jolly et al., 2010; Kuhl et al., 2010; Richardson et al., 2008; Zhong et al., 2013a).

HIV-1 cell-to-cell transmission may also influence the efficacy antiretroviral therapies. It has been reported that some antiretroviral drugs did not inhibit efficiently HIV-1 cell-to-cell spread and ongoing virus replication (Agosto et al., 2014; Duncan et al., 2013; Kim et al., 2018; Sigal et al., 2011; Titanji et al., 2013). Early studies have shown that HIV-1 cell-to-cell transmission induces more rapid apoptosis compared to cell free HIV-1 infection (Maldarelli et al., 1995), but more recently, it has been shown that HIV-1 cell-to-cell transmission to quiescent CD4⁺ T cells rather induce pyroptosis, a highly inflammatory form of programmed cell death, which was proposed to contribute to CD4⁺ T cells depletion in lymphoid tissue (Doitsh et al., 2010; Doitsh et al., 2014; Galloway et al., 2015; Monroe et al., 2014; Munoz-Arias et al., 2015). While HIV-1 ongoing replication and latency have been proposed to maintain drug-insensitive HIV-1 reservoirs during antiretroviral therapy (Pierson et al., 2000), cell-to-cell transmission of HIV-1 could also contribute to the establishment of HIV-1 latency. Since HIV-1 cell-to-cell transmission between T cells results in highly efficient infection of CD4⁺ T cells, it is reasonable to question whether this mode of viral transmission support infection of resting CD4⁺T cells and contribute viral latency. Recent studies have shown that HIV-1 cell-to-cell transmission leads to the establishment of HIV-1 latency by directly infecting resting CD4⁺ T cells (Agosto et al., 2018; Kumar et al., 2015; Schilthuis et al., 2018; Shen et al., 2013).

Different modes of virus cell-to-cell infection through different intercellular structures enabling close contacts between HIV-1-infected cells and non-infected target cells have been described over the past years. Intercellular transfer of viral material has been described mainly through establishment of the virological or infectious synapses, and also using membrane protrusions such as tunneling nanotubes (TNTs) and filopodia, phagocytosis and cell-to-cell fusion processes (Bracq et al., 2018; Chen, 2012; Jolly and Sattentau, 2004; Mothes et al., 2010; Pedro et al., 2019; Sattentau, 2008; Zhong et al., 2013b).

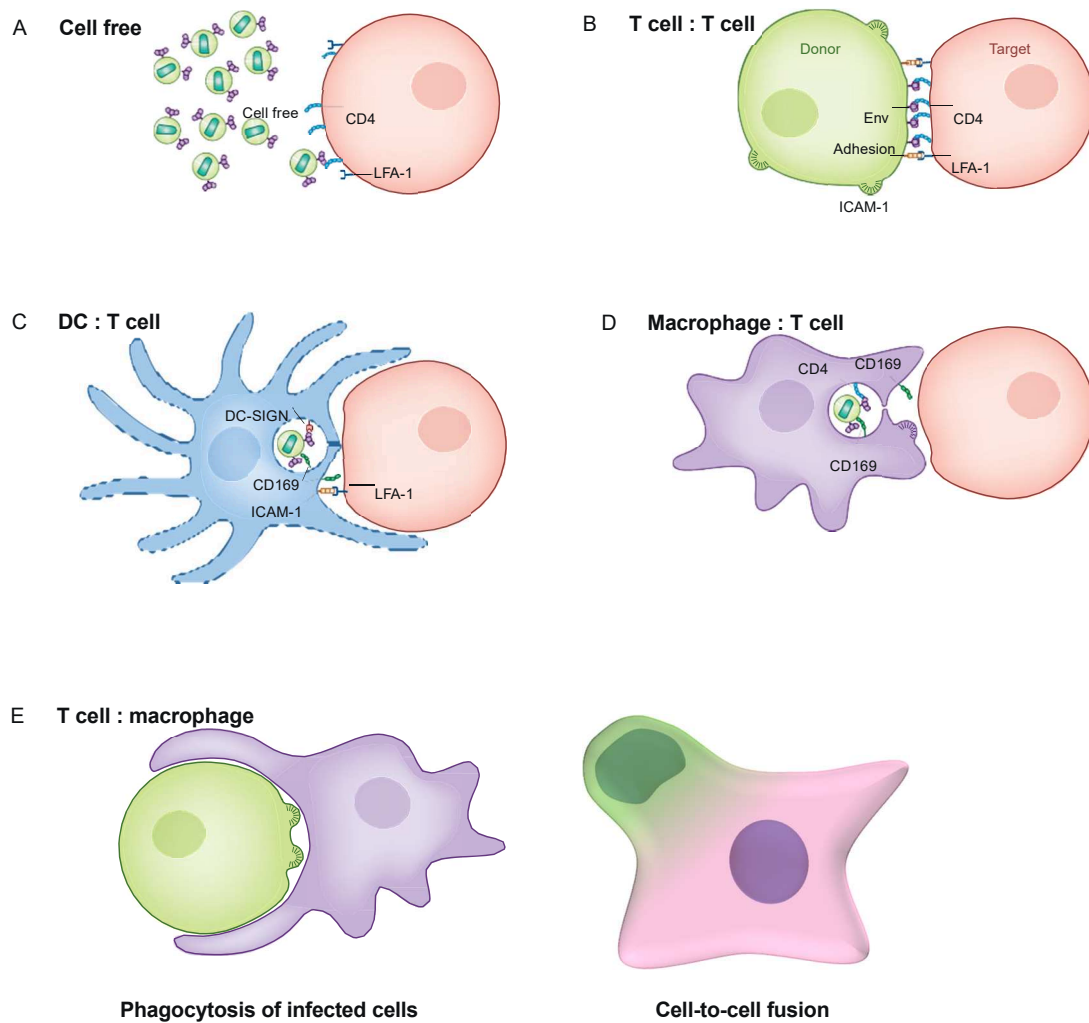


Figure 13: Routes of HIV-1 dissemination.

(A) Cell-free infection is mediated by virus particles released to the extracellular milieu from the HIV-1 infected cell. (B) The T cell-to-T-cell virological synapse (VS) forms when an HIV-1 infected CD4 T cell binds to an uninfected CD4 T cell through the engagement of viral envelope glycoprotein (Env) and CD4 receptor. Cell adhesion molecules are also recruited to the virological synapse. (C) The dendritic cell-to-T-cell infectious synapse forms when virus-carrying DC encounters an uninfected CD4 T cell and subsequently transmit virus particles to uninfected CD4 T cell. (D) Macrophage-to-T-cell infectious synapse occurs when macrophages internalize virus particles within the plasma membrane invaginations via CD169/Siglec-1. (E) T cell-to-macrophage interaction occurs either through phagocytosis of HIV-1 infected T cells or cell-to-cell fusion between an infected T cell and an uninfected macrophage. Adapted from Law et al., 2016.

4.1 The T-cell virological synapse

The formation of the virological synapse between T cells is the major and well-described route for virus cell-to-cell transmission, and was first described in the context of human T cell leukemia virus-type 1 (HTLV-I) infection as a close and organized cell-cell junction between an infected donor cell and a target cell, enabling the transfer of viral material between the two cells (Igakura et al., 2003). The virological synapse is named for its homologies with the immunological synapses formed between APCs and T cells for the antigen presentation. The formation of HIV-1 virological synapses is dependent on the interaction between the viral envelope on HIV-1-infected donor cells and the CD4 receptor on uninfected target cells (Chen et al., 2007; Jolly et al., 2004; Vasiliver-Shamis et al., 2009; Vasiliver-Shamis et al., 2008). This interaction allows the recruitment of the viral Gag polyprotein precursor to the intercellular interface and triggers the recruitment of co-receptors, CCR5 or CXCR4, adhesion molecules LFA-1, ICAM-1, and other cell surface molecules such as tetraspanins to the site of intercellular contact, for stabilization of the virological synapse and efficient viral transfer (Jolly et al., 2004; Jolly et al., 2007a; Starling and Jolly, 2016). While the recruitment of adhesion molecules to the virological synapses is well established, their specific role and requirement for HIV-1 cell-to-cell transmission remains to be determined. The cell-to-cell contacts could initiate signaling cascades in both HIV-1 infected cells and the non-infected target cells (Len et al., 2017). Additional cell surface molecules such as tetraspanins (Jolly and Sattentau, 2007), GM1 and other lipid raft-related proteins (Jolly and Sattentau, 2005) are also recruited to the virological synapses in a filaminA- and actin-dependent manner (Jimenez-Baranda et al., 2007; Jolly et al., 2004). Finally, specific re-organization of the cytoskeleton is required for the formation of the virological synapse and efficient viral transfer. Engagement of the CD4 receptor by the gp120 viral envelope glycoprotein triggers actin cytoskeleton remodeling and microtubule polarization toward the virological synapses (Jolly et al., 2007b). The formation of virological synapses between T cells is dependent of actin cytoskeleton remodeling and involves cell-to-cell adhesion, this process is facilitated by the involvement of adhesion molecules, such as ICAM-1, ICAM-3 and LFA-1 which are concentrated at the site of cell-to-cell contact. The re-organization of actin cytoskeleton and viral assembly lead to the transfer of numerous virus particles to non-infected target cells effectively increasing the efficiency of target cells infection (Del Portillo et al., 2011; Law et al., 2016; Russell et al., 2013; Zhong et al., 2013a).

The CD4-Env interaction at the virological synapse between T cells could trigger a durable adhesion at the site of cell-to-cell contact. Although the major role of Env is to mediate virus-membrane fusion, the engagement on the target cell of the CD4 receptor by Env typically does not trigger cell-to-cell fusion (Chen et al., 2007; Hubner et al., 2009; Jolly et al., 2007b). The lack of cell-to-cell fusion indicates that this fusion process is finely regulated during the formation of virological synapse between T cells (Bracq et al., 2018).

It was reported that several cell surface proteins of the tetraspanin membrane protein family, such as CD9, CD81 and CD63, are recruited at the site of cell-to-cell contact during the virological synapse formation and are proposed to be involved in the regulation of the cell fusion process (Gordon-Alonso et al., 2006). Furthermore, Ezrin, a member of the ezrin/radixin/moesin (ERM) family, is also recruited at the T cell virological synapse and may contribute to the inhibition of cell-to-cell fusion (Roy et al., 2014).

In addition to Env/CD4 interactions, other adhesion molecules expressed on cell surface could also facilitate the formation of the T cell virological synapse. The leukocyte function-associated antigen 1 (LFA-1) and its binding partners, including the intercellular adhesion molecule 1 (ICAM-1) and ICAM-3, have been shown to promote HIV-1-induced virological synapse formation (Jolly et al., 2007a; Rudnicka et al., 2009; Vasiliver-Shamis et al., 2008). Blocking antibodies targeting these adhesion molecules on cell surface could abrogate virological synapse-mediated conjugate formation and inhibit cell-to-cell transfer of HIV-1. However, contradictory findings reveal that HIV-1 cell-to-cell transmission between infected T cells and non-infected T cells could occur in the absence of these adhesion molecules; in some cases, blocking of cell surface LFA-1 from binding to ICAMs even enhances HIV-1 cell-to-cell transmission (Puigdomenech et al., 2008). Thus, the role of adhesion molecules in the formation of the T cell virological synapse requires further investigation.

In addition to the LFA-1/ICAMs interactions at the virological synapse, Env expressed on HIV-1-infected donor CD4⁺ T cells could also be recognized by the integrin $\alpha 4\beta 7$ expressed on the target T cells, which has been shown to correlate with enhanced susceptibility to HIV-1 infection (Arthos et al., 2008; Cicala et al., 2009; Ding et al., 2015; Nawaz et al., 2011). When Env binds to integrin $\alpha 4\beta 7$, it colocalizes with LFA-1 and CD4 at the site of cell-to-cell contact (Arthos et al., 2008). This Env- $\alpha 4\beta 7$ interaction could trigger the activation of LFA-1 on target CD4⁺ T cells, and then the active conformation of LFA-1 promotes the formation of the virological synapse and enhance HIV-1 infection (Hioe et al., 2011) (Figure 13). LFA-1 activation by the Env- $\alpha 4\beta 7$ interaction enhances the efficiency of HIV-1 infection by promoting virological synapse formation rather than capture of cell-free viral particles (Ding et al., 2015), since it has been reported that the expression of LFA-1 on target cells leads to enhanced

cell-free infection by HIV-1 (Hioe et al., 2001). The integrin $\alpha 4\beta 7$ has also been found to be involved in the migration of lymphocytes to mucosal tissues, suggesting this process may be relevant in HIV-1 infection of CCR5 expressing memory CD4 T cell in the vaginal mucosa and gut (Joag et al., 2016; Mavigner et al., 2012). Furthermore, targeting integrin $\alpha 4\beta 7$ has been shown to efficiently reduce intravaginal SIV transmission and also protects gut-associated lymphoid tissue from SIV infection in macaques (Byrareddy et al., 2014).

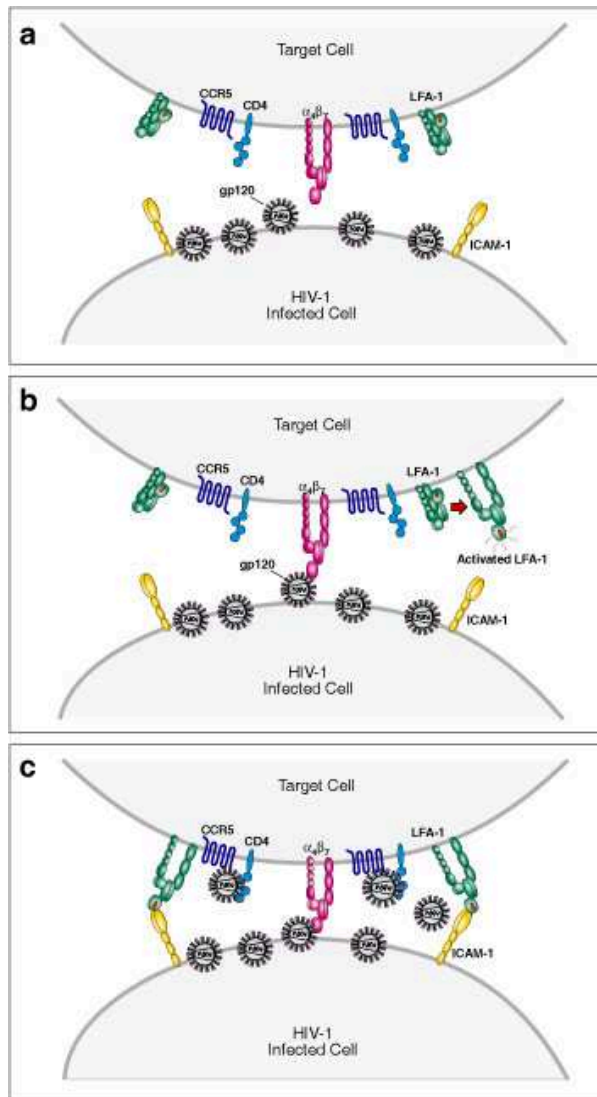


Figure 14: The formation of Virological Synapse (VS) upon engagement of integrin $\alpha 4\beta 7$ by HIV-1 envelope.

An HIV infected donor cell encounters a susceptible target cell expressing high levels of integrin $\alpha 4\beta 7$ (a). HIV-1 envelope on the cell surface of infected donor cell interacts with integrin $\alpha 4\beta 7$ on the target cell and triggers the activation of LFA-1 (b). The active conformation of LFA-1 binds to its ligand ICAM-1 (c) and promotes the formation of Virological Synapse (VS). Adapted from Cicala et al., 2010.

4.2 HIV-1 infectious synapses

The infectious synapse is formed between virus-carrying donor cells such as DCs that can significantly capture HIV-1 virions independently of CD4 receptor and without being themselves productively infected, and subsequently transfer the virus particles to the target cells through cell-to-cell contact (Kijewski and Gummuluru, 2015; McDonald, 2010; McDonald et al., 2003). The formation of the infectious synapses (ISs), is typically related to HIV-1 transmission from antigen-presenting cells (APCs) such as DCs and macrophages toward target CD4⁺T cells, and is also known as HIV-1 *trans*-infection (Arrighi et al., 2004; Geijtenbeek et al., 2000; Nikolic et al., 2011; Rodriguez-Plata et al., 2013). The formation of the infectious synapses indicates that the normal cell-to-cell interactions and the immune functions of APCs are co-opted by HIV-1 to enable efficient virus dissemination.

It seems that the formation of the infectious synapse is not triggered in the presence of the virus but is more related to a hijacking of the immunological synapse formed between APCs and CD4⁺ T cells by HIV-1 (Rodriguez-Plata et al., 2013). Thus, HIV-1 could take advantage of the normal cell-to-cell interactions and the immune functions of APCs for its own benefits, leading to efficient virus dissemination. Similarly, it has been suggested that mammary epithelial cells (MECs) and mucosal stromal fibroblasts could also capture HIV-1 without being infected and then transmit viral particles to CD4⁺ T cells (Dorosko and Connor, 2010; Neidleman et al., 2017). These studies suggest that the trans-infection mechanism likely play important role in HIV-1 transmission and dissemination *in vivo* (Hladik and McElrath, 2008).

HIV-1 can be captured through binding of the viral envelope glycoproteins to surface molecules expressed by APCs, such as mannose specific C-type lectin receptor (DC-SIGN) and SIGLEC-1, and then stored within the tetraspanin-enriched virus containing compartments (VCCs) where it could be protected from antibody neutralization and degradation (Arrighi et al., 2004; Garcia et al., 2005; Geijtenbeek et al., 2000; Gummuluru et al., 2014; Izquierdo-Useros et al., 2012b; Puryear et al., 2013; Puryear et al., 2012; Waki and Freed, 2010). The formation of the infectious synapse is mediated by the adhesion molecules LFA-1 and ICAM-1 for stabilization of intercellular interactions between virus-carrying APCs and target CD4⁺ T cells (Rodriguez-Plata et al., 2013; Sanders et al., 2002), but interactions between the MHC and TCR could also modulate DC-mediated trans-infection process (Rodriguez-Plata et al., 2013). After the formation of conjugates, the APCs recruit a number of molecules to the contact sites, while the CD4 receptor and CXCR4 or CCR5 co-receptors required for virus entry are also recruited at the site of the cell-cell contact in the target CD4⁺ T cells (Akiyama et al., 2015; McDonald et al., 2003). Rearrangements of the actin

cytoskeleton also likely play important role in HIV-1 transmission across the infectious synapse. Recent studies have shown that dynamin-2 and tetraspanin-7 control nucleation and cortical stabilization of actin to maintain HIV-1 onto DC dendrites for efficient cell-to-cell transfer from virus-carrying DCs to target CD4⁺ T cells when these cells are co-cultured, and simultaneously infected with HIV-1 (Menager and Littman, 2016).

4.2.1 HIV-1 trans-infection by plasmacytoid DCs (pDCs)

Plasmacytoid DCs (pDCs) could respond to a variety of enveloped viruses, including HIV-1 and HCV, with production of type I interferon (IFN) and type III interferon (IFN- λ) (Fitzgerald-Bocarsly et al., 2008; Mesev et al., 2019). Productive infection of pDCs by HIV-1 is less efficient than cDCs, even if both subpopulations express CD4 receptor and co-receptors (Smed-Sorensen et al., 2005). Cell-associated viruses are more potent stimulators of pDCs than cell-free viral particles. This immune activation results in the recruitment of pDCs and other types of immune cells to the sites of viral infection, which may facilitate viral transfer from infected pDCs to CD4⁺ T cells in the SIV–macaque model (Li et al., 2009). During HIV-1 infection, IFN secretion by pDCs could promote maturation of pDCs and cDCs and also inhibit HIV-1 replication in CD4⁺ T cells (Li et al., 2014; Miller and Bhardwaj, 2013; Royle et al., 2014). However, pDCs has been shown to efficiently transmit viral particles to CD4⁺ T cells, which may exacerbate T cell depletion (Evans et al., 2011; Fong et al., 2002; Lore et al., 2005).

4.2.2 HIV-1 *trans*-infection of CD4⁺ T by conventional DCs (cDCs)

The major role of conventional DCs (cDCs) is to scavenge for invading pathogens and migrate from the site of microbial infection to lymph nodes for presentation of foreign antigens to lymphocytes that initiate adaptive immunity. Paul Cameron and colleague first described that blood DCs efficiently capture HIV-free particles without being infected and produces vigorous cytopathic infection when co-cultured with non-infected CD4⁺T cells (Cameron et al., 1992). Cameron and Steinman also provide the first evidence that murine DCs, which are refractory to HIV infection, could also capture HIV and transfer viral particles to human CD4⁺ T cells as efficiently as human blood-derived DCs (Cameron et al., 1992). Taking together, these data clearly

demonstrate that DCs can capture HIV efficiently without becoming infected and transfer virions to CD4⁺ T cells, a process referred to as *trans*-infection. The mechanism by which DCs can mediate trans-infection of CD4⁺ T cells has been further described by McDonald and coll. who found that HIV-1 captured by DCs could rapidly re-localize to the site of cell-to-cell contact when the virus-carrying DC encounters a non-infected CD4⁺ T cell (McDonald et al., 2003). The CD4 receptor, co-receptors CCR5 and CXCR4 expressed on the target T cells are subsequently recruited to the interface, while the DCs concentrate viral particles to this infectious synapse to enhance viral transfer from DCs to CD4⁺ T cells (McDonald et al., 2003).

Although it has been well accepted that HIV-1 productive infection of DCs is inefficient due to the low levels of CD4 and co-receptor expression and abundance of restriction factors such as SAMHD1 (Laguetta et al., 2011), some studies have shown that cDCs could support some level of HIV-1 infection *in vitro*, but DC maturation could result in significant inhibition of HIV infection (Cameron et al., 2007; Martin-Gayo et al., 2015; Smed-Sorensen et al., 2005). In fact, early studies of HIV-DC interactions could observe infection of DCs *in vivo* (Frankel et al., 1996). *In vitro*, DCs can be productively infected using large amount of VSV-G-pseudotyped HIV-1 particles and are also capable of transmitting viral particles to mediate efficient infection of CD4⁺ T cells, a process referred as *cis*-infection (Burleigh et al., 2006; Lambert et al., 2008).

Recent studies have proposed a model on two temporal phases of HIV-1 transfer from DCs to T cells. The first-phase transfer (within 24 h) depends on highly efficient virus uptake through pattern recognition receptors, such as DC-SIGN and Langerin, which results either in proteolytic degradation of internalized viral particles in the late endosomes or immediate virus transfer to CD4⁺ T cells across the infectious synapse *in trans* (Martin et al., 2010; McDonald et al., 2003). Subsequently, a second-phase transfer occurs when DCs are productively infected over a longer period (around 96 h), and this process is mediated by viral fusion at the plasma membrane of DCs and *de novo* replication of HIV-1 in DCs. These productively infected DCs could then transfer *de novo* virus to CD4⁺ T cells across the virological synapse *in cis* (Burleigh et al., 2006; Nobile et al., 2005; Turville et al., 2004). In addition, Nasr et al. also found that uptake and transfer of HIV to CD4⁺ T cells from *ex vivo* Langerhans cells (LCs) occurred in two successive phases as described above (Nasr et al., 2014).

Immature DCs scavenge for potential pathogens in mucosal tissues, and they efficiently internalize and cross-present foreign antigens to CD4⁺ T cells. HIV-1 can benefit this process to facilitate efficient infection of CD4⁺ T cells through dendritic cell-specific intercellular adhesion molecule-3-binding nonintegrin (DC-SIGN), which is a C-type lectin receptors highly expressed on mucosal dendritic cells, and also a subset of macrophages such as M2a macrophages (Geijtenbeek et al., 2000; Kwon et

al., 2002). DC-SIGN is highly expressed on immature DCs and could mediate rapid internalization of HIV-1 particles for efficient *trans*-infection of CD4⁺ T cells. DC-SIGN binds to HIV-1 gp120 with much higher affinity than for the CD4 receptor, indicating that DC-SIGN expressed on immature DCs could be of specific importance when few viral particles are present, such as in early HIV primo-infection (Curtis et al., 1992; Geijtenbeek et al., 2000; Izquierdo-Useros et al., 2014).

Earlier studies have identified DC-SIGN as a critical HIV binding factor on immature DCs, which interacts efficiently with the viral Env, but maturation of iDCs with lipopolysaccharide (LPS) could markedly enhance the ability of DCs to capture viral particles and mediate HIV-1 *trans*-infection of CD4⁺ T cells (Izquierdo-Useros et al., 2007; Sanders et al., 2002), while DC-SIGN has been shown to significantly down-regulated upon DC maturation (Dominguez-Soto et al., 2005; Martinez-Nunez et al., 2009). These observations suggest that DC-SIGN may be dispensable for *trans*-infection by mature DCs (Wang et al., 2007). Through transcriptome analysis of mature DCs to identify the cell surface molecules which may be involved in this DC-SIGN-independent *trans*-infection process by mature DCs, Izquierdo-Useros and colleagues showed that the sialic acid-binding Ig-like lectin 1 (Siglec-1, or CD169) was significantly up-regulated upon DC maturation and was considered as a primary candidate for HIV capture receptor (Izquierdo-Useros et al., 2012b; Puryear et al., 2013). It has been further demonstrated that Siglec-1 can bind to HIV-1 particles efficiently through recognition of glycosphingolipids and also mediate HIV-1 *trans*-infection of CD4⁺ T cells (Izquierdo-Useros et al., 2012a; Izquierdo-Useros et al., 2014; Izquierdo-Useros et al., 2012b). The glycosphingolipid GM3, a host cell-derived glycosphingolipid which is incorporated into viral particles membranes, is essential for CD169-mediated capture of HIV-1 and *trans*-infection by mature DCs (Akiyama et al., 2014; Puryear et al., 2012). However, the GM3-CD169 binding is independent of the viral Env, while DC-SIGN could recognize gp120 directly for sequestration and infection (Yu et al., 2014). Upon virus capture, Siglec-1 could mediate internalization of virus particles into deep plasma membrane invaginations of DCs, leading to a decrease of the efficiency of broadly neutralizing antibodies against gp120 from neutralizing *trans*-infection (Akiyama et al., 2015).

During the formation of the immunological synapse, binding of the T-cell receptor to the MHC-peptide complex expressed on the cell surface of APCs ensures T-cell activation by transducing signals that cause transcriptional up-regulation of numerous genes, polarized secretion of cytokines or cytotoxic granules, and T cell-proliferation (Huppa and Davis, 2003). The interaction between LFA-1 and ICAM-1 is essential for the formation of immunological synapse. HIV-1 could benefit from this adhesive interaction to mediate *trans*-infection of CD4⁺ T cells by virus-carrying dendritic cells (Rodriguez-Plata et al., 2013; Sanders et al., 2002). Both ICAM-1 and LFA-1 have been

shown to be significantly up-regulated upon DC maturation, and this cell-cell contact mediated by ICAM-1-LFA-1 interaction is co-opted by HIV-1 for efficient transmission (Sanders et al., 2002). Antibody blockade and overexpression studies have shown that ICAM-2 and ICAM-3 are dispensable for DC-mediated HIV-1 transmission to CD4⁺ T cells (Wang et al., 2009).

HIV-1 transmission from virus-carrying DCs to CD4⁺ T cells might also occur by exosome-associated viruses. In this process, HIV-1 particles are rapidly internalized by mature DCs into late endosomes, multivesicular bodies (MVBs), which are endosomal compartment enriched in tetraspanins, such as CD9, CD81, CD82 and CD63, not present in immature DCs (Garcia et al., 2008; Garcia et al., 2005). Furthermore, the intraluminal vesicles found in tetraspanin-enriched MVBs could fuse with the plasma membrane and be released as exosomes, which could also be captured together with HIV particles by mature DCs and can be efficiently transferred to CD4⁺ T lymphocytes in a gp120-independent manner (Izquierdo-Useros et al., 2009; Piper and Katzmann, 2007). Recent studies have also shown that HIV RNA and proteins could be packaged into these exosomes and transported between different cells (Mukhamedova et al., 2019; Patters and Kumar, 2018). These findings suggest that retroviruses could mimic exosomes and could be captured by dendritic cells to evade the host immune system (Izquierdo-Useros et al., 2009; Piper and Katzmann, 2007).

4.2.3 HIV-1 *trans*-infection of CD4⁺ T cells by macrophages

Similar to dendritic cells, HIV-1 infection of macrophages is largely inefficient compared to CD4⁺ T cells due to low levels of expression of the CD4 receptor and high levels of expression of restriction factors, such as SAMHD1 and TRIM5 α . Infectious viral particles can persist within the virus-containing compartments (VCCs) of HIV-1 infected macrophages and retain infectivity for extended time, and could also be then transferred to activated peripheral blood lymphocytes (PBLs) 6 weeks after viral replication was blocked by the protease inhibitor indinavir sulfate (Sharova et al., 2005). Therefore, HIV-1-infected macrophages are able to transfer infectious viral particles to non-infected CD4⁺ T cells, which could reduce viral sensitivity to the reverse-transcriptase inhibitors (RTIs) and some broadly neutralizing antibodies (bNAbs) (Carr et al., 1999; Duncan et al., 2014). Finally, it has been demonstrated that macrophages can also capture both HIV-1 and murine leukemia virus (MLV) cell-free particles in a CD169-dependent manner *in vivo* and then mediate *trans*-infection of proliferating lymphocytes with MLV (Sewald et al., 2015).

The sialic acid-binding Ig-like lectin 1 (Siglec-1, or CD169) is an I-type lectin which could recognize gangliosides and capture the virus. Sampling by CD169/ Siglec-1 has been proposed as an early step of host immune responses against viral infection. Similar to the C-type lectin DC-SIGN, HIV-1 could co-opt CD169/ Siglec-1 for its own benefit to efficiently infect and disseminate in the host. The expression of CD169 on cell surface is significantly increased on monocytes and macrophages upon HIV-1 infection (Rempel et al., 2008; van der Kuyl et al., 2007; Zou et al., 2011). The mannose receptor (MR), the first C-type lectin receptors (CLR) described in myeloid cells (Stahl et al., 1980), is also capable of capturing HIV-1 as well as invading pathogens, including *Mycobacterium tuberculosis* and *Candida albicans* (Allavena et al., 2004). The MR expressed on macrophages could capture large amounts of HIV cell-free particles and facilitate viral transmission to CD4⁺ T cells *in trans* (Nguyen and Hildreth, 2003). MR-mediated *trans*-infection can be significantly blocked using inhibitors of MR binding, such as D-mannose, mannan, EDTA, as well as soluble mannose-binding lectin (Nguyen and Hildreth, 2003).

Although it was initially proposed that the C-type lectin DC-SIGN is not expressed on macrophages *in vitro*, recent studies indicate that DC-SIGN is strongly expressed on M2a macrophages and could facilitate HIV-1 entry and viral DNA synthesis in these macrophage subtype (Cassol et al., 2013). In addition, they can efficiently transmit both X4 and R5 HIV-1 to the CD4⁺ T cells in a DC-SIGN-dependent manner (Cassol et al., 2013). Intriguingly, it has been shown that monocyte-derived macrophages isolated from HIV-1-infected nonprogressors (NP) are not able to mediate *trans*-infection of autologous CD4⁺ T cells by HIV-1, compared to the monocyte-derived macrophages isolated from HIV-1 seronegatives (SN) and rapid progressors (PR) (DeLucia et al., 2018). This impairment of *trans*-infection efficiency from NP macrophages was associated with a significant decrease of the number of macrophages expressing DC-SIGN, namely M2a macrophages (DeLucia et al., 2018). These findings suggest that macrophage polarization may affect macrophage *trans*-infection efficiency. Together, these observations indicate that capturing of HIV-1 by lectin receptors expressed on macrophages, including type-I lectin CD169/ Siglec-1, C-type lectin receptor DC-SIGN and mannose receptor (MR), may facilitate viral *trans*-infection of CD4⁺ T cells and enhance virus dissemination *in vivo*.

Finally, it was also reported that antibody blockage of the scavenger receptor CD36 significantly inhibits viral release from HIV-1 infected macrophages and also prevent HIV-1 transmission from infected macrophages to CD4⁺ T cells (Berre et al., 2013). CD36 is a transmembrane glycoprotein, a member of class B scavenger receptor family, and is expressed on smooth muscle cells, endothelial cells, adipocytes, and macrophages (Kuniyasu et al., 2003; Matsumoto et al., 2000; Swerlick et al., 1992; Talle et al., 1983). CD36 is found in the VCCs of HIV-1 infected macrophages and has

been shown to be essential for efficient release of viral particles (Berre et al., 2013).

4.3 Tunnelling nanotubes (TNTs) and filopodia

The role of membrane protrusions in intercellular communications has been widely investigated (Buszczak et al., 2016). A large diversity of membrane protrusions have been described, both *in vivo* (Chinnery et al., 2008; Lou et al., 2012; Seyed-Razavi et al., 2013) and *in vitro* (Sherer et al., 2007), and play key roles for information transmission between different types of cells from physiological systems, such as T cells, myeloid cells and neurons (Eugenin et al., 2009a, b; Gousset and Zurzolo, 2009; Hase et al., 2009; Onfelt et al., 2006; Onfelt et al., 2004; Rustom et al., 2004; Wang et al., 2011).

There are two different types of nanotubes which have been described, corresponding to open-ended nanotubes (also known as tunnelling nanotubes, TNTs) and close-ended nanotubes. Intercellular communications involving TNTs were first observed as F-actin-containing membrane extensions which are able to connect distant cells during minutes to hours (Rustom et al., 2004). The TNTs are dynamic and fragile structures extended up to 100 μm in length with diameters ranging from 50 to 200 nm, and are not attached to the substratum (Rustom et al., 2004; Sowinski et al., 2008). Several studies have shown that HIV-1 co-opts TNT networks to move from one cell to another cell, leading to efficient virus cell-to-cell transfer. The frequency of TNT formation is not affected by the virus in T cells but these structures could allow for rapid spreading of HIV-1 between T cells (Sowinski et al., 2008).

Furthermore, virus particles could be also transferred by surfing along the surface of TNTs between infected and non-infected T cells (Sowinski et al., 2008). Similarly, HIV-1 dissemination through TNTs has also been reported between macrophages, in which virus particles can be transferred through intracellular vesicles derived from the endoplasmic reticulum or the Golgi apparatus (Kadiu and Gendelman, 2011a, b). Moreover, HIV-1 infection has been shown to induce the formation of these intercellular structures in macrophages for efficient viral dissemination in macrophages (Eugenin et al., 2009a). In addition, the HIV-1 Nef auxiliary protein has been reported to be responsible for the formation of TNTs in the THP-1 macrophage-like cell line as well as in primary monocyte-derived macrophages. Nef can alter the localization of the scaffolding protein M-Sec, which has been shown to be a key regulator of TNT formation by a still undefined mechanism (Hase et al., 2009; Hashimoto et al., 2016; Kadiu and Gendelman, 2011a; Xu et al., 2009).

Another route of viral cell-to-cell transmission through membrane extension

involving the formation of filopodia was first described for the transmission of the murine leukemia virus (MLV) (Sherer et al., 2007). Filopodia are also F-actin-rich thin plasma membrane extensions that are involved in a large variety of cellular functions, such as adhesion to the extracellular matrix, chemo-migration, or the formation of cell-cell contacts (Mattila and Lappalainen, 2008). In DCs, after the engagement of DC-SIGN, HIV-1 can mediate the activation of the CDC42 small GTPase and the remodeling of actin cytoskeleton to promote filopodia extension that allows efficient virus transmission to neighboring CD4⁺ T cells (Nikolic et al., 2011). By budding at the tip of filopodia in DCs, HIV-1 are able to tether concomitantly several non-infected CD4⁺ T cells, leading to viral transfer and infection of the target CD4⁺ T cells (Aggarwal et al., 2012).

4.4 Phagocytosis

Apoptosis, a unique form of programmed cell death, is closely related to recognition and clearance by phagocytosis. Removal of apoptotic cells is essential for tissue development and homeostasis (Gordon and Pluddemann, 2018). It has been proposed that HIV-1 can infect macrophages by virus cell-to-cell transfer through capture and engulfment of infected T cells (Baxter et al., 2014; Calantone et al., 2014; DiNapoli et al., 2017). HIV-1 proviral DNA and rearranged TCR DNA could be detected in myeloid cells possibly attributed to phagocytosis of apoptotic HIV-1 infected T cells by myeloid cells (DiNapoli et al., 2017). However, cellular DNA of apoptotic cells undergoes nuclease digestion prior to phagocytosis by myeloid cells, which could cleave both viral DNA and cellular DNA into small fragments rendering thus difficult for PCR amplifying of intact rearranged TCR DNA or HIV-1 proviral DNA in myeloid cells (Stevenson, 2015). Therefore, the precise mechanisms for HIV infection of macrophages through phagocytosis of infected T cells require further investigations.

4.5 The formation of multinucleated giant cells (MGCs)

The formation of syncytia, the fusion between infected and uninfected cells, is the simplest mechanism of virus cell-to-cell dissemination which can be reproduced *in vitro* for paramyxoviruses herpesviruses, and some retroviruses, leading to the formation of multinucleated giant cells (MGCs). For example, paramyxoviruses, including respiratory syncytial virus (RSV) and measles virus (MV), can induce the formation of

MGCs *in vitro* and *in vivo* for efficient viral cell-to-cell spread (Fugier-Vivier et al., 1997; McChesney et al., 1997; Takeuchi et al., 2003). Similarly, alpha-herpesviruses express a number of fusogenic glycoproteins and are able to induce MGC formation (Cole and Grose, 2003). Finally, HIV-1 and HTIV-1 can also form syncytia in tissue culture (Benovic et al., 1998; Ceccaldi et al., 2006). In fact, syncytium formation is mediated by engagement of CD4 on target cell with Env expressed by infected donor cells during HIV-1 infection (Bracq et al., 2018; Lifson et al., 1986). Earlier studies have shown that HIV-1 infected multinucleated macrophages and multinucleated DCs could also be detected *in vivo* in different tissues of HIV-1-infected patients (Frankel et al., 1996; Granelli-Piperno et al., 1995). The presence of HIV-1-infected MGCs expressing specific DC markers has been found at the surface of nasopharyngeal tonsils of HIV-1-infected individuals (Frankel et al., 1996). Similarly, HIV-1-infected multinucleated giant macrophages were found *in vivo* in different tissues during HIV-1 infection, including spleen, genital, lymph nodes, lungs, digestive tracts, as well as the central nervous system (CNS)(Bracq et al., 2018; Frankel et al., 1996; Harbison et al., 2014; Koenig et al., 1986; Symeonides et al., 2015; Vicandi et al., 1999). However, the relevance of syncytium formation for HIV-1 spread *in vivo* is less clear. Recent studies using humanized mice model and 3D cultures suggest that multinucleated giant cells formed by HIV-1 induced cell-to-cell fusion are viable and probably contribute to virus dissemination (Bracq et al., 2017; Compton and Schwartz, 2017; Law et al., 2016; Murooka et al., 2012; Symeonides et al., 2015).

Chapter 5: Cell fusion and multinucleated giant cells

The fusion of plasma membranes and formation of multinucleated giant cells are a universal, active and frequent phenomenon in living cells. Cell fusion and formation of multinucleated giant cells (MGCs) are evolutionarily conserved among eukaryotes (Ogle et al., 2005). Under certain homeostatic and developmental conditions, mammalian cells can undergo cell fusion and give rise to multinucleated giant cells with specialized functions. At the cellular level, cell fusion of sperm and egg is essential for fertilization in mammals. At the organ level, cell fusion of myoblasts is important for the formation of skeletal muscle with increased myofibre size to improve contractile strength, and it can also take place during the formation of the placenta (i.e. the fusion of trophoblast cells) (Buchrieser et al., 2019; Ogle et al., 2005).

The multinucleated giant cells (MGCs) have been morphologically classified into several different types, including osteoclasts (OCs), Langhans giant cells (LGCs), foreign body giant cells (FBGCs) and Touton giant cells (Brodbeck and Anderson, 2009; Brooks et al., 2019a; Pereira et al., 2018; Quinn and Schepetkin, 2009) (Figure 14). These different types of multinucleated giant cells (MGCs) are all derived from cells of monocyte/macrophage-lineage, but varied on their functional characteristics, the composition and arrangement of intracellular organelles. The Langhans giant cells are typically circular in shape, with the Golgi apparatus and other intracellular organelles surrounded by the peripherally located nuclei (less than 20 nuclei per cell). In contrast, Foreign-body giant cells contain numerous nuclei (up to 100-200 nuclei) which distributed in a diffused pattern throughout the cytoplasm. Touton giant cells are distinguished by multiple, clustered nuclei that are located in the center and surrounded by a foamy cytoplasm with accumulated lipids. Since all these MGCs variants are derived from the monocyte/macrophage precursors, their functional and morphological heterogeneity appears to be determined by different tissue location and the local factors present in the microenvironment.

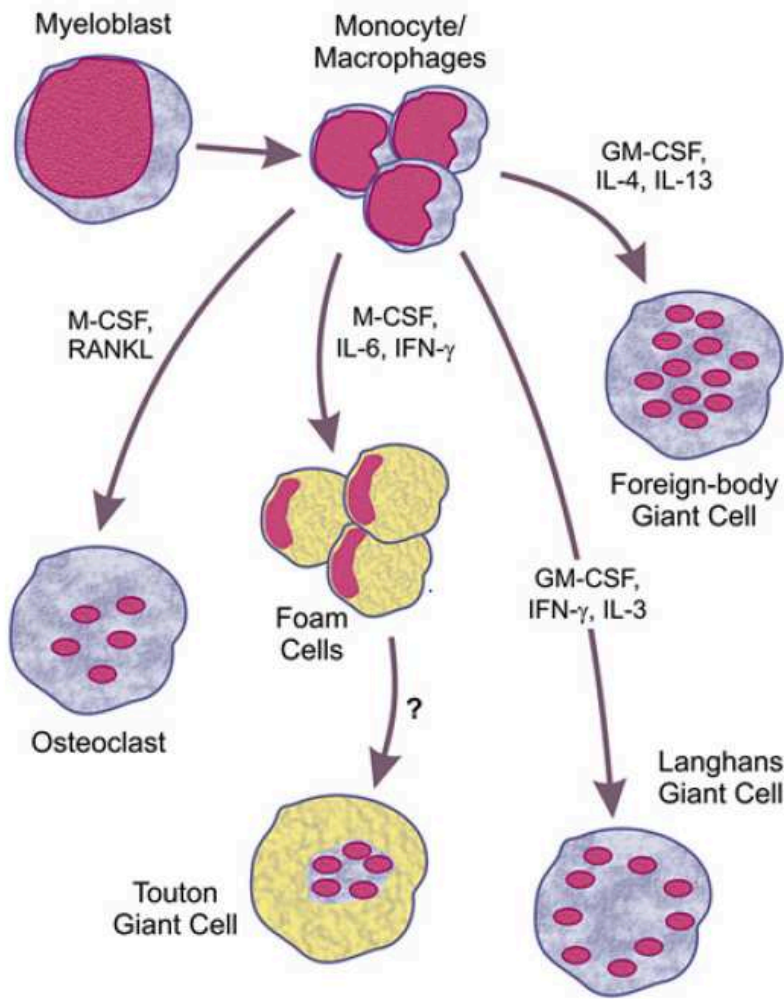


Figure 15: Different types of multinucleated giant cells (MGCs)

The multinucleated giant cells (MGCs) have been morphologically classified into several different types, including osteoclasts (OCs), Langhans giant cells (LGCs), foreign body giant cells (FBGCs) and Touton giant cells. Pathways leading to formation of different types of multinucleated giant cells (MGCs) are shown. Major cytokines involved in the cell fusion of monocyte/macrophage precursors are indicated. Adapted from Quinn et al., 2009.

5.1 Osteoclasts

Osteoclasts (OCs) are multinucleated giant cells involved in bone resorption via proteolytic degradation and acid decalcification, leading to maintenance of bone matrix homeostasis and ensuring continuous remodelling of the bones. They are specialized phagocytic cells of the bone, originating from macrophage fusion, and were initially proposed as one of the members of mononuclear phagocyte system (van Furth et al.,

1972). Bone-resorbing OCs arise by fusion of haematopoietic stem cells-derived monocytic precursors in the presence of RANKL and M-CSF (Ash et al., 1980; Lacey et al., 1998; Shalhoub et al., 2000). The receptor activation of NF- κ B ligand (RANKL) belongs to the tumor necrosis factor (TNF) family and has been considered to be an essential cytokine for osteoclastogenesis. RANKL is also expressed on the osteoblasts and their precursors, and its expression is enhanced by the osteoclast-stimulating agents such as TNF- α and parathyroid hormone. Yagi and colleagues have identified dendritic cell-specific transmembrane protein (DC-STAMP) as a key factor for macrophage fusion and the formation of osteoclasts using a knockout mouse model (Yagi et al., 2005). Osteoclast multinucleation was completely blocked in DC-STAMP knockout mice despite normal expression of the osteoclast specific markers and cytoskeletal structure (Yagi et al., 2005). Moreover, a genetic variant of *TM7SF4* gene, found in the patients with Paget's disease of bone (PDB) and which encodes for DC-STAMP, significantly increases the nuclei number of multinucleated cells and simulates osteoclastogenesis in PDB (Laurier et al., 2017).

CCR2 and its ligand CCL2 are common chemoattractant for osteoclasts, and both were found to be required for osteoclast multinucleation (Kyriakides et al., 2004; Sul et al., 2012). Indeed, significantly fewer osteoclasts and FBGCs were found when isolated from the bone marrow of CCR2 and CCL2 knockout mice, compared with the levels in wild-type mice (Khan et al., 2016). The signaling adaptor DNAX activating protein 12 (DAP12) is a transmembrane protein expressed on NK cells and myeloid cells and could mediate signalling for a variety of cell-surface receptors (Turnbull and Colonna, 2007). DAP12 has been shown to be a common mediator of macrophage fusion, the overexpression of DAP12 in macrophages could enhance macrophage fusion, while the DAP12 gain-of-function transgenic mice displayed spontaneous multinucleated giant cells (MGCs) formation in the lung (Helming et al., 2008; Kaifu et al., 2003). Furthermore, the triggering receptor expressed on myeloid cells 2 (TREM2), one of the DAP12-associated receptors, has also been shown to be required for macrophage multinucleation (Helming et al., 2008). It has been further demonstrated that TREM2 is essential for macrophage fusion, activation, and osteoclast multinucleation (Cella et al., 2003; Humphrey et al., 2004; Mocsai et al., 2004; Otero et al., 2012; Turnbull et al., 2006).

5.2 Langhans Giant Cells

The Langhans giant cells (LGCs) are frequently found in association with tuberculosis induced granulomas and are originally described by Theodor

Langhans(Brodbeck and Anderson, 2009). Langhans giant cells (LGCs) are formed by cell fusion of multiple macrophages and contain a limited number of nuclei (about 10–20 nuclei per cell) which are located on the cell periphery surrounding the Golgi apparatus and other intracellular organelle (Okamoto et al., 2003; Puissegur et al., 2007). The mechanisms regarding LGCs formation and functional property of these LGCs remain poor understood. Recent studies using an in vitro human tuberculous granulomas model indicates that high virulence mycobacteria (e.g., *Mycobacterium tuberculosis*) but not the avirulent mycobacteria (e.g., *Mycobacterium smegmatis*) induces the fusion of granulomas macrophages and formation of large LGCs (Lay et al., 2007; Puissegur et al., 2004). Further studies revealed an important role of mycobacterial glycolipids in LGCs formation. The proinflammatory glycolipids, including phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM) and trehalose-6,6'-dimycolate (TDM) are able to induce cell fusion of granuloma macrophages into Langhans giant cells (LGCs) whereas anti-inflammatory lipoarabinomannan do not(Puissegur et al., 2007). Lipomannan (LM) induced the formation of LGCs reminiscent of those multinucleated giant macrophages (MGCs) detected in vivo in tuberculosis patients, and this cell fusion process occurs through Toll-like receptor 2 (TLR2) signaling pathway, and is dependent on the expression β_1 integrin and ADAM9(Puissegur et al., 2007). ADAM9 belongs to the ADAMs (membrane proteins with a disintegrin and metalloprotease domain) transmembrane glycoproteins family and has been shown to play important role in monocyte fusion and the formation of MGCs in vitro (Namba et al., 2001). The β_1 integrins and β_2 integrins mediate macrophage adhesion and are necessary for macrophage fusion and MGCs formation (McNally and Anderson, 2002). Since the tuberculous granulomas containing LGCs are generally surrounded by T cells(Philips and Ernst, 2012), further study using an in vitro LGCs formation system revealed activated T cells play an important role in LGCs formation through the CD40–CD40L axis and IFN- γ secretion(Sakai et al., 2012). Dendritic cell-specific transmembrane protein (DC-STAMP), a key regulator of osteoclast formation, has also been shown to be involved in the formation of LGCs (Sakai et al., 2012). The functional properties of LGCs are strongly resembles those of mature dendritic cells, which exhibit reduced phagocytosis efficiency, despite high levels expression of major histocompatibility complex (MHC) class II molecules on plasma membrane(Lay et al., 2007). The LGCs detected in *M. tb*-induced granulomas seem to be involved in the destruction of bacilli that have been ingested macrophages and small multinucleated cells (MCs), and are thought to restrict mycobacteria spreading within granulomas(Brooks et al., 2019b; Cronan et al., 2016; Lay et al., 2007).

5.3 Foreign Body Giant Cells

The foreign body giant cells (FBGCs) are most commonly found at the tissues interfacing with foreign materials such as implanted biomedical devices, biomaterials and prostheses (Anderson et al., 2008). FBGCs are highly variable in cell size and exhibit up to 200 nuclei that distributed in a diffused pattern throughout the cytoplasm. The number of nuclei within FBGCs varies greatly from ten to even hundreds and exhibit no particular patterns of nuclear arrangements, with numerous nuclei randomly scattered throughout the cytoplasm (McNally and Anderson, 1995; Zhao et al., 1992). Once foreign material is exposed to the immune system, macrophages adhere to the surface of implanted biomaterials and fuse to form destructive foreign body giant cells (FBGCs), which are thought to mediate foreign body response (FBR), a chronic inflammatory response against foreign material, leading to the resorption and degradation of foreign material (Anderson et al., 2008; Brodbeck and Anderson, 2009; Major et al., 2015; Miron et al., 2016). FBGCs are able to secret reactive oxygen species (ROSs) and also inflammatory cytokines such as TGF- β 1, potentially leading to oxidative damage and degradation of implanted devices (Hernandez-Pando et al., 2000; Wiggins et al., 2001). Early studies have shown that interleukin-4 (IL-4) and interleukin-13 (IL-13) mediate macrophage fusion and formation of FBGCs through up-regulation of the mannose receptors (CD206) on fusing macrophages (Anderson et al., 2008; DeFife et al., 1999; Kurachi et al., 1994; McNally and Anderson, 1995). It has also been reported that IL-4 could induce the expression of dendritic cell-specific transmembrane protein (DC-STAMP) and E-cadherin via STAT6 signaling, and promotes the formation of LBGs (Moreno et al., 2007; Van den Bossche et al., 2009). DC-STAMP is a cell surface molecule that has been shown to be essential for macrophage fusion and formation of osteoclasts and FBGCs in vivo and in vitro (Kukita et al., 2004; Miyamoto et al., 2012; Yagi et al., 2005; Yagi et al., 2006). E-cadherin has been shown to be required for macrophage adhesion and fusion during IL-4-induced FBGCs formation (Moreno et al., 2007). Other studies have reported that vitronectin (VN), a glycoprotein of hemopexin family and component of the extracellular matrix, acts as a critical matrix adhesion substrate that supports IL-4-induced FBGCs formation (Kurachi et al., 1994; McNally et al., 2008). Other studies have demonstrated that the chemotactic factor CCL2/MCP-1, signal transducer DAP12 and the small-GTPase Rac1 are also involved in macrophage fusion and foreign body giant cells formation (Helming et al., 2008; Jay et al., 2007; Kyriakides et al., 2004). More recently, it has been demonstrated that the transient receptor potential vanilloid 4 (TRPV4), a member of transient receptor potential (TRP) vanilloid subfamily, play an important role in macrophage fusion and FBGCs formation, and genetic deficiency of TRPV4 results in

reduced macrophage fusion in vitro and impaired FBGC formation in vivo(Goswami et al., 2019).

5.4 Touton Giant Cells

Touton giant cells (TGCs), originating from cell fusion of foam cells, contain multiple, clustered nuclei that are located in the center and surrounded by a foamy cytoplasm with accumulated lipids(Aterman et al., 1988; Dayan et al., 1989). Foam cells are a specific type of macrophages which could ingest low-density lipoproteins (LDL) and form lipid-filled cytoplasm (da Silva et al., 2016; Funk et al., 1993; Oh et al., 2012; Yu et al., 2013b). TGCs are most commonly detected in lesions with cholesterol and lipid deposits, and are usually associated with Juvenile xanthogranuloma (JXG) and xanthomas (Consolaro et al., 2009; Kodama et al., 1988). However, the fusion molecules involved in the formation of TGCs remain poorly investigated.

Objectives of the project

Myeloid cells, including macrophages, dendritic cells and bone osteoclasts, play pivotal roles during infection as demonstrated in HIV-infected patients, simian immunodeficiency virus (SIV)-infected macaques, and more recently in HIV-1-infected Humanized mice (Bracq et al., 2018; Costiniuk and Jenabian, 2014; Fischer-Smith et al., 2008; Frank et al., 1996; Frankel et al., 1996; Honeycutt et al., 2016; Lewin-Smith et al., 1999; Stevenson, 2015). They are emerging as target cells of HIV-1 involved in virus transmission, dissemination, and establishment of persistent tissue virus reservoirs. Myeloid cells are poorly infected by cell-free viruses due to low levels of expression of viral entry receptors and high levels of expression of restriction factors, such as SAMHD1 and APOBEC3 family proteins (Berger et al., 2011b; Laguette et al., 2011; Lee et al., 1999; Peng et al., 2007).

While infection of HIV-1 cellular targets with cell-free viruses has been largely documented, HIV-1 cell-to-cell transmission is much more efficient and probably represents the dominant mode of infection *in vivo*. Importantly, this mode of virus dissemination may enable virus to escape elimination by the immune system, including neutralizing antibodies, and antiretroviral drugs (Abela et al., 2012; Chen et al., 2007; Dufloo et al., 2018; Ganesh et al., 2004; Gombos et al., 2015; Gupta et al., 1989; Li et al., 2017; Malbec et al., 2013; Massanella et al., 2009; McCoy et al., 2014; Reh et al., 2015; Zhong et al., 2013a) It may also overcome the inhibitory effects of several anti-viral restriction factors for HIV-1 such as TRIM5 α and tetherin/BST2 under certain circumstances (Casartelli et al., 2010; Coleman et al., 2011; Giese and Marsh, 2014; Jolly et al., 2010; Kuhl et al., 2010; Richardson et al., 2008; Zhong et al., 2013a).

HIV-1 cell-to-cell transfer between CD4⁺ T cells has been largely documented, but the mechanisms regarding virus cell-to-cell infection of myeloid cells remain poorly investigated. Myeloid cells, including subsets of blood monocytes, dendritic cells (DCs), macrophages and bone osteoclasts (OCs), all express the CD4 receptor and CCR5 and CXCR4 co-receptors required for HIV-1 entry, and represent natural host cell targets of the virus.

The general goal of this PhD project presented here was to investigate and characterize the cellular and molecular mechanisms involved in cell-to-cell transfer of HIV-1 from infected T cells for virus spreading and productive infection of myeloid cells, including, macrophages, dendritic cells and bone osteoclasts. Until now, HIV-1 entry phenotype these in target cells is mainly defined by *in vitro* cell-free viral infection of transformed cell lines expressing the CD4 receptor and a co-receptor, either CCR5 or CXCR4 (Joseph et al., 2014; Joseph and Swanstrom, 2018), but this definition does

not fully reflect the natural mode of HIV-1 spreading *in vivo* and was not related to the capacity of HIV-1 for cell-to-cell dissemination between primary lymphoid and myeloid target cells. Therefore, the last part of this PhD work was to investigate and revisit the role of co-receptor usage and cellular tropism in the context of HIV-1 cell-to-cell transfer and spreading in primary cell targets.

RESULTS

Article 1: T cells-macrophages fusion triggers multinucleated giant cell formation for HIV-1 spreading

1. Abstract

HIV-1-infected macrophages participate in virus dissemination and establishment of virus reservoirs in host tissues, but the mechanisms for virus cell-to-cell transfer to macrophages remain poorly investigated. Here, we reveal the mechanisms for cell-to-cell transfer from infected T cells to macrophages and virus spreading between macrophages. We show that contacts between infected T lymphocytes and macrophages lead to cell fusion for fast and massive transfer of CCR5-tropic viruses to macrophages. Through the merging of viral material between T cells and macrophages, these newly formed lymphocyte/macrophage fused cells acquire the ability to fuse with neighboring noninfected macrophages. Together, these two-step envelope-dependent cell fusion processes lead to the formation of highly virus-productive multinucleated giant cells reminiscent of the infected multinucleated giant macrophages detected in HIV-1-infected patients and SIVinfected macaques. These mechanisms represent an original mode of virus transmission for viral spreading and a new model for the formation of macrophage virus reservoirs during infection.

2. Article summary

We reveal a very efficient mechanism involved in cell-to-cell transfer from infected T cells to macrophages and subsequent virus spreading between macrophages by a two-step cell fusion process. Infected T cells first establish contacts and fuse with macrophage targets. The newly formed lymphocyte-macrophage fused cells then acquire the ability to fuse with surrounding uninfected macrophages, leading to the formation of infected multinucleated giant cells that can survive for a long time, as evidenced *in vivo* in lymphoid organs and the central nervous system. This route of infection may be a major determinant for virus dissemination and the formation of macrophage virus reservoirs in host tissues during HIV-1 infection.



T Cell-Macrophage Fusion Triggers Multinucleated Giant Cell Formation for HIV-1 Spreading

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ABSTRACT HIV-1-infected macrophages participate in virus dissemination and establishment of virus reservoirs in host tissues, but the mechanisms for virus cell-to-cell transfer to macrophages remain unknown. Here, we reveal the mechanisms for cell-to-cell transfer from infected T cells to macrophages and virus spreading between macrophages. We show that contacts between infected T lymphocytes and macrophages lead to cell fusion for the fast and massive transfer of CCR5-tropic viruses to macrophages. Through the merge of viral material between T cells and macrophages, these newly formed lymphocyte-macrophage fused cells acquire the ability to fuse with neighboring noninfected macrophages. Together, these two-step envelope-dependent cell fusion processes lead to the formation of highly virus-productive multinucleated giant cells reminiscent of the infected multinucleated giant macrophages detected in HIV-1-infected patients and simian immunodeficiency virus-infected macaques. These mechanisms represent an original mode of virus transmission for viral spreading and a new model for the formation of macrophage virus reservoirs during infection.

IMPORTANCE We reveal a very efficient mechanism involved in cell-to-cell transfer from infected T cells to macrophages and subsequent virus spreading between macrophages by a two-step cell fusion process. Infected T cells first establish contacts and fuse with macrophage targets. The newly formed lymphocyte-macrophage fused cells then acquire the ability to fuse with surrounding uninfected macrophages, leading to the formation of infected multinucleated giant cells that can survive for a long time, as evidenced *in vivo* in lymphoid organs and the central nervous system. This route of infection may be a major determinant for virus dissemination and the formation of macrophage virus reservoirs in host tissues during HIV-1 infection.

KEYWORDS HIV-1, T lymphocytes, macrophages, cell-to-cell transfer, cell fusion

Besides CD4⁺ T lymphocytes and dendritic cells, macrophages are cellular targets of human immunodeficiency virus type 1 (HIV-1) and play crucial roles in the pathophysiology of infection (1–5). The presence of infected macrophages has been evidenced *in vivo* in HIV-1-infected patients and simian immunodeficiency virus (SIV)-infected macaques, as well as in humanized mice, where macrophages can sustain HIV-1 productive infection and HIV-1 can persist in tissue macrophages even in mice treated with antiretroviral therapy (6–8). Several specialized functions of macrophages, such as cytokine production, phagocytosis, and migration, are affected by HIV-1 infection (9–14). In addition to latently infected CD4⁺ T cells, infected macrophages also participate in virus dissemination and establishment of persistent virus reservoirs

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in numerous host tissues, including lymph nodes, spleen, lungs, genital and digestive tracts, and the central nervous system (CNS) (4, 5, 15). Virus access to the CNS is indeed mainly related to the migration of infected perivascular monocytes/macrophages through the blood-brain barrier and can result in a massive infiltration of macrophages and microglial cells, often detected as multinucleated giant cells (MGCs) (1, 16–19).

In vitro, macrophages derived from blood monocytes, which express both CD4 and the CCR5 and CXCR4 coreceptors required for virus entry, can be productively infected by HIV-1 (2, 20). However, almost all the data on macrophage infection and from analyses of the different steps of the virus life cycle in this cell type reported so far were obtained by assays performed using cell-free virus particles. Only one study evaluating the possibility of productive infection of macrophages via the selective capture of healthy or rather dying HIV-1-infected CD4⁺ T lymphocytes was recently reported (21).

In contrast, efficient virus dissemination in T lymphocytes by cell-to-cell transfer of virus particles between T cells or from infected macrophages or dendritic cells to T cells, mainly through the formation of the so-called virological synapse (22–26) but also through other membrane structures, such as filopodia or nanotubes (27–31), has been documented. This ability of HIV-1 to spread to T cells by cell-to-cell contacts is the major determinant for virus dissemination, as evidenced *in vivo* in animal models (32, 33). Moreover, these intercellular routes of infection are, at least *in vitro*, several orders of magnitude more efficient than T cell infection with cell-free virus particles (24, 25). Importantly, this mode of virus dissemination may enable virus to escape elimination by the immune system and antiretroviral drugs (15, 20, 22, 34–37).

Whereas infection of T lymphocytes via cell-to-cell transfer was largely investigated, there is a paucity of knowledge of the mechanisms that control infection and dissemination to macrophages by cell-to-cell transfer (21). However, it is obvious that macrophages can establish tight intercellular contacts with infected T lymphocytes, as observed in some tissues, such as lymphoid organs, colon, and brain, in HIV-infected patients and in SIV-infected monkeys (2, 19, 38). The aim of the present study was to investigate how HIV-1 is transferred from infected T cells to macrophages through cell-to-cell contacts for virus dissemination and productive infection of macrophage targets. We show here, for the first time, that the establishment of close contacts between infected T lymphocytes and macrophage targets leads to heterotypic cell fusion for the fast and efficient transfer of viral material, which subsequently triggers viral envelope-dependent homotypic fusion of macrophages able to generate new infectious particles for the intercellular dissemination of HIV-1. Altogether, our data reveal a new mechanism employed by HIV-1 for spreading between its different cell targets and an original model for the formation of macrophage virus reservoirs during HIV-1 infection.

RESULTS

Productive infection of macrophages through HIV-1 cell-to-cell transfer from infected T cells. To analyze whether infected T lymphocytes could mediate cell-to-cell virus transfer to macrophages, we used Jurkat cells or purified primary CD4⁺ T cells as donor infected T cells and macrophages derived from blood monocytes (MDMs) as target cells (Fig. 1A). Jurkat or primary T cells were infected with CCR5-tropic (YU2 and NLAD8 strains) or CXCR4-tropic (NL4.3 strain) viruses (Fig. 1B to D, green bars). T cells that had been infected with HIV-1 for 36 h were cocultured for 6 h with MDMs using different MDM/T cell ratios. After elimination of the T cells by extensive washing (98.5% T cell removal; data not shown), MDMs were fixed and stained for intracellular Gag and cell surface CD11b and CD3, and the percentage of Gag-positive (Gag⁺) cells among the CD11b⁺ cells was quantified by flow cytometry to assess the transfer of viral material in MDMs. After 6 h of coculture with YU2- and NLAD8-infected Jurkat or primary T cells, the transfer of viral material was detected in a significant percentage (10 to 40%) of MDMs (Fig. 1B to D, blue bars). By comparison, a very low percentage of transfer of the YU2 and NLAD8 viruses was detected when infected T cells were separated from MDMs by a virus-permeable Transwell membrane (Fig. 1C and D, red

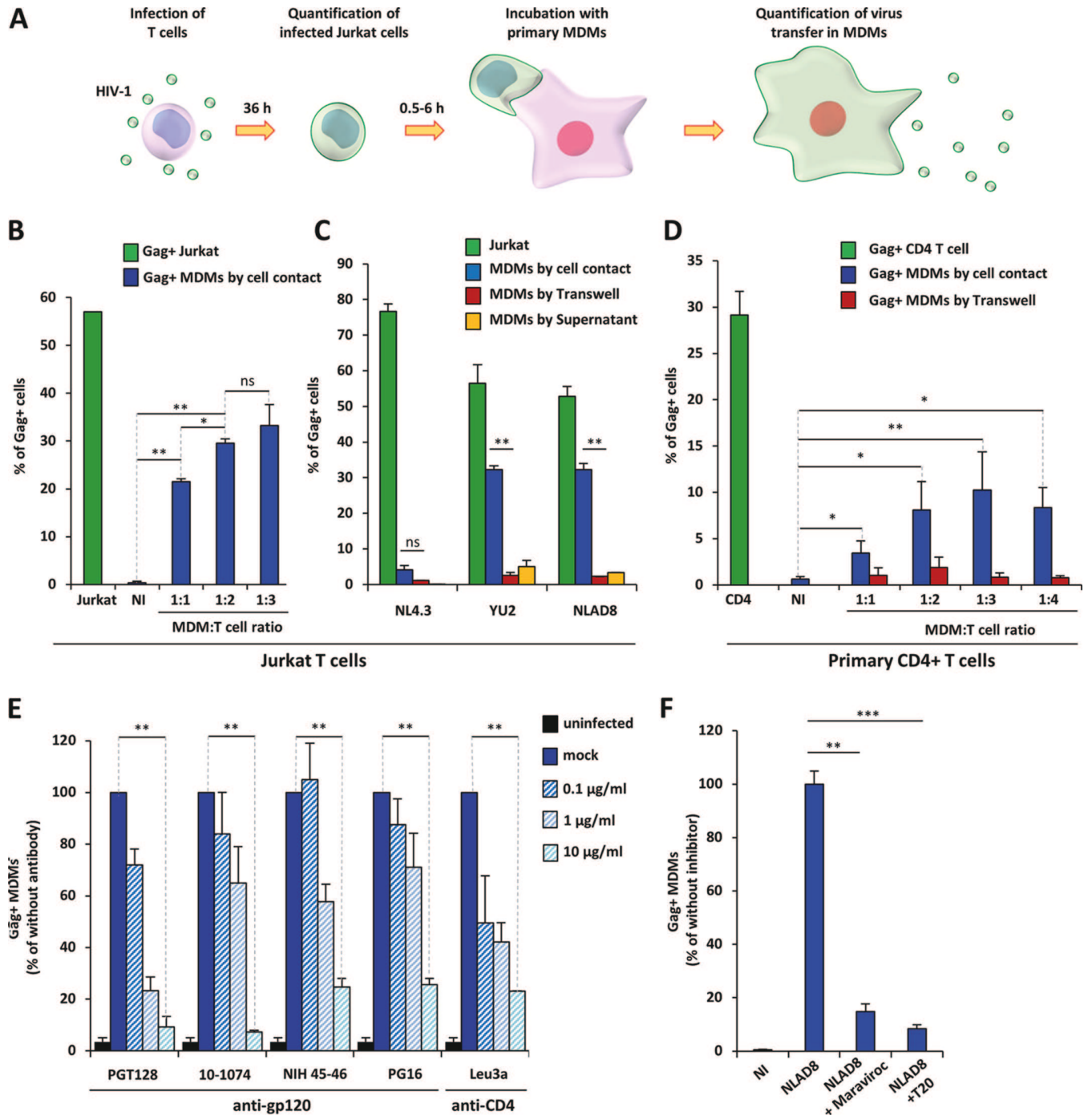


FIG 1 HIV-1 transfer from infected T cells to macrophages. (A) Experimental protocol. (B) Jurkat cells were infected with the NLAD8 strain, and the percentage of infected cells was evaluated 36 h later by flow cytometry after Gag staining (green bars). Infected Jurkat cells were cocultured for 6 h with MDMs at the indicated cell ratio (1:1, 1:2, or 1:3 MDM/Jurkat cell ratio). After elimination of the Jurkat cells, the percentage of CD11b⁺/Gag⁺ MDMs was quantified by flow cytometry (blue bars). As a negative control, noninfected (NI) Jurkat cells were cocultured with MDMs. (C) Jurkat cells were infected with the NL4.3, YU2, or NLAD8 strain, and the percentage of infected cells was evaluated 36 h later by flow cytometry (green bars). The infected Jurkat cells were then cocultured with MDMs directly (blue bars) or through a Transwell membrane (red bars) for 6 h. In parallel, culture supernatants from Jurkat cells collected during the 6-h coculture with MDMs were used to infect autologous MDMs (yellow bars). The percentage of CD11b⁺/Gag⁺ MDMs was evaluated by flow cytometry. (D) Purified primary CD4⁺ T cells were infected with the YU2 strain, and the percentage of infected cells was evaluated 36 h later by flow cytometry (green bar). Infected T cells were then cocultured either directly (blue bars) or through a Transwell membrane (red bars) with autologous MDMs using different cell ratios (1:1, 1:2, 1:3, or 1:4 MDM/T cell ratios) for 6 h. After elimination of T cells, the percentage of CD11b⁺/Gag⁺ MDMs was quantified by flow cytometry. As a negative control, noninfected CD4⁺ T cells were cocultured with MDMs. (E and F) NLAD8-infected Jurkat cells were pretreated with anti-gp120 antibodies or T20, while MDMs were pretreated with anti-CD4 or maraviroc. Infected T cells were cocultured with MDMs for 6 h, and virus transfer to MDMs was quantified as described above. The results are expressed as the percentage of Gag⁺ MDMs relative to the number of Gag⁺ MDMs determined without antibodies or inhibitors. The results are the means from 5 independent experiments performed with MDMs from 5 donors. Error bars represent 1 SEM. Statistical significance was determined using a paired Student's *t* test (ns, not significant [*P* > 0.05]; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

bars) or when MDMs were infected with cell-free viruses produced by T cells during the 6 h of coculture (Fig. 1C, yellow bars). Since virus transfer in CD11b⁺ MDMs was optimal at the 1:2 MDM/T-cell ratio (Fig. 1B and D), this ratio was used in subsequent experiments.

Interestingly, the NLAD8 CCR5-tropic viral strain, which differs from the NL4.3 CXCR4-tropic strain only by substitution of a fragment of the gp120 envelope glycoprotein from the ADA CCR5-tropic strain (39, 40), was efficiently transferred to MDMs, whereas the NL4.3 viral strain was not (Fig. 1C, blue bars). This indicates that the process for virus transfer to MDMs is related to a mechanism dependent on the tropism and coreceptor usage of the viral envelope (Env). To confirm that HIV-1 Env contributed to virus transfer, we tested various viral entry inhibitors, such as anti-gp120 neutralizing antibodies, an anti-CD4 Leu3 antibody, the fusion inhibitor T20, and the CCR5 antagonist maraviroc. As shown in Fig. 1E and F, all the entry inhibitors blocked virus transfer to MDMs, demonstrating that virus cell-to-cell transfer to MDMs is Env dependent.

We next investigated whether the transfer of viral material leads to the productive infection of MDMs. Infected Jurkat or primary CD4⁺ T cells were cocultured for 6 h with MDMs as described above and eliminated by washing, and the percentage of Gag⁺ MDMs was evaluated 1, 6, 9, 12, and 15 days later (Fig. 2A and D). Again, a very low level of virus transfer was observed with NL4.3-infected Jurkat cells, whereas both the YU2 and NLAD8 macrophage-tropic strains propagated efficiently in MDMs, as evidenced by the level of Gag⁺ MDMs detected during the 15-day observation period after the initial transfer from infected T cells. As expected, high levels of viral p24 production were detected in the supernatants from MDMs cocultured with YU2- and NLAD8-infected T cells (Fig. 2B and E). We checked that the virions produced by MDMs were fully infectious by infecting the TZM-bl reporter cell line (Fig. 2C and F). Additionally, virus dissemination and production in MDMs were inhibited by the reverse transcriptase inhibitor zidovudine (AZT) (Fig. 2G, day 6, and H) without affecting the initial virus transfer to MDMs (Fig. 2G, 6 h).

Visualization of cell contacts and virus transfer between infected T cells and macrophages. To visualize virus transfer, fluorescence microscopy was performed first using infected Jurkat cells cocultured for 30 min, 2 h, or 6 h with MDMs preloaded with the CellTrace Far Red dye before fixation and intracellular Gag staining. As shown in Fig. 3A and B, infected T cells established contacts with MDMs, leading to the visualization of Gag⁺ dots in the cytoplasm of the MDMs after 30 min of coculture (Fig. 3A, top row, 0.5 h). Virus transfer subsequently resulted in the accumulation of larger Gag⁺ puncta (Fig. 3A, middle row, 2 h). Intriguingly, a diffuse cytoplasmic staining was observed in almost all Gag⁺ MDMs after 6 h of coculture (Fig. 3A, bottom row, 6 h); at this time point, all the Gag⁺ MDMs contained at least 2 nuclei. In agreement with this finding, fluorescence quantification from images showed a significant increase in the level of intracellular Gag staining of MDMs from 0.5 h to 6 h of coculture (Fig. 3B). Similarly, infected primary CD4⁺ T cells also established contacts with autologous MDMs (leading to the accumulation of intracytoplasmic Gag⁺ dots in MDMs after 2 h of coculture) (data not shown), exhibited a diffuse cytoplasmic Gag staining, and contained several nuclei after 6 h of coculture with infected T cells (see Fig. 6G).

We also performed live-cell imaging using Jurkat cells infected with fluorescent HIV-1–green fluorescent protein (GFP) and cocultured with MDMs preloaded with CellTrace. As shown in Fig. 3C (see also Movie S1 in the supplemental material), we could visualize contacts between infected T cells and MDMs, showing a continuous discharge of fluorescent viral material (white arrows) into MDMs during the first hour of coculture. Interestingly, longer live-cell experiments showed a rapid and massive diffusion of the virus-associated fluorescence from infected T cells to the cytoplasm of MDMs, strongly suggesting that these cell contacts lead to the fusion of infected T cells with the MDM targets (Fig. 3D and Movie S2). Since our experiments were carried out 36 h after T cell infection, when less than 2% of infected T cells were apoptotic (data not shown), we did not observe phagocytosis of infected T cells by MDMs, as recently reported (21). In addition, virus transfer from infected T cells to macrophages was not

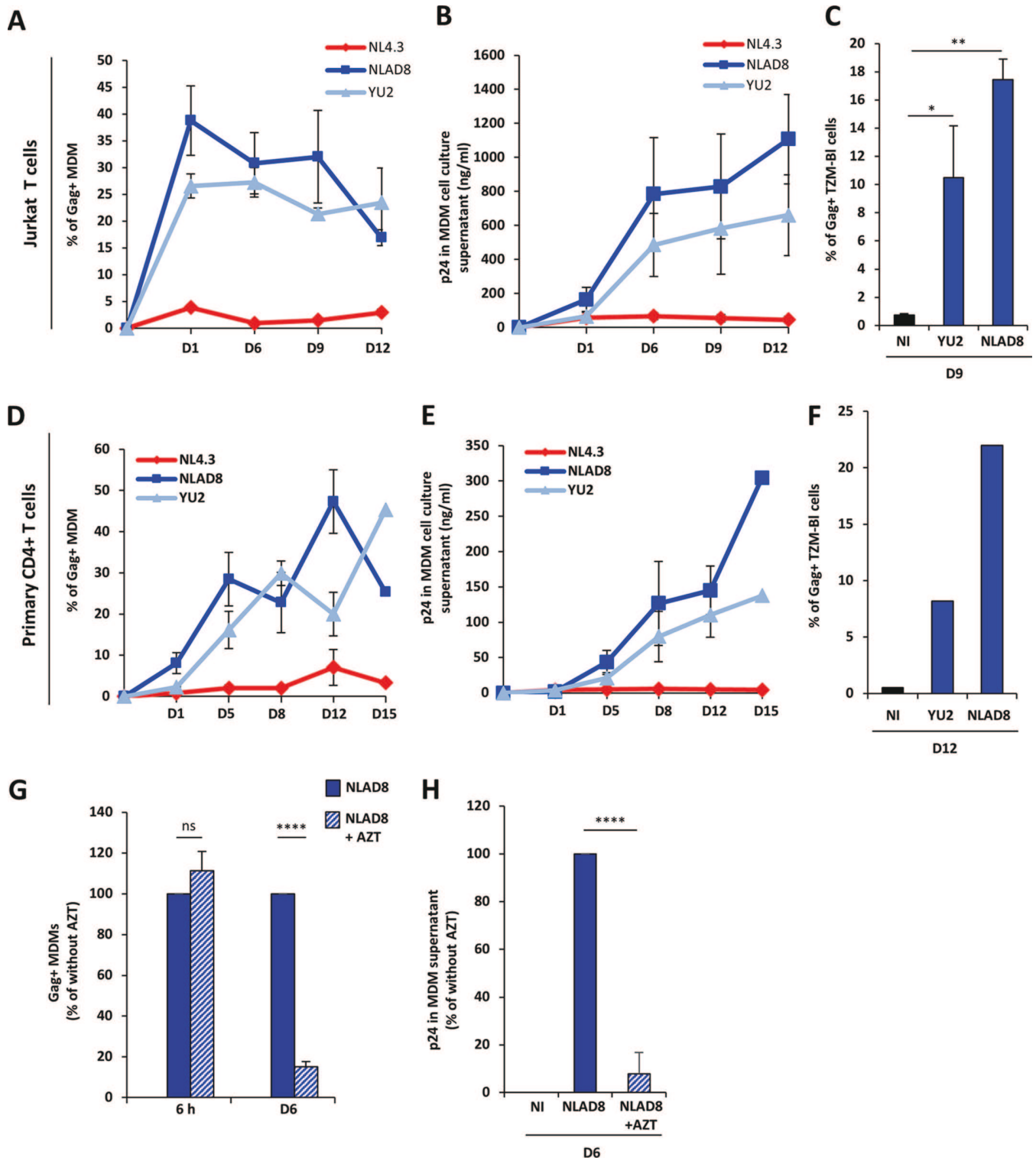


FIG 2 Productive infection of macrophages by virus cell-to-cell transfer from infected T cells. (A to F) NLAD8-infected Jurkat cells (A to C) or primary CD4⁺ T cells (D to F) were cocultured with MDMs for 6 h and eliminated, and the percentage of CD11b⁺/Gag⁺ MDMs was then evaluated 1, 6, 9, 12, or 15 days later by flow cytometry (A and D). In parallel, cell culture supernatants from MDMs were collected and p24 was quantified (B and E). Culture supernatants (100 ng of p24) of MDMs collected 9 days (C) or 12 days (F) after the coculture with YU2- or NLAD8-infected Jurkat (C) or CD4⁺ T (F) cells were used to infect TzM-bl cells, and the percentage of Gag⁺ TzM-bl cells was evaluated 48 h later by flow cytometry. (G and H) NLAD8-infected Jurkat cells were cocultured for 6 h with MDMs pretreated or not pretreated with AZT. (G) The percentage of CD11b⁺/Gag⁺ MDMs was then evaluated just after coculture (6 h) and 6 days later. (H) In parallel, culture supernatants of MDMs were collected 6 days after coculture and p24 was quantified. The results shown in panels A, B, D, E, G, and H are the means from 5 independent experiments performed with MDMs from 5 donors, while the results shown in panels C and F are representative of those from 3 independent experiments. Error bars represent 1 SEM. Statistical significance was determined using one-way analysis of variance (ns, not significant [$P > 0.05$]; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). D, day.

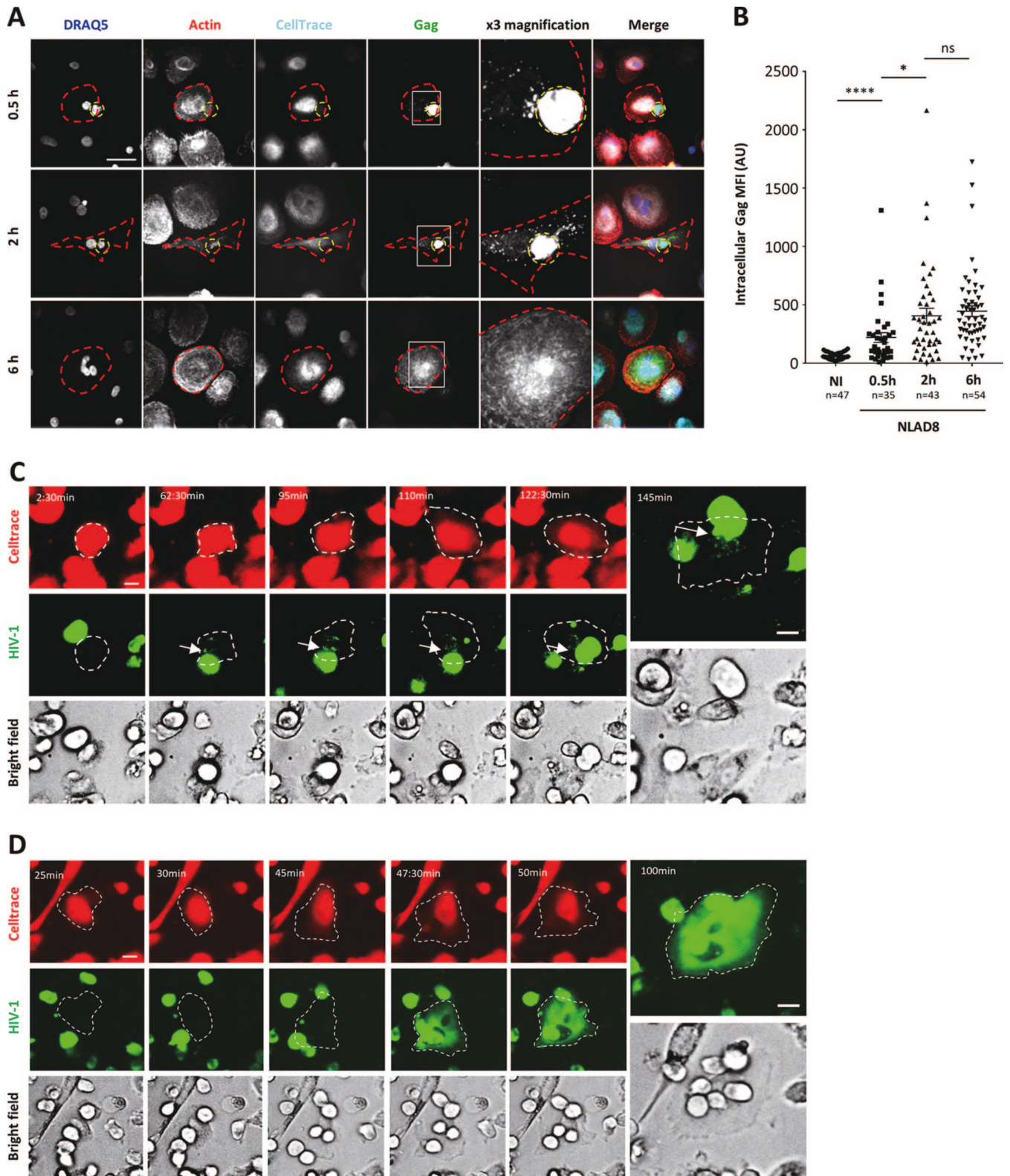


FIG 3 Fluorescence microscopy analysis of intercellular contacts and virus transfer between infected T cells and macrophages. (A and B) NLAD8-infected Jurkat cells were cocultured for 0.5, 2, or 6 h with MDMs prestained with CellTrace. Cells were then fixed, stained with anti-Gag, phalloidin, and DRAQ5, and analyzed by confocal microscopy. (A) Images of a 5- μ m-thick medial stack are shown. Infected donor T cells and MDM targets are indicated by dashed yellow and red lines, respectively. The areas in the white squares in the Gag column were magnified three times, and the images are shown in the $\times 3$ magnification column. Bar, 25 μ m. (B) The intracellular Gag mean fluorescence intensity (MFI) was quantified as indicated in the Materials and Methods section. Each dot corresponds to 1 cell, and the number of cells analyzed (*n*) is indicated. Horizontal bars represent the mean \pm 1 SEM. Statistical significance was determined by an unpaired *t* test (ns, not significant [$P > 0.05$]; *, $P < 0.05$; ****, $P < 0.0001$). AU, absorbance units. (C) Jurkat cells infected with HIV-1R5-GFP (green) were cocultured with

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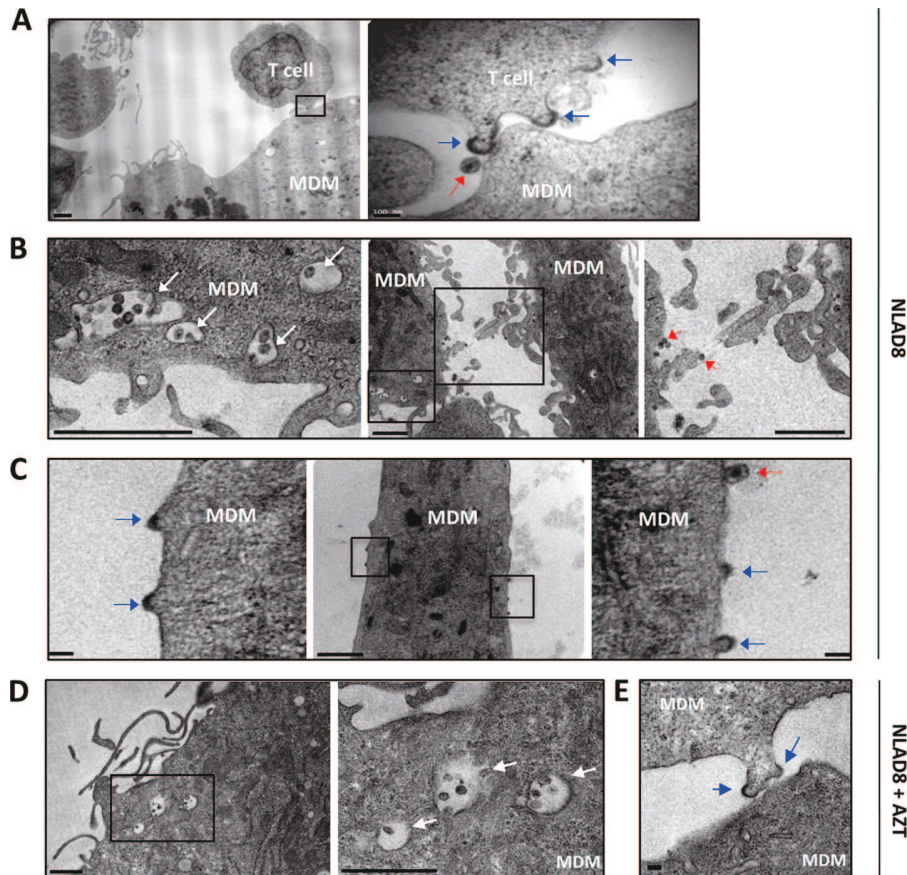


FIG 4 Transmission electron microscopy analysis of virus transfer to macrophages. NLAD8-infected Jurkat cells were cocultured for 6 h with MDMs with (D and E) or without (A to C) AZT (5 μ M). Cells were then fixed and dehydrated. Ultrathin sections were cut, stained, and observed with a transmission electron microscope. Blue arrows, assembling and budding viruses; red arrows, mature virions; white arrows, MDM cytoplasmic membrane compartments containing mature viruses (B and D). In panel A, the right image (bar, 100 nm) corresponds to a higher magnification of the boxed area in the left image (bar, 1 μ m). In panel B, the left and right images (bars, 1 μ m) correspond to higher magnifications of the small and larger boxed areas in the middle image (bar, 1 μ m), respectively. In panel C, the left and right images (bars, 100 nm) correspond to higher magnifications of the left and right boxed areas in the middle image (bar, 1 μ m), respectively. In panel D, the right image (bar, 1 μ m) corresponds to a higher magnification of the boxed area in the left image (bar, 1 μ m). The bar in panel E is 100 nm. The images shown are representative of those obtained from analysis of MDMs from 3 independent donors.

significantly affected by high concentrations of latrunculin A, an actin-binding drug that prevents F-actin polymerization and that is known to totally inhibit phagocytosis at a low concentration, in our experimental system (data not shown). Of note, we did not observe the formation of Gag⁺ T cell syncytia in our experimental system.

Transmission electron microscopy was also used to visualize the contacts between infected T cells and MDMs after 6 h of coculture. As shown in Fig. 4A, electron-dense material corresponding to viral buds protruding from the plasma membrane of the donor T cell (blue arrows), as well as mature virions (red arrow), was observed at the site of contact with MDMs. Interestingly, we also observed the accumulation of mature viruses in cytoplasmic membrane compartments of MDMs

FIG 3 Legend (Continued)

MDMs that had previously been plated onto an Ibidi dish and labeled with CellTrace (red). Fluorescence images were acquired using a 20 \times air objective on a spinning-disk microscope every 2.5 min for 145 min. A 5- μ m-thick medial stack of representative images is shown, and the time lapse is indicated. Bars, 25 μ m. The discharge of viral material (arrows) into MDMs (dashed lines) from infected T cells is shown. (D) Jurkat cells infected with HIV-1R5-GFP (green) were cocultured with MDMs that had previously been labeled with CellTrace (red). Fluorescence images were then acquired using a 20 \times air objective on a spinning-disk microscope every 2.5 min for 100 min. A 5- μ m-thick medial stack of representative images is shown, and the time lapse is indicated. Bars, 25 μ m. The labeled MDM target is indicated with a dashed line.

(Fig. 4B, white arrows). More surprisingly, electron-dense material reminiscent of Gag assembly and virus budding (blue arrows) and mature virions (red arrows) were observed at the plasma membrane of MDMs as well as between MDMs which established tight contacts (Fig. 4B and C), suggesting that virus assembly and budding took place at the cell surface of MDMs only 6 h after coculture with infected T cells, before *de novo* virus production. In agreement with this finding, virus-containing cytoplasmic compartments as well as plasma membrane viral buds were still observed when MDMs were treated with AZT during coculture with infected T cells (Fig. 4D and E). In contrast, we could not observe such virus-containing compartments or viral buds 6 h after infection of MDMs with cell-free viruses (data not shown). These observations could result from the fusion of infected T cells with MDMs revealed by live-cell imaging, suggesting plasma membrane exchanges and the merge of Gag and Env material between infected T cells and MDM targets.

Virus transfer to macrophages through heterotypic cell fusion with infected T cells. Because we observed from immunofluorescence images that almost all Gag⁺ MDMs contained at least 2 nuclei after virus transfer (Fig. 3A, bottom row, 6 h), we first quantified the number of nuclei in MDMs after coculture with infected Jurkat cells (Fig. 5A). Before coculture, more than 90% of the MDMs contained 1 nucleus, whereas about 50% of the Gag⁺ MDMs contained at least 2 nuclei (mean nucleus number = 1.83) after 2 h of coculture, and this percentage increased to 99% (mean nucleus number = 3.24) after 6 h (Fig. 5A). Similarly, MDMs cocultured for 6 h with autologous infected primary CD4⁺ T cells contained several nuclei (mean nucleus number = 5.36; Fig. 5B), suggesting that these Gag⁺ multinucleated MDMs are generated through cell fusion events with infected T cells, as documented by live-cell imaging analysis (Fig. 3D and Movie S2).

To test this hypothesis, infected Jurkat cells were preloaded with the CellTracker 7-amino-4-chloromethylcoumarin (CMAC) dye and cocultured with MDMs as described above. After 6 h of coculture, all Gag⁺ MDMs contained several nuclei, including at least 1 CellTracker dye-stained nucleus (Fig. 5C and D), demonstrating cell fusion between infected T cells and MDMs. This cell fusion between infected T cells and MDMs was mediated by viral envelope-receptor interactions, since it was totally blocked by anti-gp120 neutralizing antibodies and the fusion inhibitor T20 (Fig. 5E and F). Finally, immunofluorescence staining confirmed that all Gag⁺ MDMs contained membrane- and cytoplasm-specific T cell markers, such as CD3, CD2, and Lck, after 6 h of coculture with infected Jurkat cells (Fig. 6A to F) or primary CD4 T cells (Fig. 6G to H).

Together, these results demonstrate that HIV-1 is mainly transferred from infected T cells to macrophages through a heterotypic envelope-dependent cell fusion process leading to the formation of lymphocyte-macrophage fused cells (LMFCs).

Virus dissemination through homotypic cell fusion between macrophages. Interestingly, since we observed that a large majority of the Gag⁺ newly formed LMFCs contained more than 2 nuclei after 6 h of coculture (Fig. 5A and B), we explored whether LMFCs could fuse with surrounding MDMs, leading to the formation of Gag⁺ multinucleated giant cells. After coculture for 6 h with infected Jurkat cells to allow initial cell fusion and virus transfer, LMFCs cultured for 1 or 5 days after elimination of infected T cells indeed contained more than 2 nuclei, with the average nucleus number being 4.8 and 7.9, respectively (Fig. 7A to C). To confirm LMFC-MDM fusion, infected T cells were cocultured for 6 h with MDMs and eliminated, and autologous noninfected MDMs preloaded with the CellTrace dye were added. Twenty-four hours later, Gag⁺ MDMs with several nuclei were also labeled with CellTrace (Fig. 7D), resulting from the fusion of Gag⁺ LMFCs with neighboring uninfected MDMs to form Gag⁺ multinucleated giant cells (MGCs). Fusion of Gag⁺ MDMs with surrounding MDMs was also observed when prelabeled noninfected MDMs were added 5 days after coculture and elimination of infected T cells (data not shown). Since the initial fusion with infected T cells led to the presence of T cell-specific markers at the cell surface of LMFCs (Fig. 6), we hypothesized that the latter cells could also express the viral envelope and explored whether the fusion of Gag⁺ LMFCs with MDMs was mediated by viral envelope-

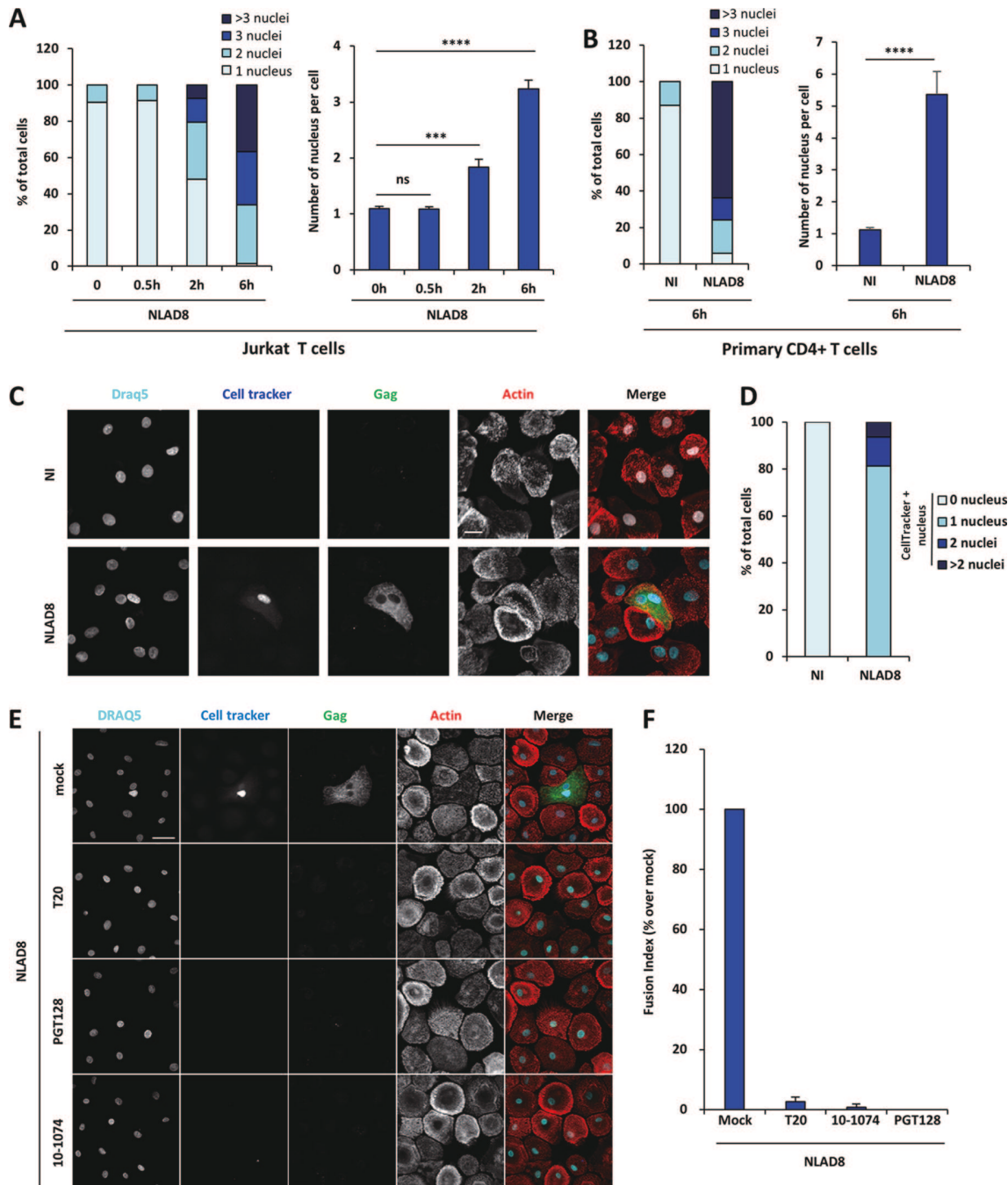


FIG 5 Virus transfer to macrophages by cell fusion with infected T cells. (A and B) NLAD8-infected Jurkat (A) or primary CD4⁺ T (B) cells were cocultured with MDMs for 0.5, 2, or 6 h. After elimination of T cells, MDMs were stained with anti-Gag, phalloidin, and DAPI. Cells were analyzed by confocal microscopy, and the number of nuclei per cell was analyzed from images of at least 50 cells. The results are expressed as the percentage of cells with 1, 2, 3, or more than 3 nuclei (left) and as the mean nucleus number per cell (right). Error bars represent 1 SEM. Statistical significance was determined by the Mann-Whitney U test (ns, not significant [$P > 0.05$]; ***, $P < 0.001$; ****, $P < 0.0001$). (C and D) NLAD8-infected Jurkat cells that had been prelabeled with CellTracker were cocultured for 6 h with MDMs. (C) After elimination of T cells, MDMs were fixed, stained with anti-Gag, phalloidin, and DRAQ5, and analyzed by confocal microscopy. Bar, 25 μm . (D) The number of CellTracker-positive (CellTracker⁺) nuclei per cell was analyzed from images of at least 50 cells. The results are expressed as the percentage of cells with 1, 2, or more than 2 CellTracker-positive nuclei. (E and F) NLAD8-infected Jurkat cells that had been prelabeled with CellTracker were pretreated with anti-gp120 antibodies (PGT128 or 10-1074) or T20 for 1 h and cocultured with MDMs for 6 h in the presence of the inhibitors. Cells were then fixed, permeabilized, stained with anti-Gag, phalloidin, and DRAQ5, and analyzed by confocal microscopy. (E) Images were acquired and processed as described in the text. Bar, 25 μm . (F) The number of CellTracker-positive nuclei per DRAQ5-positive MDM was analyzed from images of at least 1,200 cells for each condition. The results are expressed as the fusion index, corresponding to the percentage of cells containing at least 1 CellTracker-positive nucleus relative to the number of NLAD8-infected Jurkat cells cocultured with MDMs without drugs with at least 1 CellTracker-positive nucleus. The results shown are representative of those from 4 independent experiments performed with MDMs from 4 donors. NI, noninfected Jurkat or primary CD4⁺ T cells cocultured with MDMs.

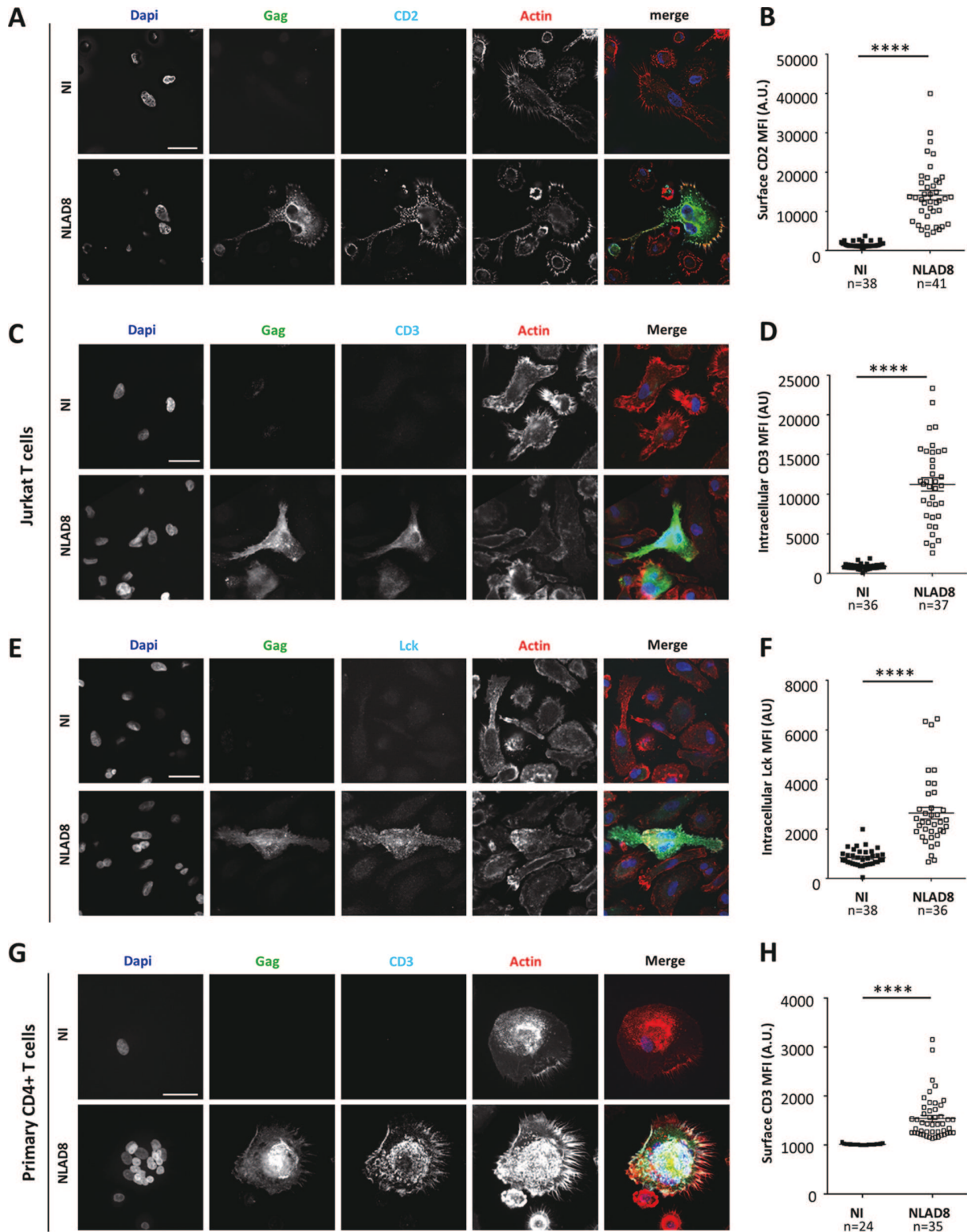


FIG 6 Macrophages express T cell-specific markers after fusion with infected T cells. NLAD8-infected Jurkat cells (A to F) or primary CD4⁺ T cells (G, H) were cocultured for 6 h with MDMs. After elimination of T cells, MDMs were stained with anti-CD2 (A and B) and anti-CD3 (G and H) before permeabilization. The cells were then permeabilized and stained with anti-Gag, anti-CD3 (C and D), anti-Lck (E and F), and DAPI. Representative images of cell surface CD2 (A), CD3 (C and G), and Lck (E) staining are shown. Bars, 25 μ m. Cell surface CD2 (B) or CD3 (H) and intracellular CD3 (D) or Lck (F) mean fluorescence intensities were quantified as indicated in the Materials and Methods section. Each dot corresponds to 1 cell, and the number of cells analyzed (*n*) is indicated. Horizontal bars represent the mean \pm 1 SEM. Statistical significance was determined by the Mann-Whitney U-test (****, *P* < 0.0001).

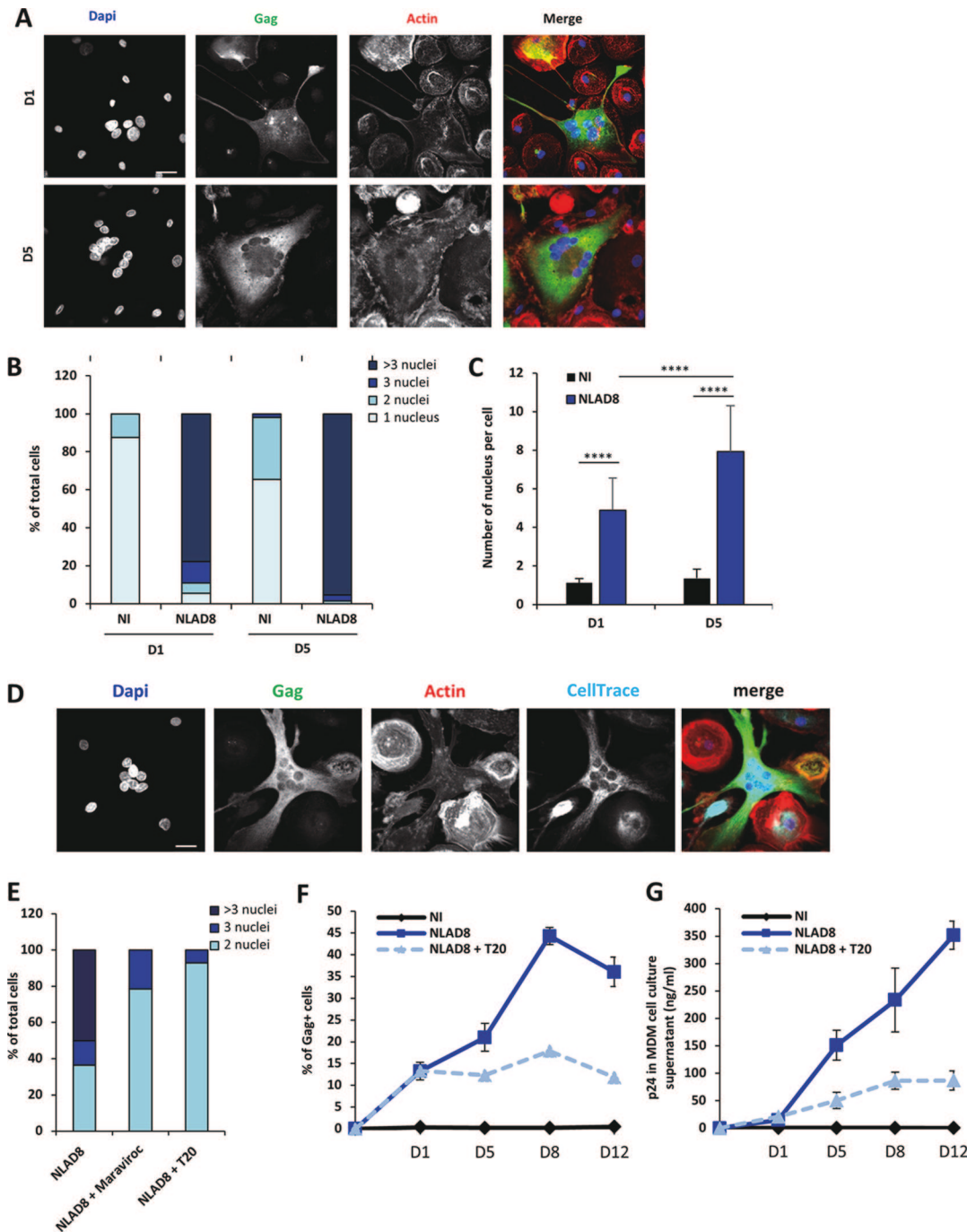


FIG 7 Viral dissemination between macrophages by homotypic cell fusion. (A to C) NLAD8-infected Jurkat cells were cocultured with MDMs for 6 h. After elimination of T cells, MDMs were cultured for 1 or 5 more days and then stained with anti-Gag, phalloidin, and DAPI. (A) Cells were analyzed by confocal microscopy. Bar, 25 μ m. (B and C) The number of nuclei per MDM was quantified from images of at least 50 cells. The results are expressed as the percentage of cells with 1, 2, 3, or more than 3 nuclei (B) and as the mean nucleus number per cell (C). Error bars represent 1 SEM. Statistical significance was determined by the Mann-Whitney U-test (****, $P < 0.0001$). (D) Infected Jurkat cells were cocultured with MDMs for 6 h. After elimination of T cells, autologous MDMs prelabeled with CellTrace were added and cultured for 1 day. MDMs were then stained with anti-Gag, phalloidin, and DAPI. Cells were analyzed by confocal microscopy. Bar, 25 μ m. (E) Infected Jurkat cells were cocultured with MDMs for 6 h. After elimination of T cells, MDMs were cultured for 1 day with or without T20 (10 μ g/ml) or maraviroc (10 μ M) before staining with anti-Gag, phalloidin, and DAPI. Cells were analyzed by confocal microscopy. The number of nuclei was analyzed from images of at least 50 cells. The results are expressed as the percentages of cells with 2, 3, or more than 3 nuclei. (F and G) Infected Jurkat cells were cocultured with MDMs for 6 h. After elimination of T cells, MDMs were cultured for 1, 5, 8, or 12 days with or without T20 (10 μ g/ml). (F) The percentage of Gag⁺ MDMs was then evaluated by flow cytometry. (G) In parallel, culture supernatants from MDMs were collected and p24 was quantified. The results shown in panels A to E are representative of those from 4 independent experiments performed with MDMs from 4 donors, while the results shown in panels F and G correspond to the means from 3 independent experiments performed with MDMs from 3 donors. NI, noninfected Jurkat cells cocultured with MDMs.

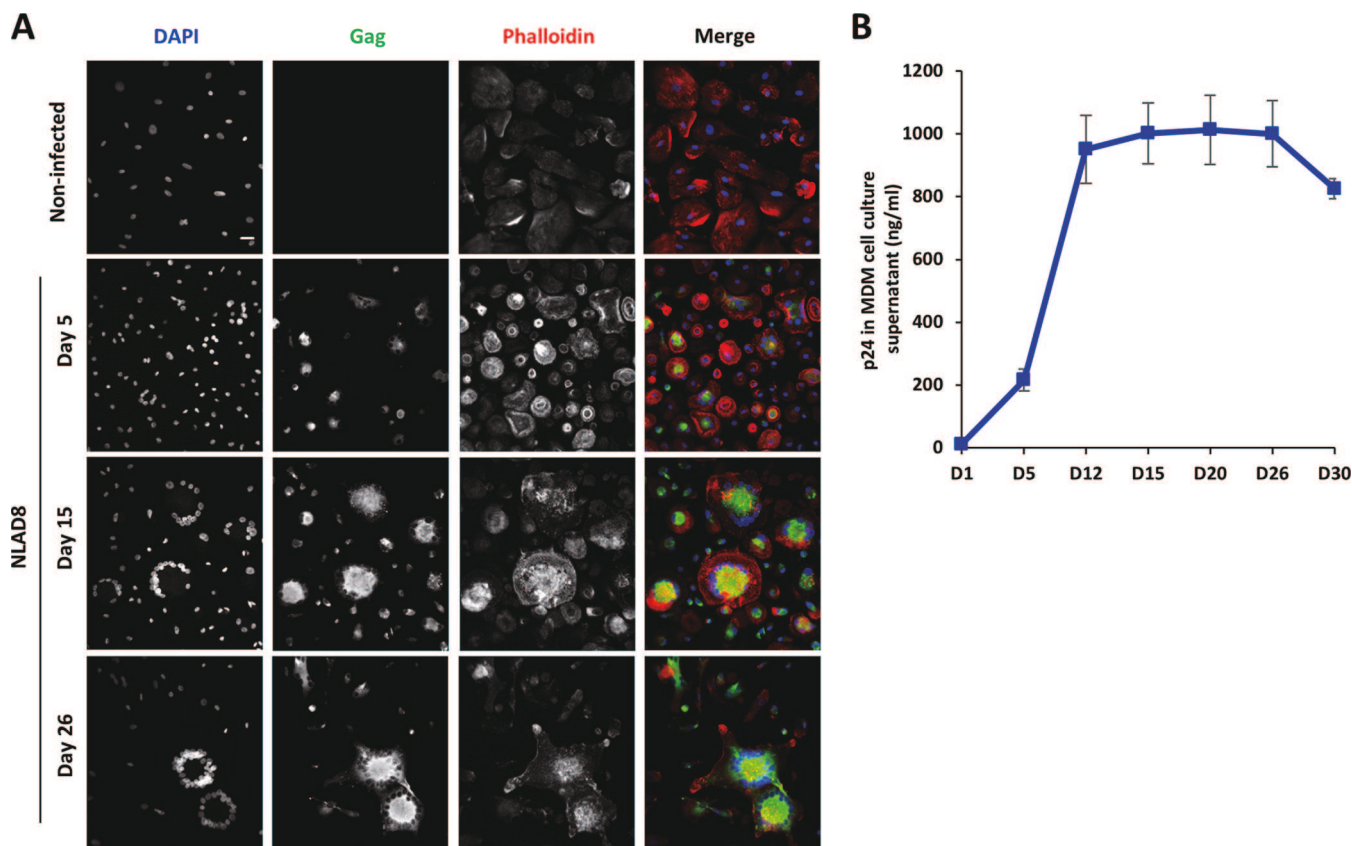


FIG 8 Formation and survival of infected multinucleated giant cells. NLAD8-infected Jurkat cells were cocultured with MDMs for 6 h and eliminated, and MDMs were then cultured for the indicated periods of time. (A) Cells were fixed, permeabilized, and stained with anti-Gag, phalloidin, and DAPI. Cells were analyzed by confocal microscopy, and images were acquired and processed as described in the text. Bar, 25 μ m. (B) Culture supernatants of MDMs were collected at the indicated days after coculture, and p24 was quantified. The results correspond to the means from 3 independent experiments. Error bars represent 1 SEM. Noninfected, noninfected Jurkat cells cocultured with MDMs.

receptor interactions. Following the 6-h coculture to allow initial cell fusion and virus transfer, infected T cells were eliminated and MDMs were cultured in the presence of the fusion inhibitor T20 and the CCR5 antagonist maraviroc. As expected, about 60% of Gag⁺ LMFCs cultured without inhibitors contained at least 3 nuclei 1 day later, but this percentage decreased to 20 and 10% when LMFCs were cultured with maraviroc and T20, respectively (Fig. 7E). Importantly, inhibition of LMFC fusion with neighboring MDMs by T20 after initial virus transfer led to a net decrease in virus dissemination (Fig. 7F) and virus production by LMFCs (Fig. 7G). In agreement with this finding, infected MGCs with many nuclei could be still detected after 26 days of culture (Fig. 8A) and were indeed still able to produce high levels of p24 30 days after elimination of infected T cells (Fig. 8B). These results show that the LMFC-MDM cell fusion is required for optimal virus spreading and production by long-lived multinucleated giant cells.

DISCUSSION

In the present study, we reveal the mechanisms involved in the rapid and massive cell-to-cell transfer of HIV-1 from infected T cells to MDMs and the subsequent virus spreading between MDMs by a two-step cell fusion process leading to the productive infection of MDM targets. Both cell fusion steps are mediated by viral envelope-receptor interactions at the cell surface of T cells and MDMs and are completed in less than 2 h. This route of infection may be a major determinant *in vivo* for virus dissemination to macrophages.

As evidenced by fluorescence microscopy analyses, including live-cell imaging, the first step is related to the establishment of contacts with infected T cells, resulting in the

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fusion of infected T cells with MDM targets. This cell fusion process is evidenced by the massive and rapid transfer of Gag⁺ material as well as membrane and cytosolic T cell-specific markers, such as CD2, CD3, and Lck, which thus ensures efficient virus dissemination between these two important target cells of HIV-1. We did not observe in our experimental system the formation of HIV-1-induced T cell syncytia using either Jurkat cells or CD4⁺ primary T cells as virus donor cells, suggesting that HIV-1-induced cell-to-cell fusion could be restricted to myeloid cell targets, such as macrophages, and inhibited between T cells (41–45). In agreement with this first step of T cell-to-MDM fusion for virus transfer, it was shown that myeloid cells from lymphoid tissues of SIV-infected macaques, such as spleen and lymph nodes, contain T cell markers and viral RNA and DNA originating from infected T cells (46, 47).

Electron microscopy analysis confirmed that infected T cells establish contacts with macrophages, with evidence of virus assembly at the site of cell-to-cell contacts, leading to virus transfer and the accumulation of mature virus particles in cytoplasmic membrane compartments in macrophages after only 6 h of coculture with infected T cells, before *de novo* virus production by the macrophage targets. We hypothesize that the mature and immature viruses found in these early-formed virus-containing compartments (VCCs) could result from the initial discharge of viral material before cell fusion, observed by fluorescence microscopy on both fixed and lived cells. Such early-formed VCCs have been observed in target T cells following virus cell-to-cell transfer in enclosed endocytic compartments from infected donor T cells (48–51). The formation of VCCs has also been largely documented when macrophages were infected with cell-free viruses, but they appeared later, at least 5 to 6 days after infection, and contained newly synthesized virus particles (52–57). While the exact mechanisms for the formation of these early and late VCCs need to be further investigated, such VCCs could participate in the long-term storage of HIV-1 in tissue macrophages and lead to the establishment of viral reservoirs for virus maintenance and spreading (1–5). Interestingly, some events of assembly and budding of virus particles were detected at the plasma membrane of the macrophage targets before *de novo* virus production after only 6 h of coculture with infected T cells, suggesting that the newly formed Gag⁺ lymphocyte-macrophage fused cells also expose the viral envelope at their cell surface. These cells then acquire the ability to fuse with neighboring uninfected MDMs, leading to the formation of multinucleated giant cells that can survive and produce large amounts of fully infectious viruses even after 30 days of cell culture. Similarly, the formation of HIV-1-infected giant cells was reported when macrophages were infected with cell-free viruses (14, 58), but they appeared several days after infection. Despite their large size, these infected multinucleated macrophages migrate faster than their infected mononucleated counterpart and may participate in virus dissemination (13). More importantly, these Gag⁺ multinucleated giant cells observed *in vitro* are reminiscent of the infected multinucleated giant macrophages detected *in vivo* in lymphoid organs and the CNS of HIV-1-infected patients and SIV-infected macaques (3, 17, 59–64).

We found that virus transfer to macrophages through initial T cell fusion and subsequent virus dissemination in multinucleated giant macrophages were restricted to macrophage-tropic CCR5-using viral strains. In contrast, Baxter et al. (21) reported that internalization of healthy or dying infected T cells by MDMs was related to a nonconventional mechanism independent of the viral envelope and showed that the NL4.3 CXCR4-tropic strain could be efficiently transferred to macrophages using GFP-tagged NL4.3-infected T lymphocytes as donor cells. This discrepancy could be related to the use of nondying T cells infected with the untagged wild-type replication-competent NL4.3 strain to analyze transfer in our experimental system. While initial studies suggested that virus transfer between CD4⁺ T cells at the virological synapse was independent of coreceptor usage (65, 66), subsequent reports showed that this transfer required coreceptor expression and was inhibited by coreceptor antagonists (24, 49, 67, 68). Here, we found that the initial virus transfer to macrophages was inhibited by neutralizing monoclonal antibodies targeting the HIV-1 gp120 envelope

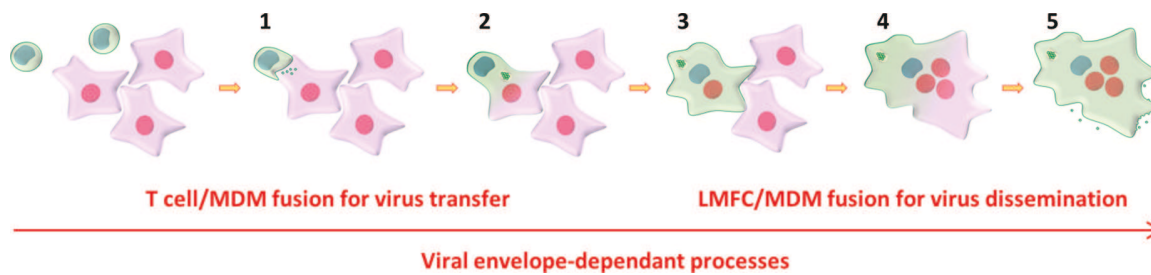


FIG 9 Model for virus cell-to-cell transfer from infected T cells to MDMs and virus spreading between MDMs. Initial virus transfer and subsequent virus spreading are mediated by a two-step cell fusion process. In the first step, infected T cells establish contacts, initially discharge viral material to MDMs (step 1), and then fuse with MDM targets (step 2), with accumulation of viruses in intracytoplasmic compartments and virus assembly and budding at the cell surface. Gag⁺ newly formed LMFCs (step 3) then acquire the ability to fuse with surrounding uninfected MDMs, leading to the formation of Gag⁺ multinucleated giant cells (step 4) that could survive for a long time to produce infectious viruses (step 5).

glycoprotein as well as by the Leu3a antibody targeting the CD4 receptor, indicating that early interactions between T cells and recipient macrophages involve recognition of CD4 by gp120. Virus transfer to macrophages leading to the formation of infected lymphocyte-macrophage fused cells was also blocked by the fusion inhibitor T20, targeting the viral transmembrane gp41 envelope glycoprotein, and the CCR5 antagonist maraviroc. These results indicate that virus transfer and T cell fusion with macrophages are mediated by initial viral envelope-receptor and -coreceptor interactions. In addition, we show that the second cell fusion step between Gag⁺ lymphocyte-macrophage fused cells and neighboring MDMs, required for virus dissemination and virus production by multinucleated giant cells, is also dependent on the viral envelope, since it is inhibited by T20 and maraviroc. While our data clearly show that both cell fusion processes for virus transfer and dissemination in macrophages are dependent on the viral envelope, we cannot exclude the possibility that other mechanisms related to the propensity of macrophages to mediate homotypic cell fusion could also participate in the cell fusion processes for virus transfer and dissemination. It would be interesting to investigate the potential role in HIV-1-mediated T cell-MDM and MDM-MDM fusions of some effector proteins known to be involved in programming of macrophages into a fusion-competent state and then in cell fusion for the formation of multinucleated giant macrophages (for a review, see reference 69).

Our analyses indicate that this cell-to-cell transfer of virus from infected T cells to macrophages is largely more efficient than virus infection with cell-free viruses and is certainly the most potent experimental system to infect macrophages *in vitro* described so far (1–5). Indeed, we failed to detect a significant transfer of viral material when macrophages were directly infected with the cell-free virus particles released by infected donor T cells during the time of coculture with macrophages. In sharp contrast, virus transfer from the infected CD4⁺ T cells evaluated here resulted in a robust productive infection of macrophages, as evidenced by the high levels of virus production in the supernatant of the multinuclear giant macrophages detected by monitoring of these macrophages for 30 days.

In summary, our results show that HIV-1 can be efficiently transferred to macrophages from infected donor T cells, without implying internalization of infected CD4⁺ T cells by the macrophage targets (21). While the two mechanisms are not mutually exclusive, we reveal that a novel fast and very efficient mechanism is involved in cell-to-cell transfer from nondividing infected T cells to macrophages and subsequent virus spreading between macrophages by a two-step cell fusion process (the model is shown in Fig. 9). In the first step, infected T cells establish tight contacts and initially discharge viral material to MDMs, resulting in the fusion of infected T cells with MDM targets. The newly formed Gag⁺ lymphocyte-macrophage fused cells then acquire the ability to fuse with surrounding noninfected MDMs, leading to the formation of infected multinucleated giant cells that can survive for a long time in host tissues to produce

infectious virus particles, as has been shown *in vivo* in lymphoid organs and the CNS of HIV-1-infected patients and SIV-infected macaques (3, 17, 59–64). Similarly, the first step related to the initial T cell-to-MDM fusion agrees with results showing that myeloid cells from lymphoid tissues of SIV-infected macaques contain T cell markers and viral DNA originating from infected T cells (46, 47). These *in vivo* observations support the importance of the molecular mechanisms revealed here, which contribute to a better understanding of virus dissemination from infected T cells to macrophages and the formation of long-lived macrophage viral reservoirs in host tissues.

MATERIALS AND METHODS

Plasmids and reagents. The proviral plasmids pNL4-3 and pNLAD8 were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID. The proviral plasmid HIV-1R5-GFP was a gift of Michael Schindler (Munich, Germany) (70), while pYU-2 and the plasmid encoding the vesicular stomatitis virus (VSV) envelope G glycoprotein (pVSVg) have been described previously (71). The following antibodies were used: phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (clone ICRF44; BD Biosciences), PE-Cy7-conjugated anti-CD11b (clone ICRF44; BioLegend), RD1- or FITC-conjugated anti-Gag (clone KC57; Beckman Coulter), anti-CD4 (clone Leu3a; BioLegend), anti-CD3 (clone UCHT1; BioLegend), and anti-CD2 (TS2/18 clone; a gift from Andres Alcover, Paris, France [72]). Alexa Fluor 647-conjugated phalloidin (Life Technologies) was also used. The following reagents were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: anti-gp120 antibodies (PG16 and NIH45-46), HIV-1 CAp24 hybridoma (183-H12-5C), HIV-Ig, maraviroc, T20, and AZT. Anti-gp120 antibodies (10-1074 and PGT128) were gifts from Paul Zhou (Shanghai, China).

Cell culture. Cells of the HEK293T, T2M-bl, and Jurkat cell lines were obtained from the ATCC collection. HEK293T and T2M-bl cells were maintained in Dulbecco minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU of penicillin/ml, and 100 μ g of streptomycin/ml (ATB; Invitrogen). Jurkat cells were maintained in RPMI 1640 complete culture medium supplemented with 10% FCS and ATB. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy anonymous donors by density gradient sedimentation using Histopaque (Sigma-Aldrich), and monocytes were purified using a CD14-positive selection kit (CD14 microbeads, Miltenyi) according to the manufacturer's guidelines. Blood samples from anonymous healthy donors were purchased from the Etablissement Français du Sang Paris-Saint-Antoine-Croizatier, Paris, France. The monocytes were differentiated into macrophages for 8 days in RPMI 1640 culture medium supplemented with 20% FCS, ATB, and 25 ng/ml of granulocyte-macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (Miltenyi). Human primary CD4⁺ T cells were isolated from PBMCs by density gradient sedimentation using Histopaque and then purified by negative selection (CD4⁺ T cell isolation kit; Miltenyi) following the manufacturer's recommendation. CD4⁺ T cells were activated for 3 days in RPMI 1640 medium containing 20% fetal bovine serum (FBS), interleukin-2 (IL-2; Miltenyi) at 10 U/ml, and phytohemagglutinin-P (PHA-P; Sigma-Aldrich) at 5 μ g/ml. After activation, the CD4⁺ T cells were kept in RPMI 1640 medium supplemented with 20% FBS and IL-2. All cells were grown at 37°C under 5% CO₂.

Viral production, titration, and infection. Replication-competent HIV-1 strains YU2, NL4.3, and NLAD8 and HIV-1R5-GFP were produced in HEK293T cells by cotransfection of the proviral plasmid in combination with pVSVg using the calcium phosphate precipitation technique as described previously (71). The amounts of CAp24 produced were determined by enzyme-linked immunosorbent assay (ELISA; Innogenetics). The viral titer was determined by flow cytometry (Accuri C6; BD Biosciences) using Jurkat cells (J77 clone) as described previously (71).

Virus transfer and dissemination. To study the transfer from Jurkat or primary CD4⁺ T cells to MDMs, T cells were infected using a multiplicity of infection (MOI) of 0.5 for 16 h. The cells were then washed and cultured for 20 h. After washing, the T cells were cocultured at a ratio of 2:1 (unless stated otherwise in the text or figure legends) with MDMs, seeded at a density of 0.5×10^6 cells/well, for 0.5 to 6 h. To remove T cells, MDMs were then washed extensively with phosphate-buffered saline (PBS) once, with PBS containing 10 mM EDTA, and then 2 more times with PBS. MDMs were harvested or cultured for several days and then collected. Cells were then surface stained using anti-CD11b and anti-CD3 antibodies and then fixed with 4% paraformaldehyde (PFA), permeabilized using permeabilization buffer (PBS, 1% BSA, 0.1% Triton X-100), and stained with an anti-Gag (KC57, 1/500). The percentage of Gag⁺ cells among the CD11b⁺ cells corresponding to the MDM population was determined by flow cytometry. To analyze viral production, MDM culture supernatants were collected, and the amount of CAp24 produced was determined by ELISA. For MDM infection with cell-free viruses, T cells were infected as described above and incubated with MDMs for 6 h through a 0.4- μ m-pore-size Transwell membrane. Alternatively, the supernatant of producer Jurkat cells corresponding to the 6-h coculture was added to the MDMs, and the percentage of Gag⁺ cells was analyzed by flow cytometry as described above.

Infectivity assay. A total of 2×10^5 T2M-bl cells (HeLa-CD4 cells stably expressing CD4 and CCR5) were incubated with viral supernatants (200 ng of CAp24) for 18 h. The T2M-bl cells were then cultured for 2 days, fixed with 3.7% formaldehyde (Sigma), and treated with permeabilization buffer. The cells were stained with an anti-Gag antibody, and the percentage of Gag⁺ cells was determined by flow cytometry.

Effect of inhibitors on virus transfer and dissemination. To analyze the effect of inhibitors on virus transfer, infected donor T cells or MDM targets were pretreated for 1 h with anti-gp120 neutralizing antibodies (PGT128, 10-1074, NIH 45-46, and PG16) or anti-CD4 (Leu3a), respectively, using 3 concentrations (0.1, 1, or 10 $\mu\text{g/ml}$) of antibodies in the presence of 10 $\mu\text{g/ml}$ of Fc-Block reagent (Sigma). Infected T cells were then cocultured for 6 h with MDMs and removed by washings, and the percentage of Gag⁺/CD11b⁺ MDMs was determined by flow cytometry. Results were expressed as the percentage of Gag⁺ MDMs relative to the number of Gag⁺ MDMs detected without antibody treatment. The effects of the CCR5 receptor antagonist maraviroc as well as the fusion inhibitor T20 on virus transfer were also tested. Infected donor T cells or MDM targets were pretreated for 1 h with T20 or maraviroc at 10 μM and 10 $\mu\text{g/ml}$, respectively. Infected T cells were then cocultured for 6 h with MDMs and removed, and the percentage of Gag⁺/CD11b⁺ MDMs was determined by flow cytometry. Results were expressed as the percentage of Gag⁺ MDMs relative to the number of Gag⁺ MDMs detected without inhibitor treatment. To show that virus transfer from infected T cells to MDMs leads to productive infection, MDM targets were pretreated with AZT (5 μM) for 2 h prior to coculture for 6 h with infected T cells. After removal of infected T cells, the percentage of Gag⁺/CD11b⁺ MDMs was determined by flow cytometry either directly after the 6 h of coculture or 6 days later, while the level of viral p24 production from MDMs was determined by ELISA after 6 days of culture. Results are expressed as the percentage of Gag⁺ MDMs or as the amount of p24 relative to that determined without AZT. To analyze the effect of inhibitors on virus dissemination between MDMs, infected T cells were first cocultured for 6 h with MDMs and removed by washings, and MDMs were cultured for 1, 5, 8, or 12 days in the presence of 10 μM T20. The percentage of Gag⁺/CD11b⁺ MDMs was then determined by flow cytometry, while the level of CAp24 production from MDMs was determined by ELISA. Results are expressed as the percentage of Gag⁺ MDMs or as the amount of CAp24 relative to that determined without T20 treatment.

Fluorescence microscopy analysis. To visualize cell contacts and virus transfer, 10⁶ Jurkat or primary CD4⁺ T cells infected with NLAD8 as described above were prelabeled with 2 μM CellTrace Far Red (Life Technologies) and cocultured for 0.5, 2, or 6 h with 0.5 \times 10⁶ MDMs that had been plated onto coverslips. MDMs were then fixed with 4% PFA, blocked for 10 min in PBS containing 1% bovine serum albumin (BSA), stained with 2 μM deep red fluorescing agent Q5 (DRAQ5; eBioscience) for 20 min in PBS, permeabilized, and stained using KC57 FITC-conjugated antibody and phalloidin-Alexa Fluor 647 (Molecular Probes) diluted in permeabilization buffer for 1 h. The coverslips were washed with PBS and mounted on slides using 10 μl of Fluoromount mounting medium (Sigma). Images were acquired on a spinning-disk (CSU-X1M1; Yokogawa) inverted microscope (DMI6000; Leica) and then processed using Fiji software (ImageJ; NIH) (73). Quantitative image analysis was performed using Fiji software, in which a region of interest was defined by using actin staining and measuring the whole fluorescence intensity of the Gag staining with respect to that for noninfected cells. To analyze fusion between infected T cells and MDMs, infected Jurkat or primary CD4⁺ T cells were cocultured for 6 h with MDMs. Cells were then fixed with 4% PFA and blocked in PBS containing 1% BSA. Anti-CD2 (TS2/18, 1/200 dilution) surface staining was performed on nonpermeabilized cells in PBS-BSA for 1 h. The coverslips were then rinsed in PBS-BSA and incubated for 1 h with anti-mouse immunoglobulin-Alexa Fluor 555 in PBS-BSA. Anti-CD3 (10 $\mu\text{g/ml}$), anti-Lck (2 $\mu\text{g/ml}$), anti-Gag (KC57-FITC, 1/200 dilution), and F-actin intracellular staining was done by incubating the coverslips with the primary antibody indicated above and phalloidin-Alexa Fluor 647 diluted in the permeabilization buffer. The coverslips were then rinsed with PBS-BSA and incubated for 1 h with the corresponding fluorescence-coupled secondary antibody. After three washes, the coverslips were mounted on microscope slides, using 10 μl of Fluoromount mounting medium with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich). Cells were examined under an epifluorescence microscope (DMI6000; Leica), and quantitative image analysis was performed using Fiji software, in which a region of interest was defined using actin staining and measuring the whole fluorescence intensity of the indicated marker (i.e., CD3, CD2, or Lck) in Gag⁺ cells with respect to that in noninfected cells. For the CellTracker experiment, NLAD8-infected Jurkat cells were labeled with 5 μM CellTracker (7-amino-4-chloromethylcoumarin [CMAC]) dye (Life Technologies) for 30 min and then cocultured for 6 h with MDMs. The cells were then fixed in 4% PFA for 20 min, blocked for 10 min in PBS containing 1% BSA, stained with 2 μM DRAQ5 for 20 min in PBS, permeabilized, and stained using KC57 FITC-conjugated antibody and phalloidin-Alexa Fluor 555 (Molecular Probes) diluted in permeabilization buffer for 1 h. The coverslips were washed with PBS and mounted on slides using 10 μl of Fluoromount mounting medium. Images were acquired and then processed as described above. To analyze the effect of inhibitors on fusion between T cells and macrophages, infected donor T cells or MDM targets were pretreated for 1 h with anti-gp120 neutralizing antibodies (PGT128, 10-1074) or T20, using a concentration of 10 $\mu\text{g/ml}$ in the presence of 10 $\mu\text{g/ml}$ of Fc-Block reagent (Sigma). Infected T cells were then labeled with 5 μM CellTracker CMAC and cocultured for 6 h with MDMs in the presence of inhibitors. Cells were then fixed and stained as described above using KC57 FITC-conjugated antibody and phalloidin-Alexa Fluor 647. Images were acquired and then processed as described above. To analyze fusion between MDMs, infected T cells were initially cocultured with MDMs for 6 h. After elimination of T cells by extensive washing, autologous MDMs prelabeled with CellTrace Far Red or CellTracker were added to the MDMs just after the 6-h coculture or 4 days later, respectively, and cultured for 1 more day. The coverslips were then washed and mounted with Fluoromount mounting medium containing DAPI or the NucRed reagent. Images were acquired and processed as described above. To analyze the effect of T20 and maraviroc on fusion between MDMs, infected T cells were initially cocultured with MDMs for 6 h. After removal of T cells by extensive washings, MDMs were cultured for 1 more day with or without T20 (10 $\mu\text{g/ml}$) or maraviroc (10 μM) and

the cells were stained for nuclei, Gag, and F actin following performance of the fixation and blocking steps described above. The number of nuclei per cell was analyzed from images of at least 50 cells. To analyze the survival of infected giant multinucleated cells in culture, infected Jurkat cells and MDMs were cocultured for 6 h, and MDMs were then cultured for up to 30 days after the removal of T cells by extensive washings as described above. Cells were then collected, fixed, and stained for Gag and F actin as described above. The coverslips were washed with PBS and mounted on slides using 10 μ l of Fluoromount mounting medium containing DAPI. Images were acquired on a spinning-disk inverted microscope and then processed using Fiji software as described above.

Live-cell imaging of HIV-1 transfer. Jurkat cells (2×10^6) infected with HIV-1R5-GFP were cocultured for 1 h with 10^6 MDMs prelabeled with CellTrace Far Red and plated onto a 35-mm Ibidi dish in RPMI 1640 medium not containing phenol red but containing 10% FBS and ATB. Images were recorded using a 20 \times air objective every 1 or 2.5 min for 1, 2, or 3 h on a spinning-disk (CSU-X1M1; Yokogawa) confocal inverted microscope (DMI6000; Leica) equipped with a heated chamber. z-stack optical sections were acquired at 0.5- μ m depth increments, and movies were analyzed using Fiji software.

Transmission electron microscopy analysis. Jurkat cells (2×10^6) infected with NLAD8 as described above were cocultured for 6 h with 10^6 MDMs that had been plated onto coverslips with or without AZT (5 μ M). In parallel, MDMs were infected by cell-free NLAD8 for 6 h. The coverslips were then fixed using 2.8% glutaraldehyde and 2% PFA for 20 min. After 2 washes in PBS, the cells were dehydrated and embedded into epoxy (Electron Microscopy Sciences). Ultrathin sections of 90 nm were cut with an ultramicrotome, stained with uranyl acetate and Reynold's lead, and observed with a transmission electron microscope (JEOL 1011). Acquisition was performed with a Gatan E51000W charge-coupled-device camera.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01237-17>.

SUPPLEMENTAL FILE 1, AVI file, 0.6 MB.

SUPPLEMENTAL FILE 2, AVI file, 0.6 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

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Article 2: Cell-to-cell spreading of HIV-1 in myeloid 1 target cells escapes SAMHD1 restriction

1. Abstract

Dendritic cells (DCs), macrophages as well as osteoclasts (OCs) are emerging as target cells of HIV-1 involved in virus transmission, dissemination, and establishment of persistent tissue virus reservoirs. While these myeloid cells are poorly infected by cell-free viruses, because of the high expression of cellular restriction factors such as SAMHD1, we show here that HIV-1 uses a specific and common cell-to-cell fusion mechanism for virus transfer and dissemination from infected T lymphocytes to the target cells of the myeloid lineage, including immature DCs (iDCs), OCs and macrophages, but not monocytes and mature DCs. The establishment of contacts with infected T cells leads to heterotypic cell fusion for the fast and massive transfer of viral material into OC and iDC targets, which subsequently triggers homotypic fusion with non-infected neighboring OCs and iDCs for virus dissemination. These two cell-to-cell fusion processes are not restricted by SAMHD1 and allow a very efficient virus spreading in myeloid cells, resulting in the formation of highly virus productive multinucleated giant cells. These results reveal the cellular mechanism for SAMHD1-independent cell-to-cell spreading of HIV-1 in myeloid cell targets through the formation of the infected multinucleated giant cells observed *in vivo* in lymphoid and non-lymphoid tissues of HIV-1-infected patients.

2. Article summary

We demonstrate that HIV-1 uses a common two-step cell-to-cell fusion mechanism for massive virus transfer from infected T lymphocytes and dissemination in myeloid target cells, including dendritic cells, macrophages, as well as osteoclasts. This cell-to-cell infection process bypasses the restriction imposed by the SAMHD1 host cell restriction factor for HIV-1 replication leading to the formation of highly virus-productive multinucleated giant cells as observed *in vivo* in lymphoid and non-lymphoid tissues of HIV-1-infected patients. Since myeloid cells are emerging as important target cells of HIV-1, these results contribute to a better understanding of the role of these myeloid cells in pathogenesis, including cell associated virus sexual transmission, cell-to-cell virus spreading, and establishment of long lived viral tissue reservoirs.

Cell-to-cell spreading of HIV-1 in myeloid target cells escapes SAMHD1 restriction

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Running title: HIV-1 cell-to-cell spreading in myeloid cells

27 **Abstract**

28

29 Dendritic cells (DCs), macrophages as well as osteoclasts (OCs) are emerging as target cells
30 of HIV-1 involved in virus transmission, dissemination, and establishment of persistent tissue
31 virus reservoirs. While these myeloid cells are poorly infected by cell-free viruses, because of
32 the high expression of cellular restriction factors such as SAMHD1, we show here that HIV-1
33 uses a specific and common cell-to-cell fusion mechanism for virus transfer and
34 dissemination from infected T lymphocytes to the target cells of the myeloid lineage,
35 including immature DCs (iDCs), OCs and macrophages, but not monocytes and mature DCs.
36 The establishment of contacts with infected T cells leads to heterotypic cell fusion for the fast
37 and massive transfer of viral material into OC and iDC targets, which subsequently triggers
38 homotypic fusion with non-infected neighboring OCs and iDCs for virus dissemination.
39 These two cell-to-cell fusion processes are not restricted by SAMHD1 and allow a very
40 efficient virus spreading in myeloid cells, resulting in the formation of highly virus-
41 productive multinucleated giant cells. These results reveal the cellular mechanism for
42 SAMHD1-independent cell-to-cell spreading of HIV-1 in myeloid cell targets through the
43 formation of the infected multinucleated giant cells observed *in vivo* in lymphoid and non-
44 lymphoid tissues of HIV-1-infected patients.

45

46 **Importance**

47

48 We demonstrate that HIV-1 uses a common two-step cell-to-cell fusion mechanism for
49 massive virus transfer from infected T lymphocytes and dissemination in myeloid target cells,
50 including dendritic cells, macrophages, as well as osteoclasts. This cell-to-cell infection
51 process bypasses the restriction imposed by the SAMHD1 host cell restriction factor for HIV-
52 1 replication leading to the formation of highly virus-productive multinucleated giant cells as
53 observed *in vivo* in lymphoid and non-lymphoid tissues of HIV-1-infected patients. Since
54 myeloid cells are emerging as important target cells of HIV-1, these results contribute to a
55 better understanding of the role of these myeloid cells in pathogenesis, including cell-
56 associated virus sexual transmission, cell-to-cell virus spreading, and establishment of long-
57 lived viral tissue reservoirs.

58

59 **Introduction**

60

61 Myeloid cells, including subsets of blood monocytes, dendritic cells (DCs), macrophages
62 and bone osteoclasts (OCs), express the CD4 receptor and CCR5 and CXCR4 co-receptors
63 required for HIV-1 entry, and represent natural host cell targets of the virus [1,2]. However,
64 most of the attention regarding HIV-1 spreading and persistence have focused on T
65 lymphocytes, even if myeloid cells are now emerging as important target cells involved in all
66 steps of HIV-1 pathogenesis and in viral persistence in tissues of infected individuals, even
67 under antiretroviral treatment [3]. Human myeloid-lineage cells play pivotal roles during
68 infection as demonstrated in HIV-infected patients, simian immunodeficiency virus (SIV)-
69 infected macaques, and more recently in HIV-1-infected Humanized mice [3–12]. While DCs
70 have been proposed as the first cells targeted by HIV-1 after sexual transmission and are
71 involved in early virus dissemination in lymphoid organs where they can transmit viral
72 particles to activated CD4⁺ T cells [13–16], macrophages participate in the formation of virus
73 reservoirs in numerous host lymphoid and non-lymphoid tissues [9,10,12,17–20]. Finally,
74 infection of OCs, which are cells specialized in bone resorption, has been studied recently,
75 and this is likely responsible for the bone disorders observed in HIV-1-infected patients
76 [21,22].

77 While HIV-1 infection with cell-free viruses has been largely documented, cell-to-cell
78 transmission probably represents the dominant mode of infection [23,24]. This propagation
79 route is very efficient and involves the different cell types targeted by HIV-1, including cells
80 of the myeloid lineage (i.e. macrophages, DCs and OCs) [22,24–26] which are not easily
81 infected by cell-free viruses, mainly because of the high expression of cellular restriction
82 factors, including SAMHD1, an enzyme that cleaves deoxynucleotide triphosphates and
83 depletes the pool of intracellular nucleotides for efficient HIV-1 replication in these

84 noncycling myeloid cells [16,27–31]. This cell-to-cell mode of infection likely plays
85 important roles at the level of genital and rectal mucosa, and then for virus spreading in
86 numerous host tissues. However, there is still a paucity in knowledge of the mechanisms that
87 control virus dissemination by cell-to-cell transfer in myeloid cell targets. Only two recent
88 reports have been published regarding virus cell-to-cell dissemination to macrophages from
89 infected T cells. While it was reported by the group of Quentin Sattentau that macrophages
90 could be infected via selective capture or phagocytosis of HIV-1-infected T cells [26], we
91 have reported that HIV-1 is mainly transferred to macrophages from infected T cells by a
92 two-step cell-fusion process [25].

93 Since myeloid cells are emerging as important target cells of HIV-1, the goal of this
94 study was to understand the mechanisms involved in cell-to-cell transfer of HIV-1 from
95 infected T cells for virus spreading in myeloid cells. We show that HIV-1 uses a two-step
96 cell-fusion process for virus transfer from infected T lymphocytes to myeloid cells, including
97 macrophages, OCs and immature DCs (iDCs), and then subsequent dissemination in these
98 target cells, leading to the formation of highly virus-productive multinucleated giant cells
99 (MGCs). These results reveal a very efficient mechanism for virus spreading in myeloid cell
100 targets and the formation of MGCs observed *in vivo* in tissues of HIV-1-infected patients.

101

102 **Results**

103

104 **Efficient HIV-1 cell-to-cell transfer between infected T cells and myeloid cells.**

105 Since we and others have reported that HIV-1 could be efficiently transferred from T
106 cells to macrophages through cell-to-cell contacts, we investigated whether HIV-1 could also
107 be transferred to other myeloid cell targets, i.e. DCs and OCs. CD14⁺ monocytes were
108 isolated from blood donors, differentiated in macrophages (monocyte-derived macrophages,
109 MDMs), OCs or immature DCs using specific cytokine cocktails, and used as target cells for
110 coculture with infected CD4⁺ T cells (i.e., Jurkat CD4⁺ T cells or autologous purified human
111 primary CD4⁺ T cells) as schematized in Fig. 1A. Differentiated cells were characterized
112 morphologically, functionally and by differential expression of specific markers
113 (supplementary Fig. S1). To analyze virus cell-to-cell transfer between infected T cells and
114 cells of the myeloid lineage, Jurkat cells or primary T cells were infected with the CCR5-
115 using macrophage-tropic (NLAD8) or the CXCR4-using lympho-tropic (NL4.3) viruses, and
116 then cocultured with MDMs, OCs or iDCs for 6 or 24 h (Fig. 1). Since MDMs and OCs were
117 strongly adherent, T cells were eliminated by extensive washes, and MDMs and OCs were
118 collected and stained for the intracellular viral Gag antigen. The percentage of Gag⁺ cells was
119 then determined by flow cytometry (Fig. 1B and C). As expected, around 15% of MDMs
120 exhibited high levels of Gag expression after 6 h of coculture with NLAD8-infected T cells.
121 Interestingly, around 50% of OCs were already Gag⁺, as soon as 6 h of coculture with
122 NLAD8-infected T cells, indicating a very efficient viral transfer from infected T cells to OCs.
123 In comparison, a very low level of viral transfer (less than 5%) was detected in MDMs or OCs
124 cocultured with NL4.3-infected T cells. Regarding viral transfer to iDCs, which are semi-
125 adherent cells, iDCs and T cells were collected after coculture (see Fig. 1A), and stained for
126 intracellular Gag and cell surface DC-SIGN. The percentage of Gag⁺ cells was then analyzed

127 in the DC-SIGN⁺ cell population (Fig. 1D and E). Significant levels of Gag⁺/DC-SIGN⁺
128 cells (20 to 50%) were detected by flow cytometry when iDCs were cocultured with either
129 NLAD8- or NL4.3-infected T cells, even if the levels of Gag⁺/DC-SIGN⁺ cells were
130 significantly higher in iDCs cocultured with T cells infected with the NLAD8 macrophage-
131 tropic virus than with NL4.3-infected T cells. Finally, we also compared virus transfer to
132 monocyte-derived DCs from the same donors before (iDCs) or after maturation (mDCs)
133 induced by bacterial lipopolysaccharide (LPS) treatment (supplementary Fig. S1E). iDCs and
134 mDCs were thus cocultured for 6 h with infected T cells and viral transfer was analyzed as
135 before after Gag and DC-SIGN staining. While both viruses were efficiently transferred to
136 iDCs, virus transfer was significantly reduced in mDCs from the same donors (Fig. 1F and G),
137 showing that iDCs are more susceptible to HIV-1 cell-to-cell transfer from infected T cells.

138 Together, the results reported in Fig. 1 show that, in addition to macrophages, other
139 myeloid cell targets, including OCs and iDCs, can support significant transfer of viral
140 material from infected T cells.

141

142 **Cell-to-cell transfer of macrophage-tropic virus by heterotypic cell fusion between T** 143 **cells and myeloid cells.**

144 Since we have previously shown that HIV-1 is mainly transferred from infected T cells to
145 macrophages through heterotypic cell fusion [25], we then performed immunofluorescence
146 analysis to visualize virus transfer from infected T cells to DCs and OCs. Infected Jurkat or
147 primary T cells were preloaded with the CellTracker dye for staining of T cell nuclei, and
148 cocultured with DCs or OCs for 6 h. Cells were then stained for intracellular Gag, and using
149 the DRAQ5 dye to stain all nuclei (not shown), before observation by fluorescence
150 microscopy (Fig. 2 and supplementary Fig. S2). After 6 h of coculture with NLAD8-infected
151 T cells, iDCs and OCs exhibited a strong diffuse cytoplasmic Gag staining (Fig. 2A and B).

152 Interestingly, more than 95% of Gag⁺ iDCs contained several nuclei with an average nucleus
153 number being 3.3 (Jurkat/iDCs) and 2.9 (Primary T cells/iDCs) when they were cocultured
154 with NLAD8-infected T cells (supplementary Fig. S2A, left and middle panels). Similar
155 results were obtained with iDCs cocultured with autologous primary T cells infected with
156 other macrophage-tropic viral strains such as YU2 and JRFL strains (supplementary Fig. S3).
157 As shown in Fig 2B, a diffuse Gag staining was also observed when OCs were cocultured
158 with NLAD8-infected T cells, and contained several nuclei. Almost all these Gag⁺
159 multinucleated iDCs and OCs contained at least 1 CellTracker dye-stained nucleus coming
160 from T cells (Fig. 2A-D, and supplementary Fig. S3B) and a total number of nuclei
161 significantly higher than uninfected cells (supplementary Figs S2A, S2B and S3C),
162 demonstrating cell-fusion between infected T cells and iDCs or OCs resulting in the
163 formation of Gag⁺ MGCs. In contrast, when iDCs and OCs were cocultured with the lympho-
164 tropic NL4.3-infected T cells, no cell-fusion was observed and they did not contain
165 CellTracker-positive nuclei (Fig. 2A-D, and supplementary Fig. S2A and B). Interestingly,
166 mononucleated Gag⁺ iDCs cocultured with NL4.3-infected T cells showed a cytoplasmic
167 dotted Gag staining (Fig. 2A and supplementary Fig. S4A), suggesting that lympho-tropic
168 viruses can be captured by iDCs and accumulate in cytoplasmic membrane compartments
169 after cell-to-cell contact, as previously shown for cell-free viruses [32,33]. Of note, dotted
170 Gag staining was also observed in a few mononucleated iDCs cocultured with NLAD8-
171 infected T cells (supplementary Fig. S4A). In agreement with this observation, we were able
172 to visualize accumulation of mature virus particles in virus containing compartments and
173 plasma membrane invaginations by transmission electron microscopy when iDCs were
174 cocultured with NLAD8- or NL4.3- infected T cells (supplementary Fig. S4F). Similarly,
175 when LPS-induced mDCs were cocultured with NLAD8- or NL4.3-infected T cells, no cell-
176 fusion with T cells was observed, and mDCs contained only a single CellTracker-negative

177 nucleus (Fig. 2E and F, and supplementary Fig. S2C). However, virus capture by mDCs,
178 indicated by cytoplasmic dotted Gag staining, was observed after coculture with NLAD8- or
179 NL4.3-infected T cells (Fig. 2E). Finally, we never observed phagocytosis events of infected
180 T cells by iDCs, mDCs or OCs, as it was reported previously for virus cell-to-cell transfer to
181 macrophages from dying infected T cells [26].

182 Together, these results indicate that viral material is massively transferred from infected
183 T cells to iDCs and OCs through heterotypic cell-to-cell fusion, and this cell-fusion transfer is
184 restricted to macrophage-tropic viral strains. By contrast, both macrophage- and lympho-
185 tropic viral strains can be captured by iDCs and mDCs from infected T cells without cell-
186 fusion. In addition, both NLAD8 and NL4.3 viral strains could also be captured by purified
187 blood monocytes without cell-fusion with infected T cells after 6 h of coculture
188 (supplementary Fig. S4B and C). Similarly, cell-to-cell transfer between infected T cells was
189 observed across the so-called “virological synapse”, but no fusion was observed since almost
190 all Gag⁺ target T cells contained only one nucleus (supplementary Fig. S4D and E),
191 confirming that the process of cell-fusion for HIV-1 transfer is specific of iDCs, OCs and
192 macrophages [25].

193 To confirm that transfer of macrophage-tropic viruses to iDCs and OCs was related to
194 cell-fusion with infected T cells, we checked that the Gag⁺ MGCs, formed upon coculture,
195 expressed specific T cell markers. Jurkat or primary CD4 T cells were infected with
196 macrophage-tropic virus strains (i.e., NLAD8, YU2 or JRFL), and then cocultured with iDCs
197 or OCs for 6 h before staining for the CD3 and CD2 T-cell markers in addition to Gag. As
198 shown in Fig. 3A-D and supplementary Figs S5A and S5B, the newly formed MGCs acquired
199 the T cell CD3 and CD2 markers resulting from plasma membrane and cytoplasmic
200 exchanges between infected T cells and iDCs/OCs through the cell-fusion process. Finally,
201 this cell-fusion between infected T cells and OCs or iDCs was strongly inhibited by the CCR5

202 antagonist maraviroc (MVC) and the fusion inhibitor T20 (Fig. 3E-H), showing that this
203 heterotypic cell-fusion is envelope-dependent and mediated by viral envelope-receptor
204 interactions.

205

206 **Virus dissemination through homotypic cell-fusion between dendritic cells.**

207 Since we observed that about half of the newly formed Gag⁺ lymphocyte/iDCs fused
208 cells contained 3 or more nuclei including at least 2 CellTracker-negative nuclei (Fig. 2A, and
209 supplementary Figs S2A and S3), we investigated whether lymphocyte/iDCs fused cells were
210 able to fuse with surrounding uninfected iDCs for virus dissemination. We first quantified the
211 number of nuclei in iDC targets after 6, 24 or 48 h of coculture with NLAD8-infected T cells.
212 After 6 h of coculture, about 95% of Gag⁺ iDCs contained more than 2 nuclei with an average
213 number of nuclei per cell of 3.5 which increased to 5.7 and 9.3 nuclei after 24 and 48 h of
214 coculture, respectively (Fig. 4A and B). Interestingly, we observed that after 24 h of coculture
215 with infected T cells, the newly formed Gag⁺ lymphocyte/iDC fused cells acquired adhesion
216 properties demonstrated by immunofluorescence analysis performed directly on coverslips,
217 since more than 98% of T cells were removed from the coculture after 4 extensive washes,
218 whereas iDCs cocultured with uninfected T cells were still non-adherent and mostly removed
219 by a single wash (supplementary Fig. S5C).

220 To demonstrate the homotypic fusion between lymphocyte/iDC fused cells and
221 surrounding uninfected iDCs, NLAD8-infected T cells were cocultured with iDCs preloaded
222 with CellTracker for 3 h, and autologous iDCs preloaded with the CellTrace dye were then
223 added for 3 additional hours before staining for intracellular Gag as schematized on Fig. 4C.
224 When iDCs were initially cocultured with uninfected or NL4.3-infected T cells, iDCs were
225 stained with either CellTracker or CellTrace and did not show diffuse cytoplasmic Gag
226 staining (Fig. 4D and E). In contrast, when iDCs were cocultured with NLAD8-infected T

227 cells, we observed that about 20% of the Gag⁺ multinucleated iDCs were stained with both
228 CellTracker and CellTrace dyes (Fig. 4D and E). These results indicate that the first
229 heterotypic fusion step between infected T cells and CellTracker-positive iDCs is followed by
230 a second homotypic fusion with uninfected CellTrace-positive iDCs leading to the formation
231 of Gag⁺ multinucleated giant iDCs.

232 We also used electron microscopy to visualize multinucleated giant iDCs after 6 h of
233 coculture with NLAD8-infected T cells (Fig. 4F, left image). More interestingly, electron-
234 dense material reminiscent of Gag assembly corresponding to virus buds (blue arrows), as
235 well as mature virions (red arrows), were observed protruding from the plasma membrane of
236 iDCs (Fig. 4F, central and right images), indicating that virus assembly and budding took
237 place at the cell surface of iDCs after only 6 h of coculture with infected T cells. These
238 observations also confirm plasma membrane exchanges and the transfer of Gag material
239 between infected T cells and iDC targets.

240

241 **Virus spreading in myeloid cells through cell-fusion escapes SAMHD1 restriction**

242 Finally, we investigated whether this virus cell-to-cell transfer and dissemination through
243 cell-fusion resulted in the productive infection of iDCs. We took advantage of the acquisition
244 of adherence properties of the MGCs formed through the two-step cell-fusion process for
245 elimination of infected T cells (see supplementary Fig. S5C) in order to monitor virus
246 production generated by iDC-derived MGCs after virus transfer. Infected T cells and iDCs
247 were cocultured for 24 h before elimination of T cells by extensive washes, and viral p24
248 released by iDCs-derived MGCs was monitored every 3 days. Gag⁺ multinucleated giant
249 iDCs containing many nuclei were still alive 9 days after T cell removal (supplementary Fig.
250 S6A), and produced high levels of p24 (Fig. 5A, blue lines). Since iDCs have the ability to
251 capture and release virus particles without virus replication [34,35] (see supplementary Fig.

252 S4A), we analyzed the effect of the reverse transcription inhibitor AZT on virus dissemination
253 in iDCs and p24 release. When iDCs were treated with AZT during and after coculture with
254 infected T cells, virus dissemination evaluated by the percentage of Gag⁺ iDCs (Fig. 5B), as
255 well as virus production (Fig. 5A, red lines), were significantly reduced, without affecting the
256 initial step of virus cell-to-cell transfer from infected T cells (supplementary Figs S6B and C).
257 These results show that virus-mediated cell-to-cell fusion of infected T cells with iDCs is a
258 very efficient process for virus dissemination through the formation of highly virus-
259 productive MGCs, suggesting that this cell-fusion process may bypass the restriction imposed
260 by host cell restriction factors such as SAMHD1 expressed in iDCs. To test this hypothesis,
261 specific siRNA were transfected in iDCs or MDMs leading to a net decrease of SAMHD1
262 expression (Fig. 5C, and supplementary Fig. S7A), and virus transfer and dissemination was
263 evaluated in SAMHD1-depleted myeloid cells infected either by NLAD8 cell-free (CF)
264 viruses or after 24 h of coculture with infected T cells (CTC). As expected, virus transfer,
265 evidenced through analysis of the percentage of Gag⁺ iDCs, was significantly increased when
266 SAMHD1-depleted iDCs or MDMs were infected by cell-free viruses (Fig. 5D, left panel, and
267 supplementary Fig. S7B, left panel). In contrast, no difference in the percentage of Gag⁺ iDCs
268 was observed when siCtrl- and siSAMHD1-transfected-iDCs or MDMs were cocultured with
269 infected T cells (Fig. 5D, right panel, and supplementary Fig. S7B, right panel). Similarly,
270 virus dissemination assessed 6 days later by p24 quantification in the cell-culture supernatant
271 was still significantly higher when iDCs or MDMs were infected by CTC compared to CF
272 viruses (Fig. 5E, and supplementary Fig. S7C), and SAMHD1 depletion did not impact on the
273 high virus production observed when iDCs or MDMs were initially cocultured with infected
274 T cells (Fig. 5F, and supplementary Fig. S7D). These results demonstrate that the restriction
275 to virus replication imposed by SAMHD1 expression through cell-free virus infection of

276 myeloid cells is bypassed through formation of myeloid cell-derived MGCs able to produce
277 high levels of virus particles.

278 Since only the dephosphorylated form of SAMHD1 is able to restrict HIV-1 replication
279 [36–38], we investigated further the SAMHD1 restriction escape mechanism by analyzing
280 SAMHD1 expression and phosphorylation in myeloid cells infected by cell-free viruses or by
281 cell-fusion with infected T cells. As usual, NLAD8-infected T cells were cocultured for 24 h
282 with MDMs before cell lysis and Western blot analysis of total and phosphorylated SAMHD1
283 in MGCs formed upon cell-fusion with infected T cells (Fig. 5G, and supplementary Fig.
284 S7E). As expected, SAMHD1 was expressed in its active dephosphorylated form for HIV-1
285 restriction when MDMs were infected with cell-free viruses (Fig. 5G, and supplementary Fig.
286 S7E, left panels). In sharp contrast, most of the SAMHD1 protein was phosphorylated when
287 MDM targets were infected by cell-to-cell fusion with infected T cells (Fig. 5G, and
288 supplementary Fig. S7E, right panels). These findings show that the two-step cell-fusion
289 process leading to MGC formation results in phosphorylation of SAMHD1 allowing efficient
290 HIV-1 replication and spreading in myeloid cells.

291

292 Discussion

293

294 In this study, we reveal that HIV-1 uses a specific and common two-step cell-fusion
295 process for cell-to-cell transfer and dissemination in the CD4⁺ target cells of the myeloid
296 lineage, including macrophages, OCs and iDCs, but not monocytes and mDCs. This two-step
297 cell-fusion mechanism bypasses the SAMHD1-related restriction to HIV-1 replication
298 observed in these myeloid cells with cell-free viruses, and may be responsible for virus
299 spreading in these cell targets *in vivo*, as observed in host lymphoid and non-lymphoid tissues,
300 including the central nervous system and bones [39–43]. In agreement, it was reported that
301 myeloid cells from spleen and lymph nodes of SIV-infected macaques contain T cell markers
302 and viral RNA and DNA originating from infected T cells [44,45].

303 We have shown here that virus cell-to-cell transfer from infected T cells is first mediated
304 by heterotypic cell fusion with macrophages, OCs or iDCs, followed by virus dissemination
305 through a second homotypic fusion with uninfected myeloid cells leading to the formation of
306 highly virus-productive MGCs. This two-step cell-fusion process is specific of these myeloid
307 cell targets, since HIV-1 cell-to-cell transfer between CD4⁺ T cells is mainly mediated by the
308 formation of the virological synapse without cell-fusion between virus-donor and target T
309 cells [46,47] (and see supplementary Fig. S4D and E). Similarly, we did not detect cell-fusion
310 for cell-to-cell transfer of HIV-1 from infected T cells to CD4⁺ monocytes or mDCs,
311 suggesting that the cell-fusion process between T cells and iDCs, OCs or macrophages is
312 finely regulated. Previous studies using cell-free viruses proposed that the lower sensitivity of
313 monocytes and mDCs to HIV-1 was related to the low expression of the CCR5 co-receptor at
314 the cell surface of these cell types [48,49], and monocytes and mDCs indeed express low
315 level of CCR5 compared to iDCs and differentiated macrophages (see supplementary Fig.
316 S1E, and data not shown). In addition, it was suggested that some cell surface molecules, such

317 as tetraspanin proteins, might have a negative regulatory role on cell-fusion and formation of
318 syncytia between infected and target T cells after formation of the virological synapse [50].
319 While our data show that interactions between the viral envelope and the CD4 receptor and
320 CCR5 co-receptor are required for the cell-fusion process leading to formation of HIV-1-
321 infected MGCs originating from either macrophages, OCs or iDCs, it would be interesting to
322 investigate the potential role of some cell surface proteins [51], such as tetraspanins, in the
323 mechanism of HIV-1-mediated cell-to-cell fusion between lymphoid and myeloid cells
324 described here. Similarly, we cannot exclude the possibility that some signaling pathways and
325 effectors involved in programming of myeloid cells into a fusion-competent state could also
326 participate in the two-step cell-fusion process for HIV-1 cell-to-cell spreading in myeloid
327 cells. Depending of the cytokine environment, it was established that cells of the myeloid
328 lineage have the propensity to mediate homotypic cell-fusion leading to the formation of
329 multinucleated OCs and MGCs in both physiological and pathological conditions [52,53].
330 While the formation in bones of functional multinucleated OCs is mainly orchestrated by the
331 RANKL cytokine, alternative activation of macrophages by IL-4 and IL-13 interleukins can
332 promote homotypic macrophage fusion and MGC formation, as observed in some
333 inflammatory disorders [53]. Therefore, it would be interesting to explore the contribution of
334 cellular pathways mobilized by these cytokines in both cell-fusion steps allowing HIV-1 cell-
335 to-cell spreading in myeloid cells.

336 HIV-1 transfer from infected T cells to myeloid cells through cell-fusion is a fast and
337 very efficient process leading to transfer of viral material toward 20 to 40% of the myeloid
338 target cells after only 6 h of coculture. This process leads to virus spreading in iDCs, OCs
339 (data not shown) or macrophages [25] through the formation of MGCs able to produce high
340 amount of viral particles over several days and weeks. In contrast, many reports indicate that
341 these myeloid cells are poorly infected by cell-free viruses, because virus replication is

342 limited by the high expression of host cell restriction factors, such as SAMHD1 [54]. When
343 non-cycling myeloid target cells are infected with cell-free viruses, SAMHD1 is expressed in
344 its dephosphorylated active form able to restrict HIV-1 replication [36-38]. In contrast, our
345 findings show that the two-step cell-fusion process results in phosphorylation of SAMHD1
346 allowing efficient virus spreading in iDCs and macrophages. Interestingly, Puigdomenech et
347 al. [55] previously suggested that HIV-1 cell-to-cell transmission from infected T cells to DCs
348 was restricted by SAMHD1 expression. Even if similar experimental system and viral strains
349 were used in both studies, the discrepancy with our findings can be explained by the gating
350 strategy used to analyze virus dissemination by flow cytometry, since the Gag+ iDC-derived
351 MGCs formed through cell-fusion during co-culture with infected T cells were certainly
352 excluded from the analysis performed by Puigdomenech and colleagues [55]. In addition, they
353 did not perform fluorescence microscopy analysis to visualize and study MGC formation
354 through cell-fusion of infected T cells with iDC targets. In contrast, our data demonstrate that
355 this cell-to-cell fusion process supports high levels of virus production by iDC-, OC- and
356 macrophage-derived MGCs, in which SAMHD1 is expressed in its inactive phosphorylated
357 form, suggesting that the Cyclin/CDK kinase complex involved in SAMHD1 phosphorylation
358 is expressed in MGCs. While further investigations are thus needed to elucidate this intriguing
359 mechanism of SAMHD1 phosphorylation in MGCs, we can speculate that the Cyclin/CDK
360 complex could be transferred from activated infected T cells through the first heterotypic cell-
361 fusion and delivering of the cellular content to the myeloid target cell. In addition, the high
362 level of dNTPs transferred from proliferative infected T cells could also help to overcome the
363 restriction activity of SAMHD1 expressed in myeloid cells. Alternatively, an attractive
364 hypothesis could be related to the maintenance of transcriptionally-active T cell nucleus after
365 the initial fusion between infected T cells and myeloid target cells (LB and SB, unpublished
366 observations). Thus, transfer of the T cell nucleus already containing integrated proviral DNA

367 may allow to bypass the reverse transcription step restricted by SAMHD1, leading to efficient
368 SAMHD1-independant viral expression by the newly fused cells formed through cell fusion
369 between infected T cells and myeloid cells.

370 While the physiological formation of OCs results from cell-to-cell fusion of
371 monocyte/macrophages [52,53], we show that multinucleated OCs are also able to fuse with
372 HIV-1-infected T cells for virus dissemination in these cell targets. Similarly, iDC targets can
373 fuse with infected T cells for HIV-1 cell-to-cell transfer followed by a second step of
374 homotypic fusion with uninfected iDCs leading to the formation of MGCs. Interestingly, it
375 has been shown that DCs were able to fuse together and to acquire new phenotypical
376 properties upon inflammatory conditions [56], in granulomatous diseases [57] or proliferating
377 disorders such as Langerhans cell histiocytosis [58]. In agreement with our results,
378 heterotypic fusion of DCs with HIV-1-infected T cells was already suggested 20 years ago by
379 the group of R.M. Steinman [7,59]. This group and others reported the presence of HIV-1-
380 infected multinucleated syncytia expressing specific markers of both DCs and T cells at the
381 surface of the nasopharyngeal tonsils, adenoid and parotid gland of HIV-1-infected patients
382 [7,60-62], but the mechanisms related to these *in vivo* observations were not further
383 investigated. Our study demonstrate that the cell-fusion process for HIV-1 cell-to-cell transfer
384 from T cells to iDCs is responsible for the formation of the multinucleated infected DCs
385 observed in infected patients.

386 In summary, the novel and very efficient two-step cell-fusion mechanism we report for
387 virus cell-to-cell spreading is specific to iDC, OC and macrophage target cells of HIV-1. First,
388 infected T cells fuse with these myeloid cells for massive viral transfer, followed by a second
389 step of homotypic fusion with uninfected myeloid cells for virus dissemination. Both steps are
390 dependent on the viral envelope and seem restricted to macrophage-tropic viral strains. While
391 HIV-1 infection of these myeloid cells by cell-free viruses is known to be strongly restricted

392 [28,63], this new mechanism of virus spreading in myeloid cells by cell-fusion escapes the
393 restriction imposed by SAMHD1 and leads to the formation of highly virus-productive MGCs
394 reminiscent of the infected MGCs originating from myeloid cells observed *in vivo* in infected
395 patients and SIV-infected macaques. These *in vivo* observations support the importance of the
396 mechanisms revealed here, which contribute to a better understanding of virus cell-to-cell
397 dissemination in myeloid cells and their roles in HIV-1 pathogenesis, including cell-
398 associated virus sexual transmission, virus spreading, and establishment of long-lived viral
399 tissue reservoirs.

400

401 **Materials and Methods**

402

403 **Plasmids and reagents.** The proviral plasmids pNL4.3 and pNLAD8 were obtained from the
404 AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, while the
405 plasmid encoding the VSV-G envelope glycoprotein (pVSVg), has been described[64]. The
406 following antibodies were used: PE- or FITC-conjugated anti-CD11b (clone ICRF44, BD
407 Biosciences), PE-Cy7-conjugated anti-CD11b (clone ICRF44, Biolegend), RD1- or FITC-
408 conjugated anti-Gag (clone KC57, Beckman coulter), anti-CD4 (clone Leu3a, Biolegend),
409 anti-CD3 (clone UCHT1, Biolegend) , anti-DC-SIGN (clone 120507, R&D systems), FITC-
410 conjugated anti-CD11c (clone B-ly6, BD Biosciences), anti-CD14 (clone M5E2, BD
411 Biosciences), APC-conjugated anti-CD83 (clone HB15, Miltenyi), Alexa 647-conjugated
412 anti-CCR5 (clone HEK/1/85a, Biolegend), PE-conjugated anti-CXCR4 (clone 12G5,
413 Biolegend); anti-CD2 (TS2/18 clone) was a gift from Dr. Andres Alcover (Paris, France)[65];
414 Alexa647- or Alexa555- conjugated phalloidin (Life Technologies) were used. The following
415 reagents were obtained from the AIDS Research and Reference Reagent Program, Division of
416 AIDS, NIAID: HIV-1 CAp24 Hybridoma (183-H12-5C), HIV-IG, Maraviroc, T20, and AZT.
417

418 **Cell culture.** HEK293T and Jurkat cell-lines were obtained from the ATCC Collection.
419 HEK293T cells were maintained in Dulbecco minimal essential medium (DMEM)
420 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU of penicillin/ml, and
421 100 µg of streptomycin/ml (ATB, Invitrogen). Jurkat cells (clone J77) were maintained in
422 RPMI 1640 complete culture medium supplemented with 10% FCS and ATB. Peripheral
423 blood mononuclear cells (PBMCs) were isolated from blood of healthy anonymous donors by
424 density gradient sedimentation using Histopaque (Sigma), and monocytes were purified using
425 the CD14-positive selection kit (CD14 microbeads, Miltenyi) according to manufacturer's

426 guidelines. Blood samples from anonymous healthy donors were purchased from
427 "Etablissement Français du Sang Paris-Saint-Antoine-Crozatier, 21 rue Crozatier, 75012
428 Paris, France" or from "Changhai Hospital, 169 changhai road, Shanghai, China". The
429 monocytes were differentiated into macrophages for 8 days in RPMI 1640 culture medium
430 supplemented with 20% FCS, ATB, and 25 ng/ml of GM-CSF and M-CSF (Miltenyi).
431 Monocytes were differentiated into osteoclasts for 4 days in RPMI 1640 culture medium
432 supplemented with 20% FCS, ATB, and 50 ng/ml of M-CSF and 30 ng/ml of RANKL, and
433 then cultured for another 6 days with 25 ng/ml of M-CSF and 100 ng/ml of RANKL allowing
434 complete maturation. The markers and functionality (bone degradation) of OCs was analyzed
435 as previously [22]. In addition, RNA level of specific OC markers was assessed by qPCR
436 analysis as described in (Souriant et al. Cell report, 2019; in press). Finally, monocytes were
437 differentiated into iDCs for 5 days in RPMI 1640 culture medium supplemented with 20%
438 FCS, ATB, and 50 ng/ml of GM-CSF and IL-4 (Miltenyi). Maturation of DCs was performed
439 using 1 µg/ml of LPS (*Escherichia coli* O55:B5, Sigma-Aldrich) for 48 h. Human primary
440 CD4⁺ T cells were isolated from PBMCs by density gradient sedimentation using Histopaque
441 and then purified by negative selection (CD4⁺ T cell isolation kit, Miltenyi) following
442 manufacturer's recommendations. CD4⁺ T cells were activated for 3 days in RPMI medium
443 containing 20% FBS, interleukin-2 (IL-2, Miltenyi) at 10 u/ml, and phytohemagglutinin-P at
444 5 µg/ml (PHA-P, Sigma-Aldrich). After activation, CD4⁺ T cells were kept in RPMI medium
445 supplemented with 20% FBS and IL-2. All cells were grown at 37°C under 5% CO₂.

446

447 **Viral production, titration, and infection.** Replication-competent HIV-1 NL4.3 and
448 NLAD8 strains were produced in HEK293T cells by cotransfection of the proviral plasmid in
449 combination with pVSVg using calcium phosphate precipitation technique as described⁵⁰.
450 Amounts of p24 produced were determined by enzyme-linked immunosorbent assay (ELISA;

451 Innogenetics). Viral titer was determined using Jurkat cells by flow cytometry (Accuri C6,
452 BD Biosciences) as described[64].

453

454 **Cell-to-cell viral transfer and dissemination.** To study virus transfer from infected Jurkat or
455 primary CD4⁺ T cells to myeloid cells, T cells were infected using a multiplicity of infection
456 (MOI) of 0.5 for 16 h, then washed and cultured for another 24 h. After washing, T cells were
457 cocultured during 3-72 h at a ratio 1:1 with MDMs, OCs or iDCs seeded at a density of
458 0.5×10^6 , 1×10^6 or 0.4×10^6 cells/well, respectively. Where indicated, iDCs or MDMs were
459 transfected with a mix of two different siRNA targeting SAMHD1 at a concentration of 100
460 nM each (Dharmacon, #L-013950-09 and #L-013950-10) using Hiperfect transfection reagent
461 (Qiagen) accordingly to the manufacturer recommendation. 24 h after transfection, a second
462 round of transfection with siRNA was performed using the same protocol, and then used to
463 coculture with infected T cells for 6 h. To remove T cells after co-culture with myeloid cells,
464 4 extensive washes with PBS were performed. MDMs, OCs or DCs were harvested or
465 cultured during several days and then collected. Cells were then surface stained using anti-
466 CD11b, anti-CD11c, anti-DC-SIGN, anti-CD2 or anti-CD3 antibodies, then fixed with 4%
467 PFA, permeabilized and stained with an anti-Gag (KC57, 1/500) using permeabilization
468 buffer (Beckman Coulter). The percentage of Gag⁺ cells among CD11b⁺, CD11c⁺ or DC-
469 SIGN⁺ cells corresponding to the MDM, OC or DC population, respectively, was determined
470 by flow cytometry or by fluorescence microscopy as described [22,25]. To analyze viral
471 production, cell-culture supernatants of iDCs were collected, and the amount of p24 produced
472 was determined by ELISA.

473

474 **Effect of inhibitors on viral transfer and dissemination in iDCs and OCs.** To analyze the
475 effect of inhibitors on virus transfer, infected donor T cells, and OC or iDC targets were pre-

476 treated for 1 h with T20 or Maraviroc at 10 μ M and 10 μ g/ml, respectively. Infected T cells
477 were then cocultured for 6 h with OCs or iDCs. For OCs, T cells were eliminated by
478 extensive washes, and the percentage of Gag⁺ OCs was determined by flow cytometry or
479 fluorescence microscopy as described^{20,24}. For iDCs, mixture of T cells and iDCs were
480 collected and the percentage of Gag⁺/CD11c⁺ iDCs was determined by flow cytometry.
481 Results were expressed as the percentage of Gag⁺ OCs or iDCs relative to that determined
482 without inhibitors. To show that virus transfer from infected T cells to iDCs led to productive
483 infection, iDC targets were pretreated with AZT (10 μ M) for 2 h prior to the coculture for 6,
484 24 or 72 h with infected T cells. After removal of infected T cells by 4 extensive washings
485 with PBS, the percentage of Gag⁺/CD11b⁺ iDCs was determined by flow cytometry directly
486 after the 6, 24 or 72 h of co-culture, while the viral p24 production from iDCs was determined
487 by ELISA after 3, 6, 9, or 12 more days of culture in the presence of AZT.

488

489 **Fluorescence microscopy analysis.** To visualize cell contacts, virus transfer and virus
490 dissemination, Jurkat or primary CD4⁺ T cells infected with NLAD8 or NL4.3 as described
491 above were pre-loaded with 5 μ M of CellTracker CMAC (Life Technologies) and cocultured
492 for 6, 24 or 48 h with OCs (plated onto coverslips), DCs, monocytes or CD4⁺ T cells. After
493 coculture, OCs were directly fixed with 4% PFA while the mixture of DCs (or monocyte or T
494 cell targets) and virus-donor infected T cells were seeded on L-polylysine-coated coverslips
495 (Sigma-Aldrich) before fixation. Cells were then blocked for 10 min in PBS containing 1%
496 BSA, stained with 2 μ M of DRAQ5 (eBioscience) for 20 min in PBS for staining of nuclei,
497 followed by surface staining of DC-SIGN (1/100 dilution) for 1 h and incubation with
498 Alexa555-coupled secondary antibody, then permeabilized and stained using KC57 FITC-
499 conjugated antibody or Phalloidin-Alexa647 (Molecular Probes) diluted in permeabilization
500 buffer for 1 h. Coverslips were then washed with PBS and mounted on slide using 10 μ l of

501 Fluoromount (Sigma) or Fluoromount containing DAPI (Sigma-Aldrich). Images were
502 acquired on a spinning disk (CSU-X1M1, Yokogawa) inverted microscope (DMI6000, Leica),
503 and then processed using the Fiji software (ImageJ, NIH)[66]. Quantitative image analysis
504 was performed using Fiji by defining a region of interest using the actin or DC-SIGN staining
505 and measuring the whole fluorescence intensity of the Gag staining, with respect to non-
506 infected cells. To analyze fusion between infected T cells and OCs or iDCs, infected Jurkat or
507 primary CD4⁺ T cells were cocultured for 6 or 24 h with myeloid cells. Cells were then fixed
508 with 4% PFA and blocked in PBS containing 1% BSA. Anti-CD3 (10 µg/mL), -CD2 (1/200
509 dilution), -DC-SIGN, -Gag (KC57-FITC, 1/200 dilution), and F-actin (with fluorescent
510 Phalloidin) intracellular staining were done by incubating coverslips with the indicated
511 primary antibody and phalloidin-Alexa647 diluted in the permeabilization buffer. Coverslips
512 were then rinsed with PBS-BSA and incubated for 1 h with the corresponding fluorescent-
513 coupled secondary antibody. After 3 washes, coverslips were mounted on microscope slides
514 using 10 µl of Fluoromount mounting medium containing DAPI. Cells were examined under
515 an epifluorescence microscope (DeltaVision), and quantitative image analysis was performed
516 using the Fiji software by defining a region of interest using the F-actin staining and
517 measuring the whole fluorescence intensity of the indicated marker (i.e. CD3, or CD2) in
518 Gag⁺ cells, with respect to non-infected cells. To analyze the effect of inhibitors on fusion
519 between T cells and OCs and iDCs, infected virus-donor T cells, or OC and iDC targets were
520 pre-treated for 1 h with T20, Maraviroc or AZT at 10 µM, 10 µg/ml and 10 µM, respectively.
521 Infected T cells were then cocultured for 6 h with OCs or iDCs in the presence of inhibitors.
522 Cells were then fixed and stained as previously using KC57 FITC-conjugated antibody and
523 phalloidin-Alexa647. Images were acquired and then processed as described above. To
524 analyze fusion between iDCs, infected T cells were initially co-cultured with iDCs preloaded
525 with Celltracker-CMAC for 3 h. Autologous iDCs pre-labeled with CellTrace-Far-red were

526 then added to the mixture of infected T cells and iDCs and cultured for 3 more hours. The
527 mixture of iDCs and T cells were then seeded on poly-lysine coverslips, fixed with PFA 4%
528 and stained with anti-Gag (KC57-FITC) and phalloidin-Alexa555, and mounted with
529 Fluoromount media. Images were acquired and processed as previously. To analyze viral
530 transfer between T cells or between T cells and monocytes, infected T cells preloaded with
531 CellTracker CMAC or CellTrace-Far-red were cocultured with unloaded T cells or monocytes
532 for 6 h. Mixture of cells were then collected and seeded on coverslips coated with poly-lysine,
533 fixed with PFA 4%, permeabilized and stain for nuclei (DRAQ5), intracellular Gag (KC57-
534 FITC) or phalloidin-Alexa647 and mounted with Fluoromount media. Images were acquired
535 and processed as previously. The number of nuclei per cell was analyzed from images on at
536 least 50 cells and then processed using Fiji as previously.

537

538 **Transmission electron microscopy analysis.** Jurkat cells infected with NLAD8 or NL4.3 as
539 described above were co-cultured for 6 h with iDCs. Cells were then collected and seeded on
540 coverslips coated with poly-lysine. The coverslips were then fixed using glutaraldehyde 2.8%
541 and 2% PFA for 20 min. After 2 washes in PBS, cells were dehydrated and embedded into
542 epoxy (Electron Microscopy Sciences) as described previously [25]. Ultrathin sections of 90
543 nm were cut with an ultra-microtome, stained with uranyl acetate and Reynold's lead and
544 observed with a transmission electron microscope (JEOL 1011) as described [25]. Acquisition
545 was performed with a Gatan ES1000W CCD camera.

546

547 **Western blot analysis.** For analysis of total and phosphorylated SAMHD1 expression in
548 siRNA-transfected iDCs or MDMs, as well as in MDM-derived MGCs, cells (10^6) were lysed
549 in 100 μ l of reducing Laemmli sample buffer (2X) supplemented with PhosSTOPTM
550 (Phosphatase Inhibitor Cocktail Tablets, Sigma-Aldrich) and cOmpleteTM (EDTA-free

551 Protease Inhibitor Cocktail, Sigma-Aldrich), and then boiled at 96°C for 10 min. Cell lysates
552 were then resolved by SDS-PAGE as described [64], and analyzed by Western blotting using
553 anti-SAMHD1 (Abcam, ab67820), anti-phospho-SAMHD1 (Cell Signaling #89930), anti-
554 HIV-1 p24 (Abcam, ab9071) and anti- β -actin (Sigma-Aldrich, clone AC-15) antibodies.

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567

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770

771 **Figure legends**

772

773 **Figure 1. HIV-1 cell-to-cell transfer from infected T cells to myeloid cells. (A)**

774 Experimental protocol. (B and C) Jurkat cells were infected with the NLAD8 or NL4.3 strains
775 for 36 h and then cocultured for 6 or 24 h with MDMs or OCs. After elimination of Jurkat
776 cells, the percentage of Gag⁺ MDMs or OCs was quantified by flow cytometry. As a negative
777 control (NI), non-infected Jurkat cells were cocultured with MDMs or OCs. (D and E) Jurkat
778 cells were infected as previously and then co-cultured with iDCs for 6 or 24 h. The percentage
779 of Gag⁺/DC-SIGN⁺ iDCs was evaluated by flow cytometry. (F and G) Jurkat cells were
780 infected and then co-cultured with iDCs or mDCs for 6 h. The percentage of Gag⁺/DC-
781 SIGN⁺ iDCs or mDCs was evaluated by flow cytometry. The results are the means of at least
782 4 independent experiments performed with MDMs, OCs, iDCs or mDCs of 4 different donors.
783 Error bars represent 1 SEM. Statistical significance was determined using Mann-Whitney U-
784 test (ns, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$).

785

786 **Figure 2. Viral transfer to immature DCs and OCs by cell fusion with infected T cells.**

787 (A and C) NLAD8- or NL4.3-infected Jurkat (A, upper images; and C, left panel) or primary
788 CD4⁺ T (A, lower images; and C, right panel) cells pre-labeled with CellTracker were
789 cocultured for 6 h with iDCs. Cells were then stained with anti-Gag and anti-DC-SIGN, and
790 analyzed by confocal microscopy (scale bar, 10 μ m) (A). T cells and iDCs are indicated by
791 plain yellow and dashed white lines, respectively. In C), the number of nuclei per DC-SIGN⁺
792 cell was quantified from images on at least 50 cells. Results are expressed as the percentages
793 of cells with 0, 1, 2, or more than 2 CellTracker(+) nuclei. (B and D) NLAD8- or NL4.3-
794 infected Jurkat (B, upper images; and D, left panel) or primary CD4⁺ T (B, lower images; and
795 D, right panel) cells pre-labeled with CellTracker were cocultured with OCs for 6 h. After

796 elimination of T cells, OCs were stained with anti-Gag and phalloidin (Actin), and analyzed
797 by confocal microscopy (scale bar, 10 μ m) (B). OCs are indicated by dashed white lines. In
798 D), the number of nuclei per cell was quantified from images on at least 50 cells. Results are
799 expressed as the percentages of cells with 0, 1, 2, or more than 2 CellTracker(+) nuclei. (E
800 and F) NLAD8- or NL4.3-infected Jurkat cells pre-labeled with CellTracker were co-cultured
801 for 6 h with iDCs (E, left images; and F, left panel) or mDCs (E, right images; and F, right
802 panel). Cells were then stained with anti-Gag and anti-DC-SIGN, and analyzed by confocal
803 microscopy (scale bar, 10 μ m) (E). T cells and DCs are indicated by plain yellow and dashed
804 white lines, respectively. In F), the number of nuclei per DC-SIGN+ cell was quantified from
805 images on at least 50 cells. Results are expressed as the percentages of cells with 0, 1, 2, or
806 more than 2 CellTracker(+) nuclei. The results shown are representative of 3 independent
807 experiments performed with DCs and OCs from 3 different donors. NI, non-infected Jurkat or
808 primary CD4+ T cells were co-cultured with OCs or DCs.

809

810 **Figure 3. Cell fusion between infected T cells and iDCs or OCs by a viral**
811 **envelope-dependent mechanism.** (A-D) NLAD8-infected Jurkat cells were cocultured for 6
812 h with iDCs (A and B) or OCs (C and D). Cells were then stained with anti-CD3, anti-Gag
813 and DAPI (nuclei), and analyzed by confocal microscopy. Representative images of
814 intracellular CD3 staining are shown (A and C) (scale bars, 25 μ m), and T cells and myeloid
815 cells (OCs or iDCs) are indicated by plain yellow and dashed white lines, respectively. In B
816 and D), intracellular CD3 mean fluorescence intensities (MFI) were quantified as indicated in
817 the Methods Section. Each dot corresponds to 1 cell, and the number of cells analyzed is
818 indicated (n). Horizontal bars represent the mean \pm 1 SEM. Statistical significance was
819 determined with the Mann-Whitney U-test (****p<0.0001). (E-H) NLAD8-infected Jurkat
820 cells were pretreated or not (mock) with Maraviroc (MVC) or T20 for 1 h and cocultured with

821 iDCs (E and F) or OCs (G and H) for 6 h in the presence of the inhibitor. Cells were then
822 stained with DRAQ5 (nuclei) and anti-Gag, in combination with anti-DC-SIGN or phalloidin
823 (Actin), and analyzed by confocal microscopy (scale bar, 10 μ m). In E and G), T cells and
824 myeloid cells (iDCs or OCs) are indicated by plain yellow and dashed white lines,
825 respectively. In F and H), the percentage of Gag⁺ iDCs or OCs (upper panels), as well the
826 number of nuclei per iDCs or OCs (lower panels) were quantified from images on at least 50
827 cells. Each dot corresponds to 1 cell, and the number of cells analyzed is indicated (n).
828 Horizontal bars represent the mean \pm 1 SEM. Statistical significance was determined with the
829 Mann-Whitney U-test (*p<0.05; **p<0.01; ****p<0.0001). The results shown are
830 representative of at least 3 independent experiments performed with iDCs and OCs from 3
831 different donors. NI, non-infected Jurkat cells were co-cultured with iDCs or OCs.

832

833 **Figure 4. Virus dissemination between iDCs by homotypic cell fusion.** (A and B) NLAD8-
834 infected Jurkat cells were cocultured with iDCs for 6, 24 or 48 h, and then stained with
835 phalloidin (Actin), anti-Gag and DAPI (Nuclei), and analyzed by confocal microscopy (A,
836 scale bar, 25 μ m). In B), the number of nuclei per iDC was quantified from images on at least
837 40 cells. Each dot corresponds to 1 cell, and the number of cells analyzed is indicated (n).
838 Horizontal bars represent the mean \pm 1 SEM. Statistical significance was determined with the
839 Mann-Whitney U-test (*p<0.05; **p<0.01; ****p<0.0001). The results shown are
840 representative of at least 3 independent experiments performed with iDCs from 3 different
841 donors. NI, non-infected Jurkat cells were cocultured with iDCs. (C) experimental protocol
842 for homotypic cell-fusion experiment. (D and E) NLAD8- or NL4.3-infected Jurkat cells were
843 cocultured with iDCs pre-labeled with CellTracker for 3 h, and autologous iDCs pre-labeled
844 with CellTrace were added and cultured for 3 additional hours. Cells were then stained with
845 anti-Gag and phalloidin (Actin), and analyzed by confocal microscopy (scale bar, 10 μ m). In

846 E), the number of single-positive CellTrace(+) and CellTracker(+), as well as double-positive
847 CellTrace(+)/CellTracker(+) iDCs were quantified from images, and the results are expressed
848 as the percentages of single- and double-positive iDCs. The number of cells analyzed is
849 indicated (n). NI, non-infected Jurkat cells were co-cultured with iDCs. (F) NLAD8-infected
850 Jurkat cells were cocultured for 6 h with iDCs, and cells were then fixed and dehydrated.
851 Ultrathin sections were cut, stained and observed with a transmission electron microscope. A
852 multinucleated iDC with nuclei indicated by red stars is shown (left image, scale bar, 2 μ m).
853 The right image (scale bar, 200 nm) corresponds to higher magnification of the area selected
854 in red on the central image (scale bar, 1 μ m). Assembling and budding viruses are indicated
855 by blue arrows, while mature virion is indicated by a red arrow. The results shown are
856 representative of 3 independent experiments performed with iDCs from 3 donors.

857

858 **Figure 5. Productive infection of iDCs by cell-to-cell fusion with infected T cells escapes**
859 **SAMHD1 restriction.** (A) NLAD8-infected Jurkat cells were cocultured for 24 h with iDCs
860 derived from 4 different donors with or without AZT. T cells were then eliminated as
861 previously by extensive washes, and iDCs were cultured with or without AZT for different
862 period of time. Cell-culture supernatants from iDCs were collected after 3, 6, 9 or 12 days and
863 viral p24 was quantified. Each experiment was performed in duplicate. NI, non-infected
864 Jurkat cells were cocultured with iDCs. (B) NLAD8-infected Jurkat cells were cocultured for
865 6, 24 or 72 h with iDCs with or without AZT and the percentage of Gag⁺/CD11c⁺ iDCs was
866 then evaluated by flow cytometry. The results are the means of 3 independent experiments
867 performed with iDCs of 3 different donors. Error bars represent 1 SEM. Statistical
868 significance was determined using Mann-Whitney U-test (ns, $p > 0.05$; ** $p < 0.01$). (C) iDCs
869 were transfected with siRNA targeting SAMHD1 (siSAMHD1) or scrambled siRNA (siCtrl)
870 used as a control. Lysates from transfected cells were analyzed by Western blot using anti-

871 SAMHD1 and anti- β -Actin antibodies. The band intensities were quantified using NIH Image
872 Software and the signal measured in siRNA-transfected cells was normalized to the signal
873 obtained for Actin. The indicated values are expressed as percentages of the signal intensity
874 relative to siCtrl (100%), and are representative of 3 independent experiments. (D-F) iDCs
875 transfected with siCtrl (blue bars) or siSAMHD1 (hatched bars) were infected by cell-free
876 NLAD8 viruses produced by Jurkat cells (CF) or by co-culture with NLAD8-infected Jurkat
877 cells (CTC) for 24 h. In D), the percentage of Gag+/DC-SIGN+ iDCs was evaluated by flow
878 cytometry directly after 24 h of infection by CF or CTC. The results are expressed as the
879 percentage of Gag+ iDCs relative to that obtained with siCtrl-transfected iDCs (100%). In E
880 and F), quantification of p24 released in cell-culture supernatants from siRNA-transfected
881 iDCs infected by CF or CTC for 24 h, and then cultured for 6 more days. The results are
882 expressed as the percentage of p24 released relative to that released by CTC infection (E), or
883 as the percentage of p24 released relative to that released by siCtrl-transfected iDCs (F).
884 Results are the means of 4 independent experiments performed with iDCs from 4 different
885 donors. Error bars represent 1 SEM. Statistical significance was determined using Mann-
886 Whitney U-test (ns, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$). (G) MDMs were infected by cell-free
887 NLAD8 viruses produced by CD4+ primary T cells (Cell-free, left panels) or by co-culture
888 with NLAD8-infected primary T cells (Cell-to-cell, right panels) for 24 h. Cell lysates were
889 then analyzed by Western blot using anti-SAMHD1, anti-phospho-SAMHD1, anti-HIV-1 p24
890 and anti- β -actin antibodies.

891

892

893

894 **Supplemental Material**

895

896 **Supplementary Figure S1. Characterization of cells of the myeloid lineage.** (A-C) CD14+
897 blood monocytes were differentiated in MDMs or in OCs, and expression of the indicated OC
898 specific factors were analyzed by RT-qPCR in A). The results are the means of 10
899 independent experiments performed with MDMs or OCs from 10 donors. In B), MDMs and
900 OCs were stained with phalloidin (green) and DAPI (blue) and analyzed by confocal
901 microscopy (scale bars, 5 μm), and the fusion index (right panel) was determined by
902 quantification of at least 500 cells from 5 different donors. In C), MDMs or OCs were seeded
903 on bovine bone slides and stained with Toluidine blue. Bone resorption pits were analyzed on
904 brightfield images (scale bars, 20 μm) and bone degradation index (right panel) was
905 quantified on 7 different donors. (D and E) Monocytes were differentiated in MDMs (grey
906 bars) or iDCs (red bars). Maturation of DCs (mDCs, green bars) was induced using LPS.
907 Expression of the indicated MDM and DC markers was analyzed by flow cytometry. The
908 results shown are representative of 4 independent experiments performed with DCs and
909 MDMs from 4 different donors. Error bars represent 1 SEM. Statistical significance was
910 determined using Mann-Whitney U-test (ns, $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

911

912 **Supplementary Figure S2. Formation of multinucleated cells through cell-fusion**
913 **between infected T cells and iDC or OC cell targets.** (A and B) NLAD8- or NL4.3-infected
914 Jurkat or primary CD4+ T cells pre-labeled with CellTracker were cocultured for 6 h with
915 iDCs (A) or OCs (B). Cells were then stained with anti-Gag, in combination with anti-DC-
916 SIGN or phalloidin (Actin), and analyzed by confocal microscopy. The number of nuclei per
917 iDC and OC cell was quantified from images on at least 50 cells. Results are expressed as the
918 percentage of cells with 1, 2, 3 or more than 3 nuclei (left panels), as the mean nucleus

919 number per cell (central panels), and as the percentage of cells with 1, 2, 3 or more than 3
920 CellTracker(-) nuclei (right panels). (C) NLAD8- or NL4.3-infected Jurkat cells pre-labeled
921 with CellTracker were cocultured for 6 h with iDCs or mDCs. Cells were then stained with
922 anti-Gag and anti-DC-SIGN, and analyzed by confocal microscopy. The number of nuclei per
923 DC-SIGN+ cell was analyzed from images on at least 50 cells. Results are expressed as the
924 percentages of iDCs or mDCs with 1, 2, 3 or more than 3 nuclei (left and central panels,
925 respectively), and as the mean nucleus number per iDCs or mDCs (right panel). The results
926 shown are representative of at least 3 independent experiments performed with DCs and OCs
927 from at least 3 different donors. NI, non-infected Jurkat or primary CD4+ T cells were co-
928 cultured with OCs or DCs. Statistical significance was determined using Mann-Whitney U-
929 test (ns, $p > 0.05$; **** $p < 0.0001$).

930

931 **Supplementary Figure S3. Transfer of macrophage-tropic viruses to iDCs by cell-fusion.**

932 Primary CD4+ T cells infected with the NLAD8, JRFL or YU2 viral strains were pre-labeled
933 with CellTracker, and cocultured for 6 h with iDCs. Cells were then stained with anti-Gag,
934 anti-DC-SIGN and DRAQ5, and analyzed by confocal microscopy (scale bar, 20 μm).
935 Representative images are shown in (A). The number of nuclei per DC-SIGN+ cell was
936 quantified from images on at least 50 cells. In B), results are expressed as the percentages of
937 cells with 0, 1, 2, or more than 2 CellTracker(+) nuclei. In C) Results are expressed as the
938 percentage of cells with 1, 2, 3 or more than 3 nuclei (left panel), as the mean nucleus number
939 per cell (central panel), and as the percentage of cells with 1, 2, 3 or more than 3
940 CellTracker(-) nuclei (right panel).

941

942 **Supplementary Figure S4. Characterization of virus capture and transfer without cell**
943 **fusion by DC, monocyte and CD4+ T cell targets.** (A) NLAD8- or NL4.3-infected Jurkat

944 cells pre-labeled with CellTracker were cocultured for 6 h with iDCs, and then stained with
945 anti-Gag, phalloidin (Actin) and DRAQ5 (Nuclei). Cells were analyzed by confocal
946 microscopy (scale bar, 10 μ m). (B and C) NLAD8- or NL4.3-infected Jurkat cells pre-labeled
947 with CellTrace were cocultured for 6 h with primary monocytes, stained with anti-Gag,
948 phalloidin (Actin) and DRAQ5 (Nuclei), and analyzed by confocal microscopy (B, scale bar,
949 10 μ m). T cells and monocytes are indicated by plain yellow and dashed white lines,
950 respectively. (D and E) NLAD8- or NL4.3-infected primary CD4⁺ T cells preloaded with
951 CellTracker were cocultured for 6 h with primary CD4⁺ T cells, stained with anti-Gag,
952 phalloidin (Actin) and DRAQ5 (Nuclei), and analyzed by confocal microscopy (D, scale bars,
953 10 μ m). In C and E), The number of nuclei per monocyte (C) or T cell (E) targets was
954 quantified from images on at least 100 cells, and the results are expressed as the percentage of
955 cells containing 1 or 2 nuclei. The results shown are representative of 3 independent
956 experiments performed with monocytes or CD4⁺ T cell targets or from 3 different donors. NI,
957 non-infected Jurkat or primary T cells were co-cultured with monocytes or primary T cell
958 targets. (F) NLAD8- or NL4.3-infected Jurkat cells were cocultured for 6 h with iDCs, and
959 cells were then fixed and dehydrated. Ultrathin sections were cut, stained and observed with a
960 transmission electron microscope. The right images (scale bar, 200 nm) correspond to higher
961 magnification of the area selected by red squares on the left images (scale bars, 0.5 μ m). The
962 results shown are representative of 3 independent experiments performed with iDCs from 3
963 donors. Virus particles are indicated by red arrows.

964

965 **Supplementary Figure S5. Characterization of iDC-derived multinucleated giant cells.**

966 (A) NLAD8-infected Jurkat cells were cocultured for 6 h with iDCs, then stained with anti-
967 CD2, anti-Gag and DAPI (Nuclei), and analyzed by confocal microscopy (scale bar, 20 μ m).
968 Intracellular CD2 mean fluorescence intensities (MFI) were quantified as indicated in the

969 Methods Section on at least 50 iDCs (right panel). Each dot corresponds to 1 cell, and the
970 number of cells analyzed is indicated (n). Horizontal bars represent the mean \pm 1 SEM.
971 Statistical significance was determined with the Mann-Whitney U-test (****p<0.0001). (B)
972 Primary CD4⁺ T cells infected with the NLAD8, JRFL or YU2 viral strains were cocultured
973 for 6 h with iDCs, then stained with anti-CD3, anti-Gag and DAPI (Nuclei), and analyzed by
974 confocal microscopy (scale bar, 20 μ m). Intracellular CD3 MFI were quantified as indicated
975 in the Methods Section on at least 50 iDCs (right panel). Each dot corresponds to 1 cell, and
976 the number of cells analyzed is indicated (n). Horizontal bars represent the mean \pm 1 SEM.
977 Statistical significance was determined with the Mann-Whitney U-test (****p<0.0001). (C)
978 NLAD8-infected Jurkat cells were cocultured with iDCs for 24 h, and 1, 2 or 4 extensive
979 washes were then performed with PBS in order to eliminate non-adherent cells. Remaining
980 adherent cells were fixed, permeabilized and stained with anti-Gag, anti-DC-SIGN and DAPI
981 (Nuclei), and analyzed by confocal microscopy (scale bar, 10 μ m). The percentages of T cell
982 removal before (No wash) or after 1, 2 or 4 washes was determined by quantification of the
983 DC-SIGN(-) (T cell nuclei, green) or DC-SIGN(+) (iDC nuclei, blue) deduced from images
984 on at least 50 cells (right panel). The results shown are representative of 4 independent
985 experiments performed with iDCs from 4 different donors. NI, non-infected Jurkat cells were
986 cocultured with iDCs.

987

988 **Supplementary Figure S6. Survival of iDC-derived MGCs and effect of AZT on MGC**
989 **formation.** (A) NLAD8-infected Jurkat cells were cocultured with iDCs for 24 h, and non-
990 adherent cells were eliminated by 4 washes with PBS. Adherent cells were then cultured for 3
991 (D3), 6 (D6) or 9 (D9) days before fixation, permeabilization and staining with anti-Gag,
992 phalloidin (Actin) and DAPI (Nuclei), and analyzed by confocal microscopy (scale bar, 10
993 μ m). (B and C) NLAD8-infected Jurkat cells were cocultured for 24 h with iDCs with or

994 without AZT. Cells were then stained with anti-Gag, anti-DC-SIGN and DRAQ5 (nuclei).
995 Cells were analyzed by confocal microscopy (B, scale bar, 10 μ m). In C), the number of
996 nuclei per iDC was quantified from images. Each dot corresponds to 1 cell, and the number of
997 cells analyzed is indicated (n). Horizontal bars represent the mean \pm 1 SEM. Statistical
998 significance was determined with the Mann-Whitney U-test (ns, $p > 0.05$). The results shown
999 are representative of 4 independent experiments performed with iDCs from 4 donors.

1000

1001 **Supplementary Figure S7. Productive infection of MDMs by cell-to-cell fusion with**
1002 **infected T cells escapes SAMHD1 restriction.** (A) MDMs were transfected with siRNA
1003 targeting SAMHD1 (siSAMHD1) or scrambled siRNA (siCtrl) used as a control. Lysates
1004 from transfected cells were analyzed by Western blot using anti-SAMHD1 and anti- β -Actin
1005 antibodies. The band intensities were quantified using NIH Image Software and the signal
1006 measured in siRNA-transfected cells was normalized to the signal obtained for Actin. The
1007 indicated values are expressed as percentages of the signal intensity relative to siCtrl (100%).
1008 (B-D) MDMs transfected with siCtrl (blue bars) or siSAMHD1 (hatched bars) were infected
1009 by cell-free NLAD8 viruses produced by Jurkat cells (CF) or by coculture with NLAD8-
1010 infected Jurkat cells (CTC) for 24 h. In B), the percentage of Gag⁺ MDMs was evaluated by
1011 flow cytometry directly after 24 h of infection by CF or CTC. The results are expressed as the
1012 percentage of Gag⁺ MDMs relative to that obtained with siCtrl-transfected MDMs (100%). In
1013 C and D), quantification of p24 released in cell-culture supernatants from siRNA-transfected
1014 MDMs infected by CF or CTC for 24 h, and then cultured for 6 more days. The results are
1015 expressed as the percentage of p24 released relative to that released by CTC infection (C), or
1016 as the percentage of p24 released relative to that released by siCtrl-transfected iDCs (D). (E)
1017 MDMs were infected by cell-free NLAD8 viruses produced by Jurkat cells (Cell-free, left
1018 panels) or by co-culture with NLAD8-infected Jurkat (Cell-to-cell, right panels) for 24 h. Cell

1019 lysates were then analyzed by Western blot using anti-SAMHD1, anti-phospho-SAMHD1,
1020 anti-HIV-1 p24 and anti- β -actin antibodies.

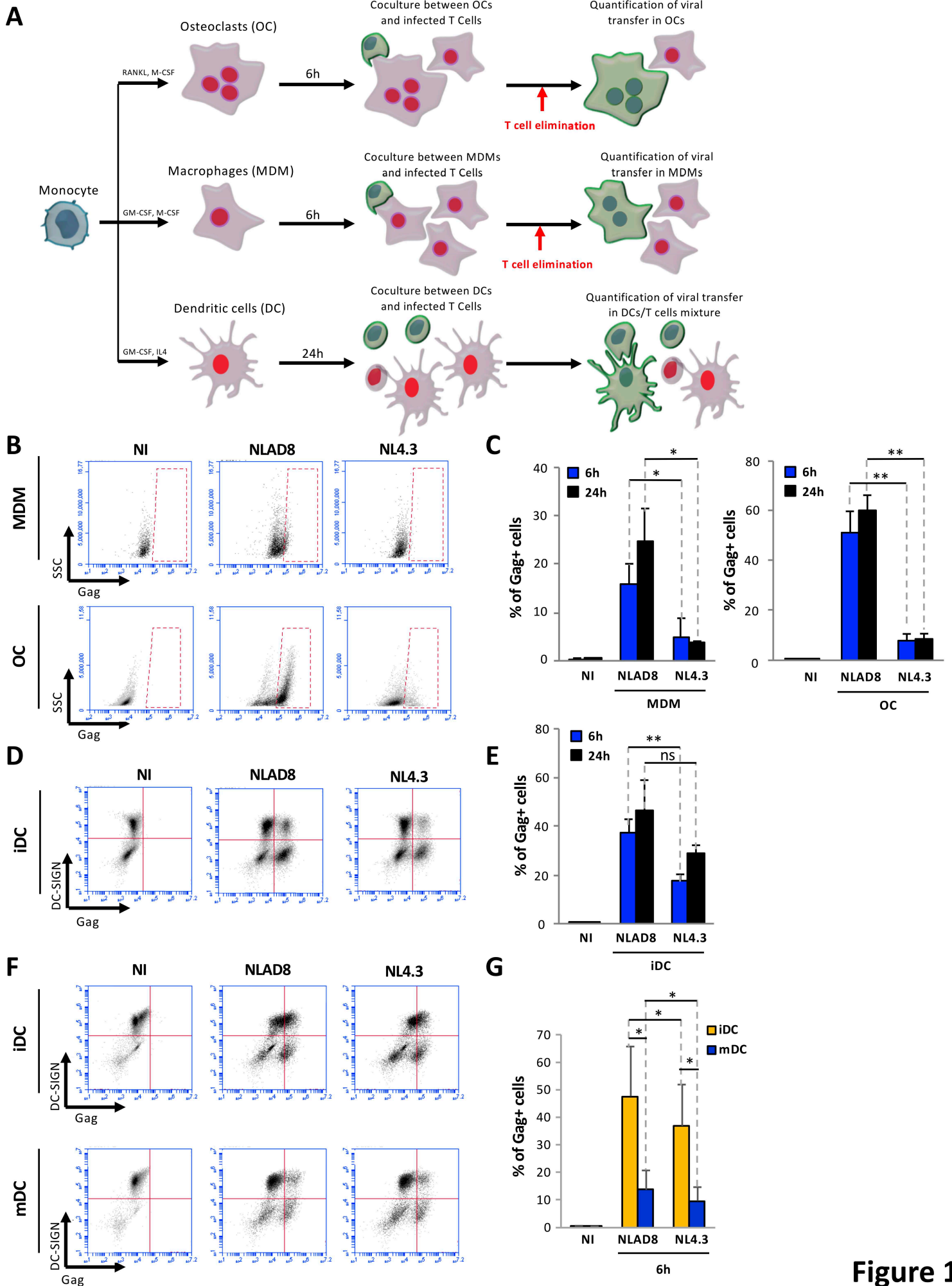


Figure 1

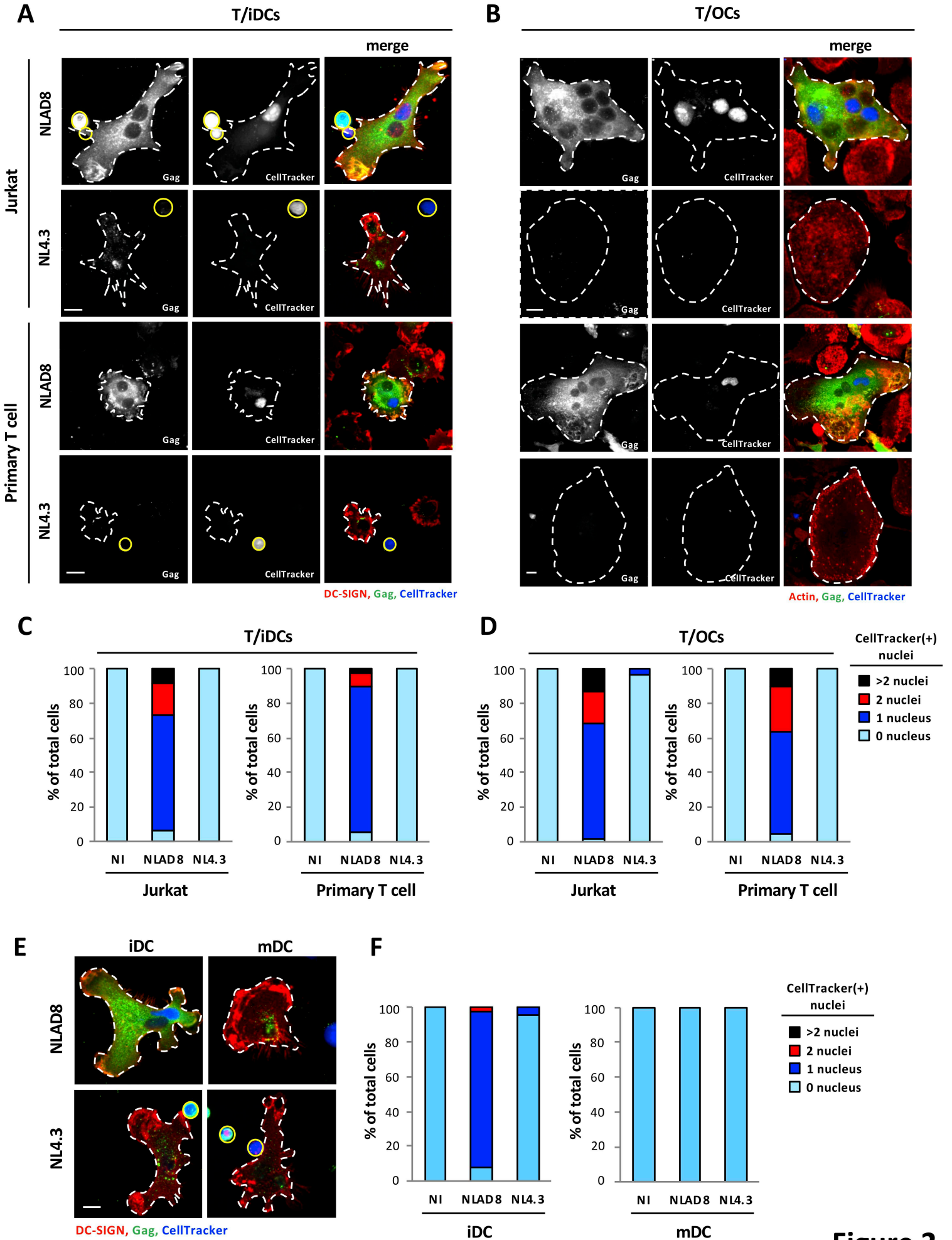


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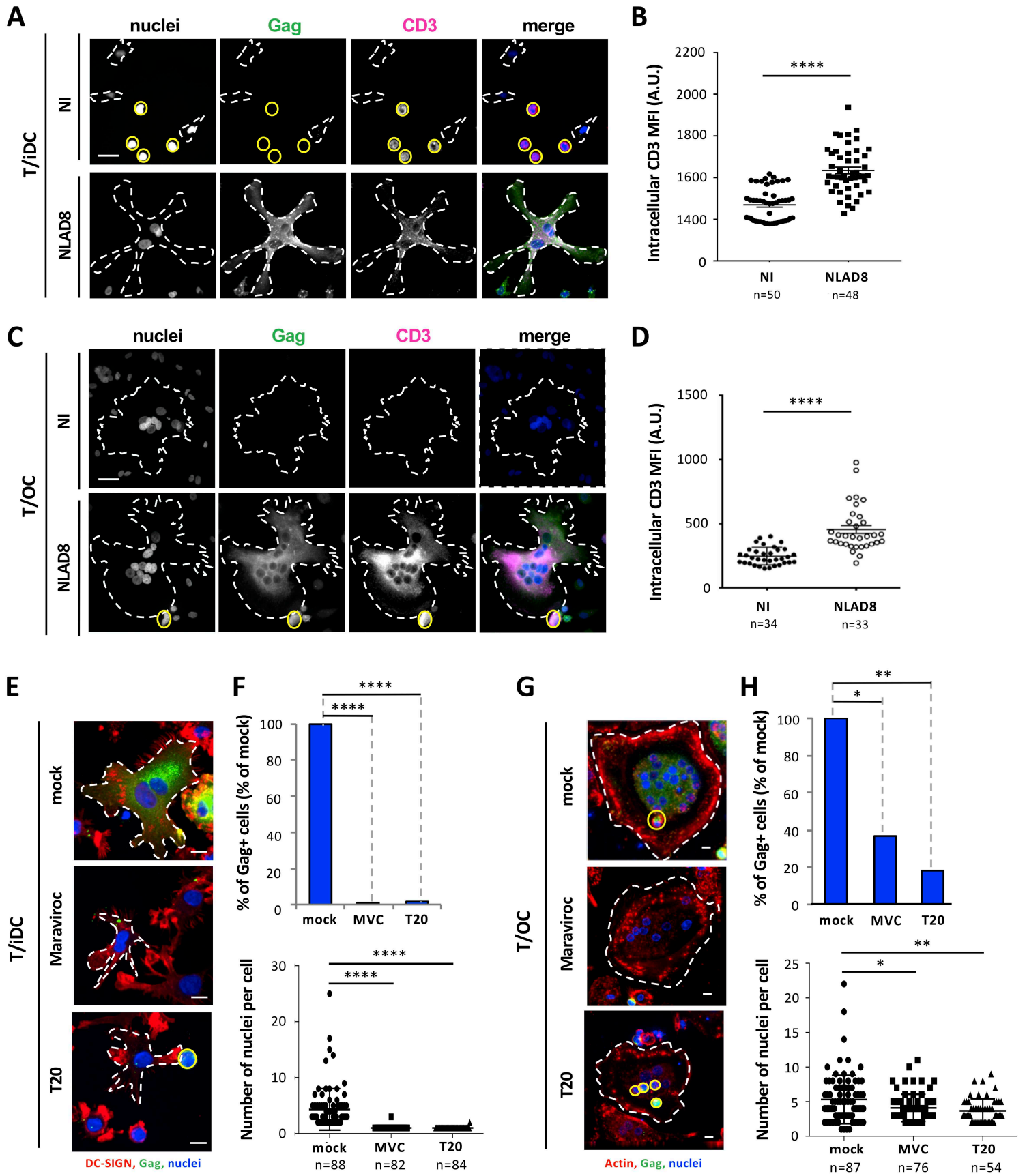
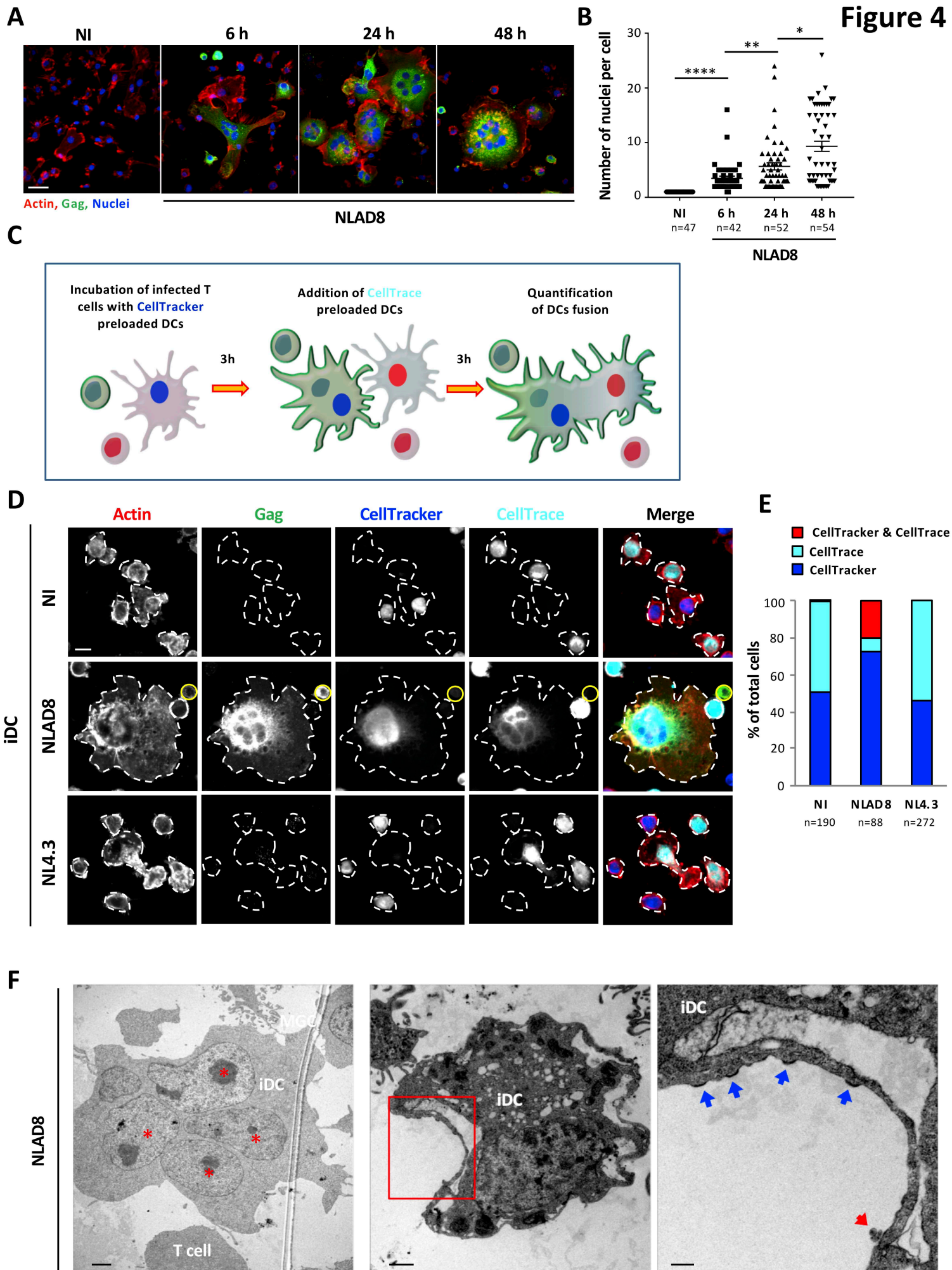
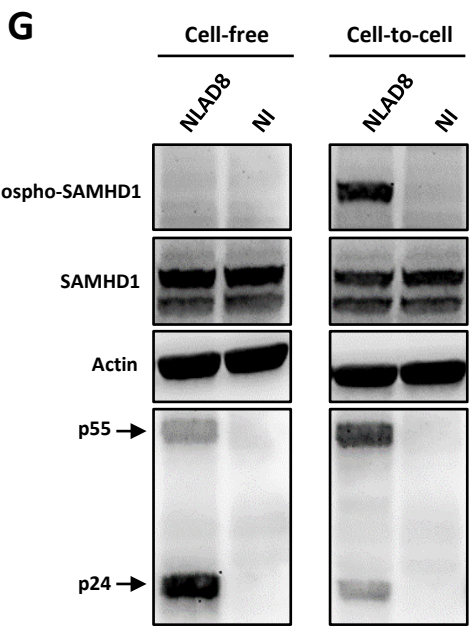
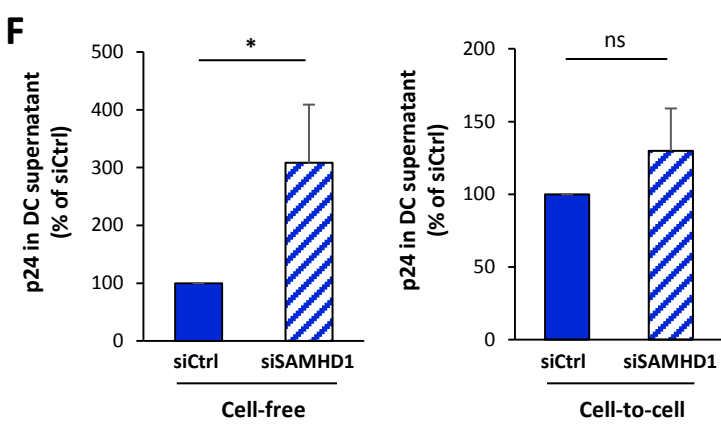
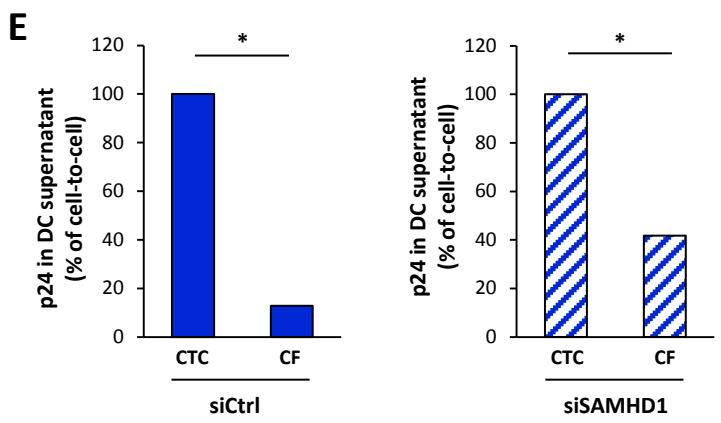
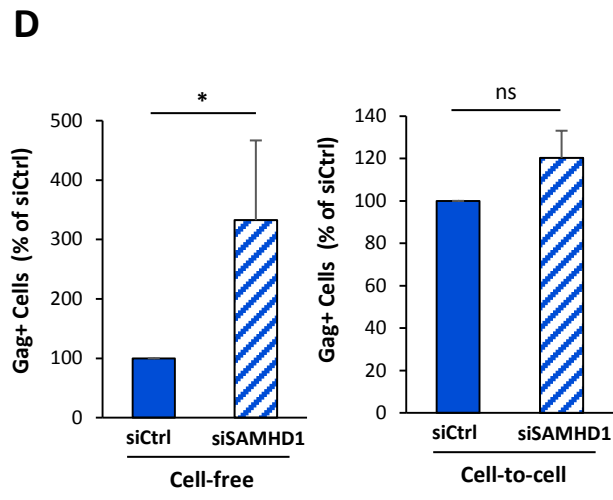
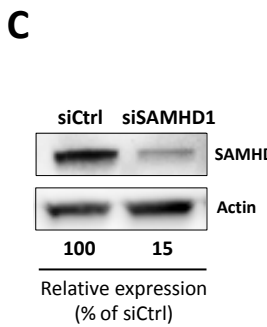
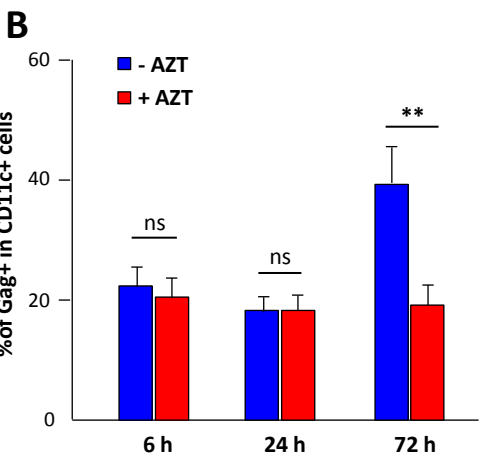
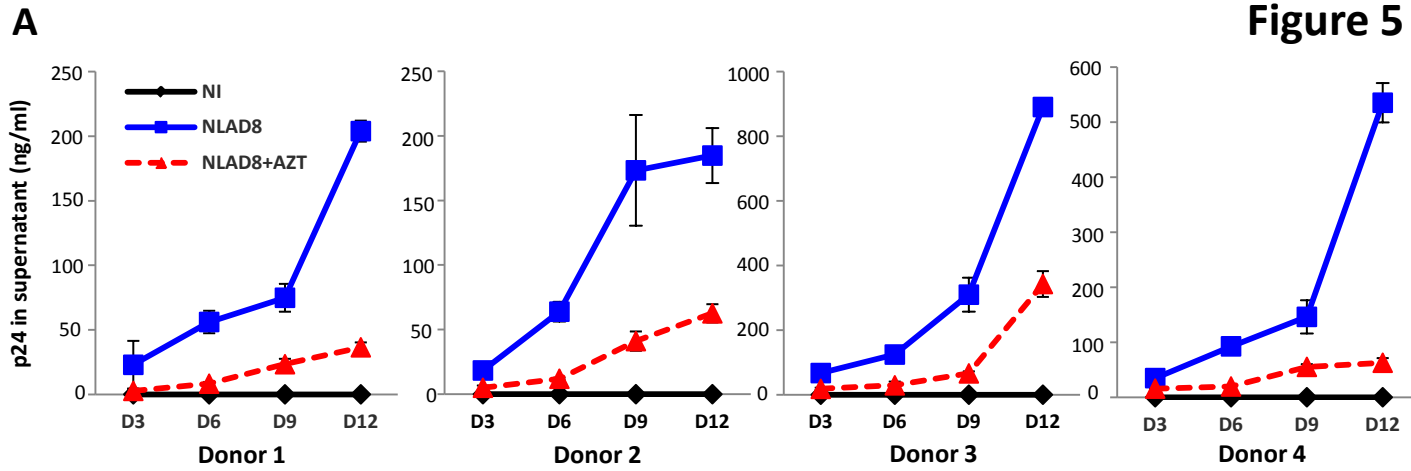
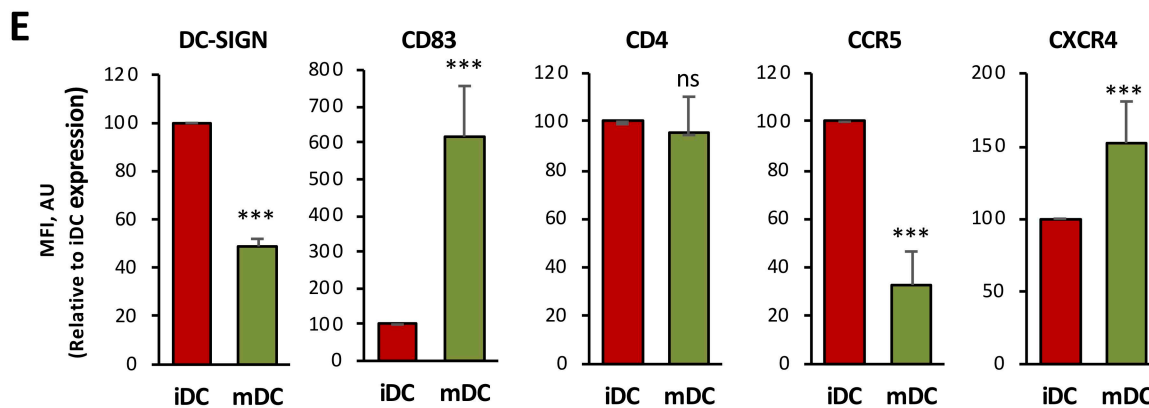
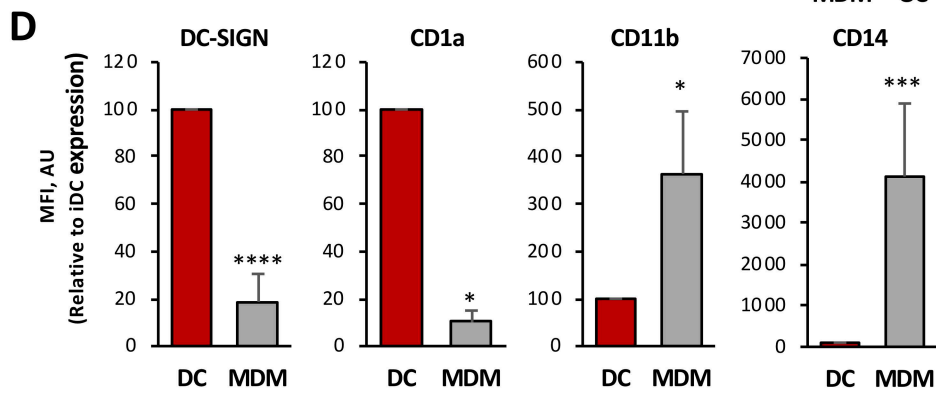
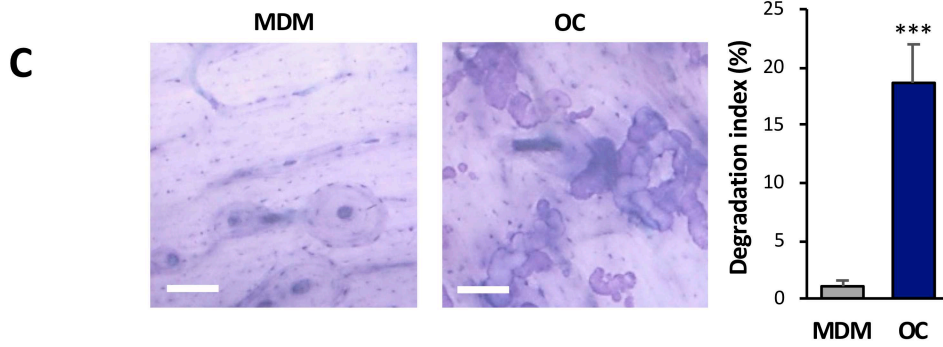
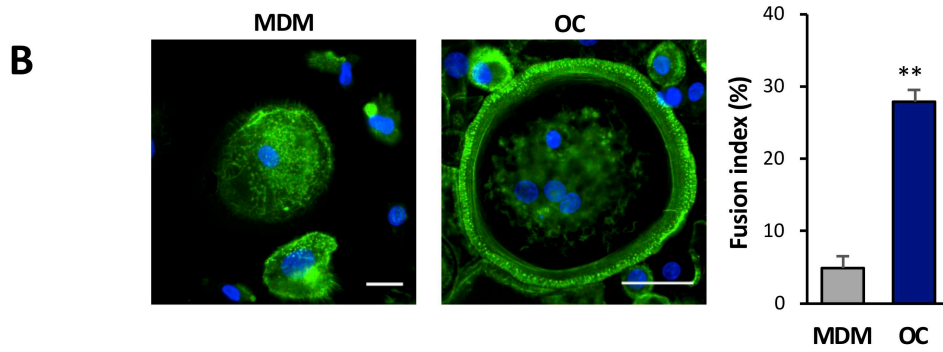
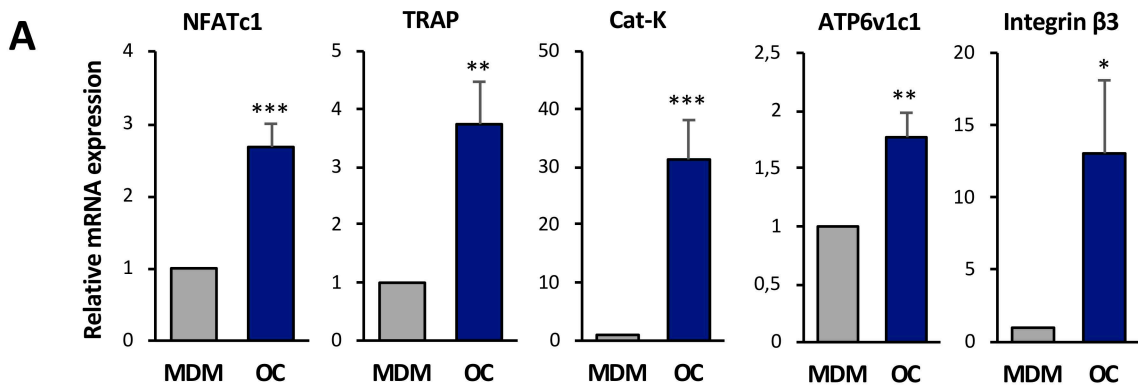


Figure 3

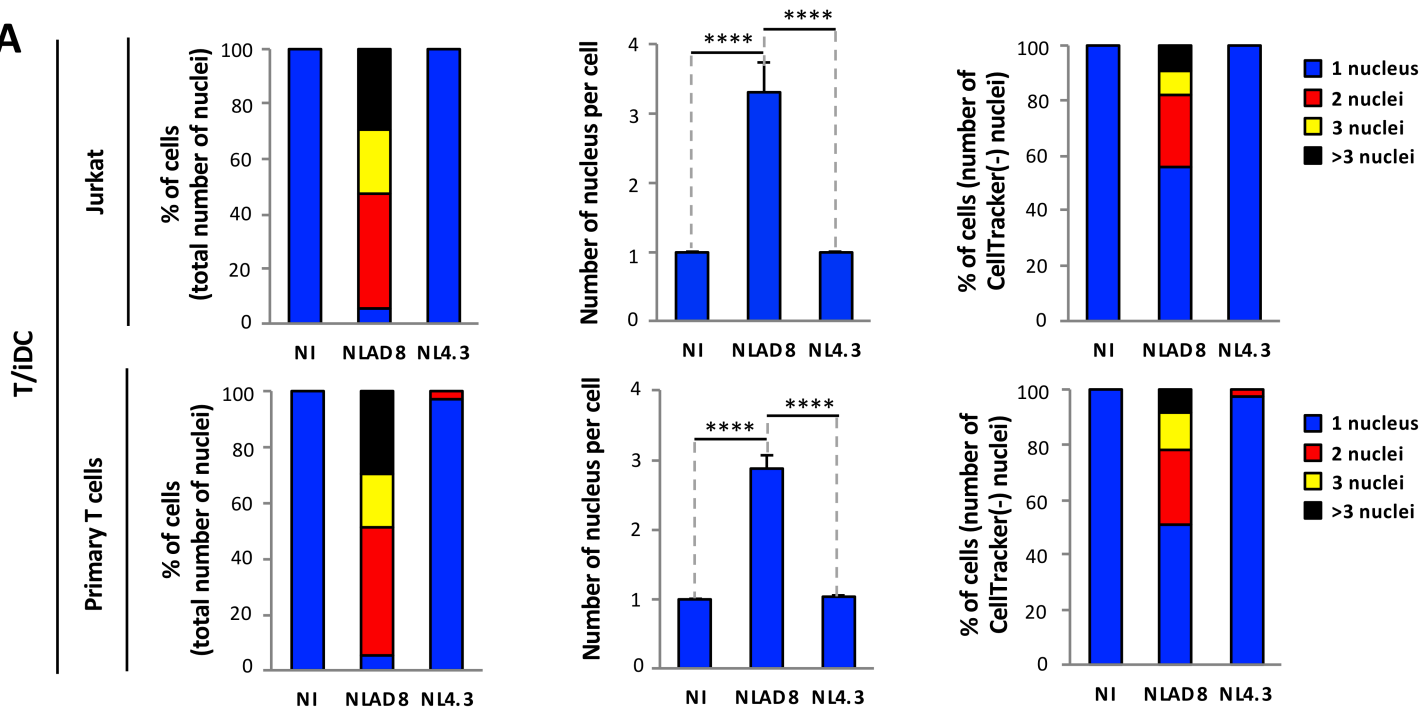
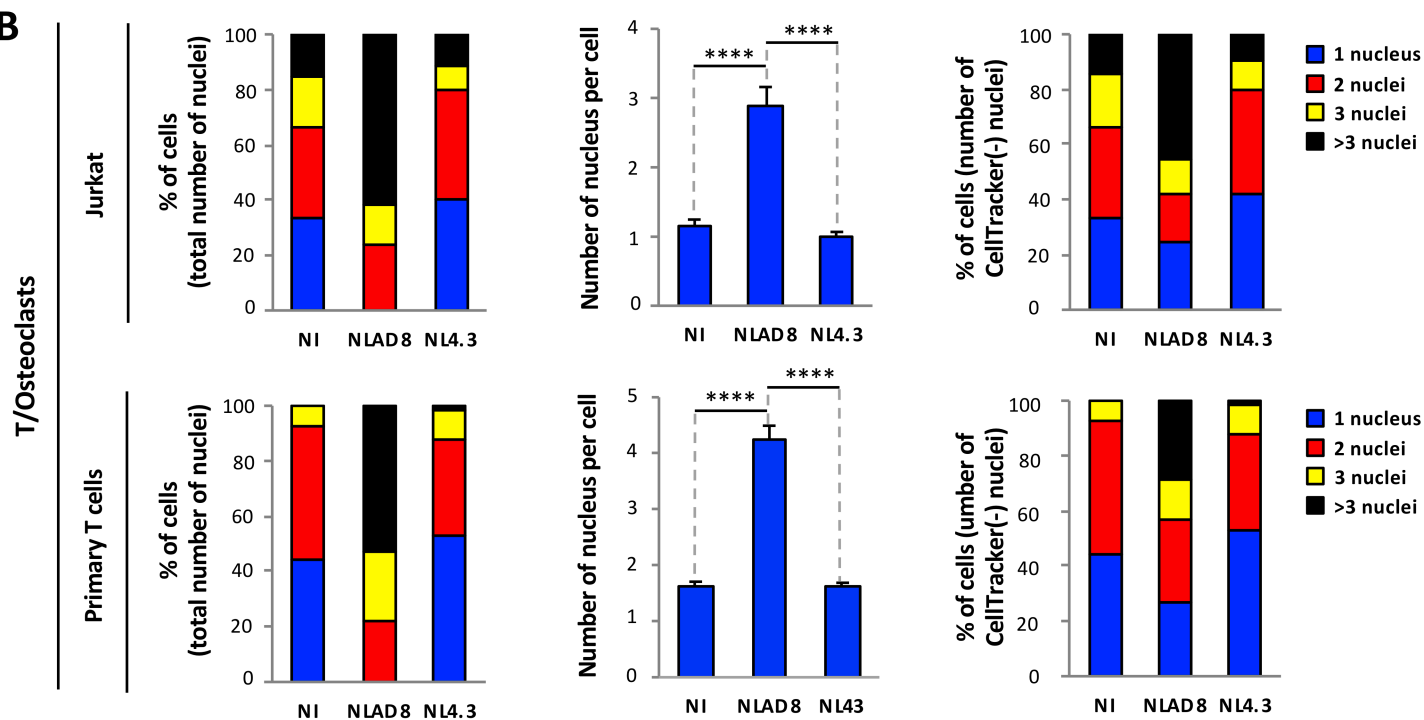
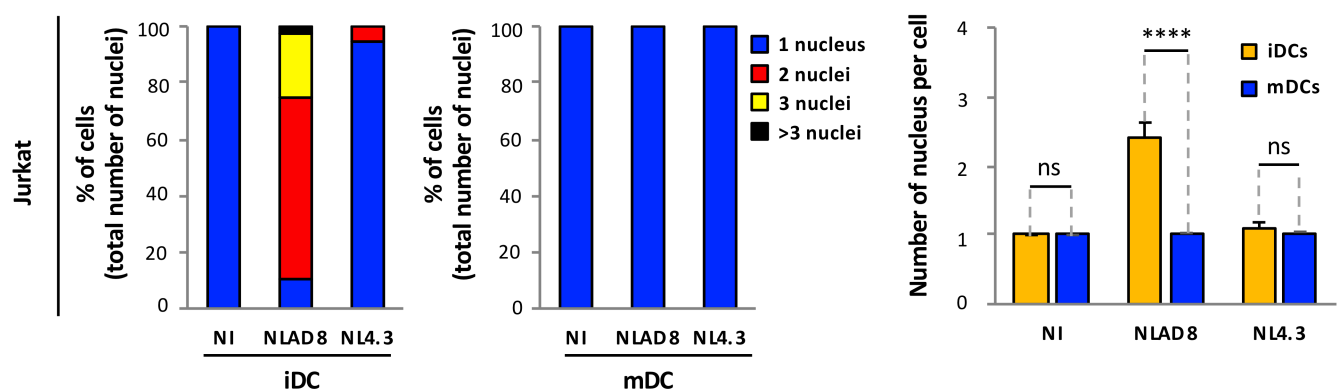
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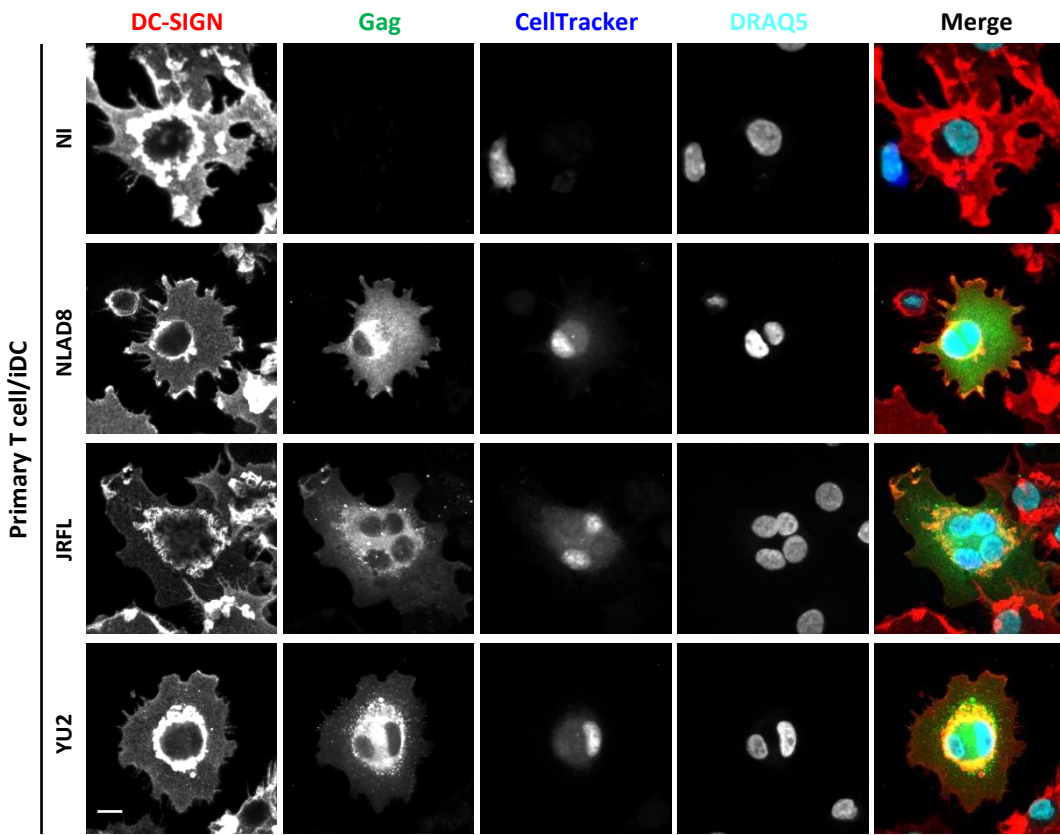
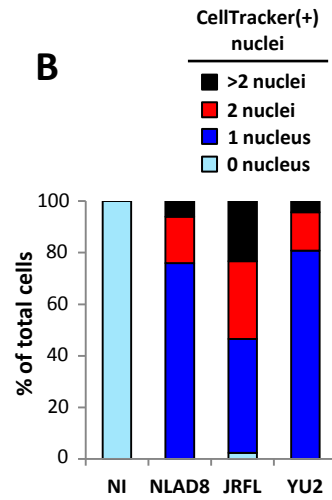
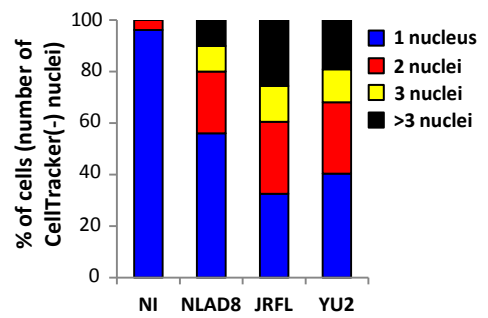
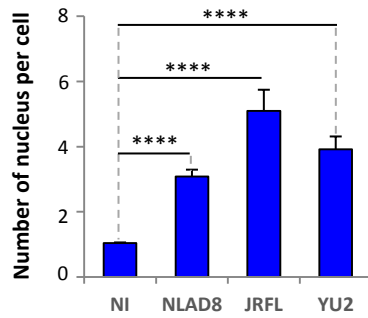
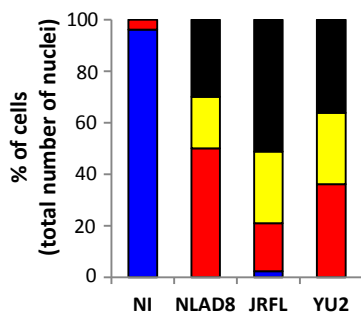


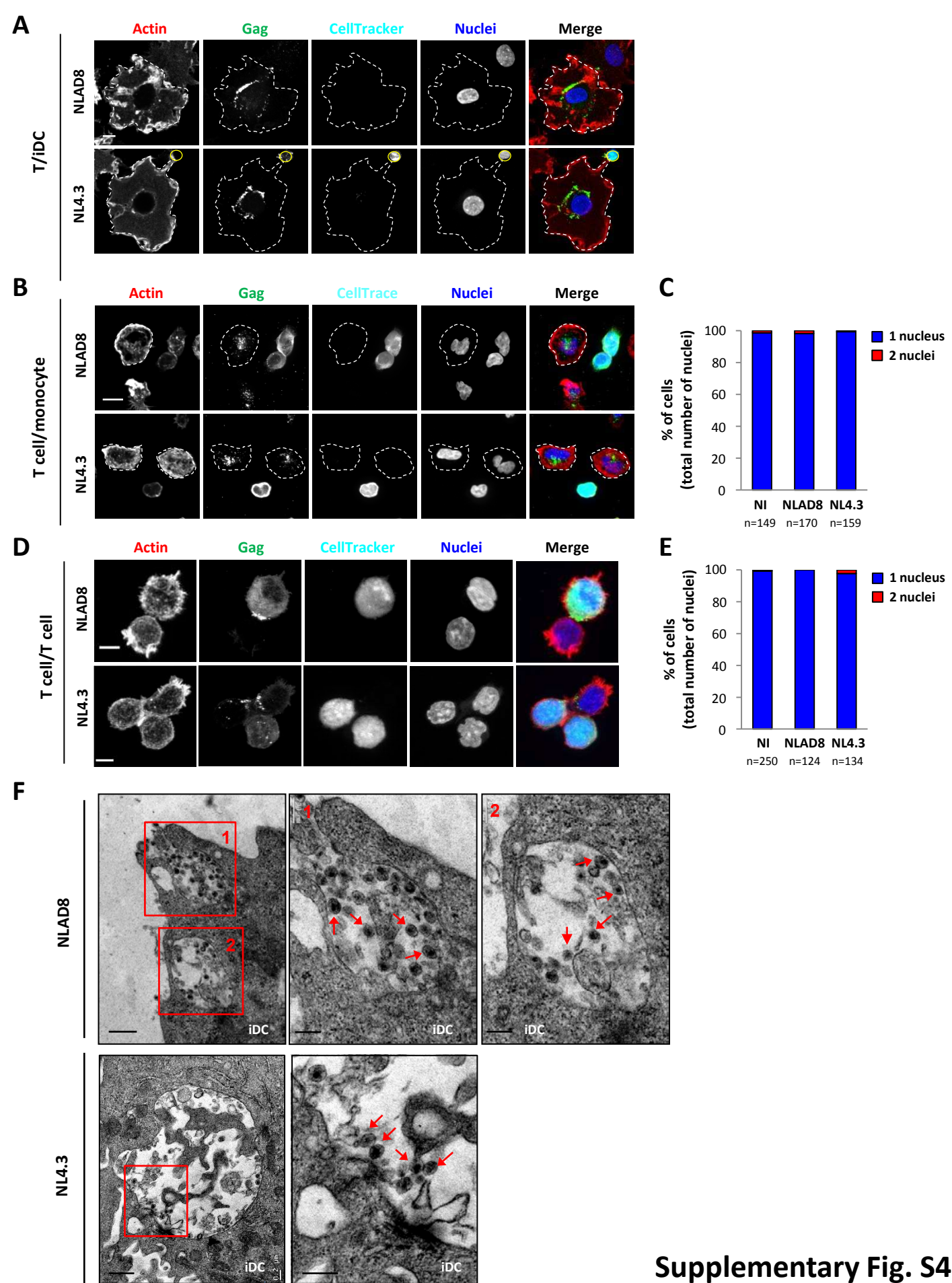


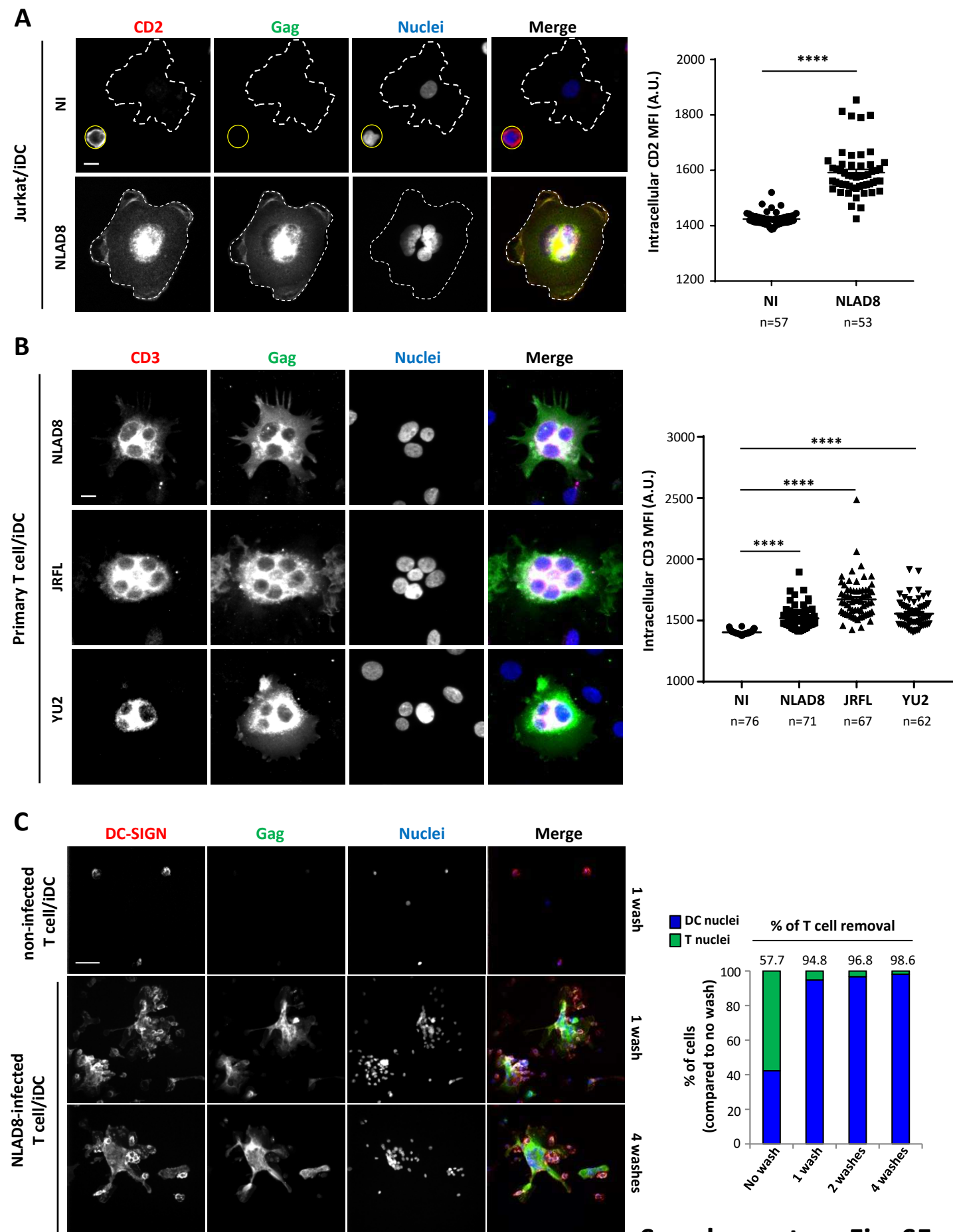


Supplementary Fig. S1

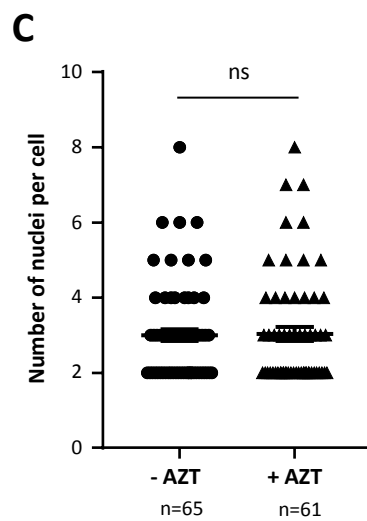
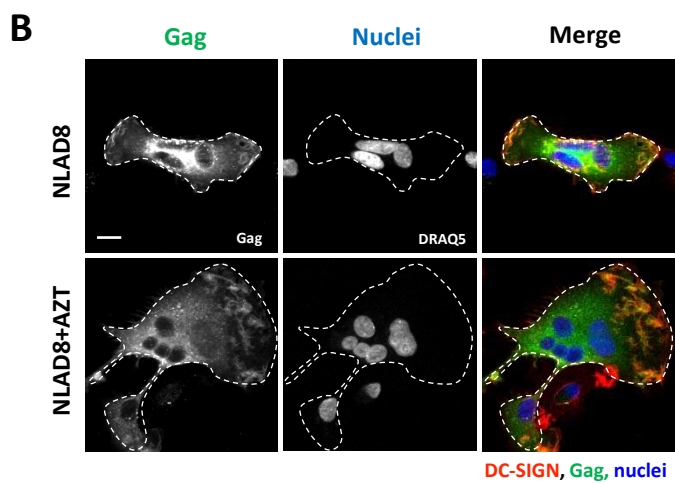
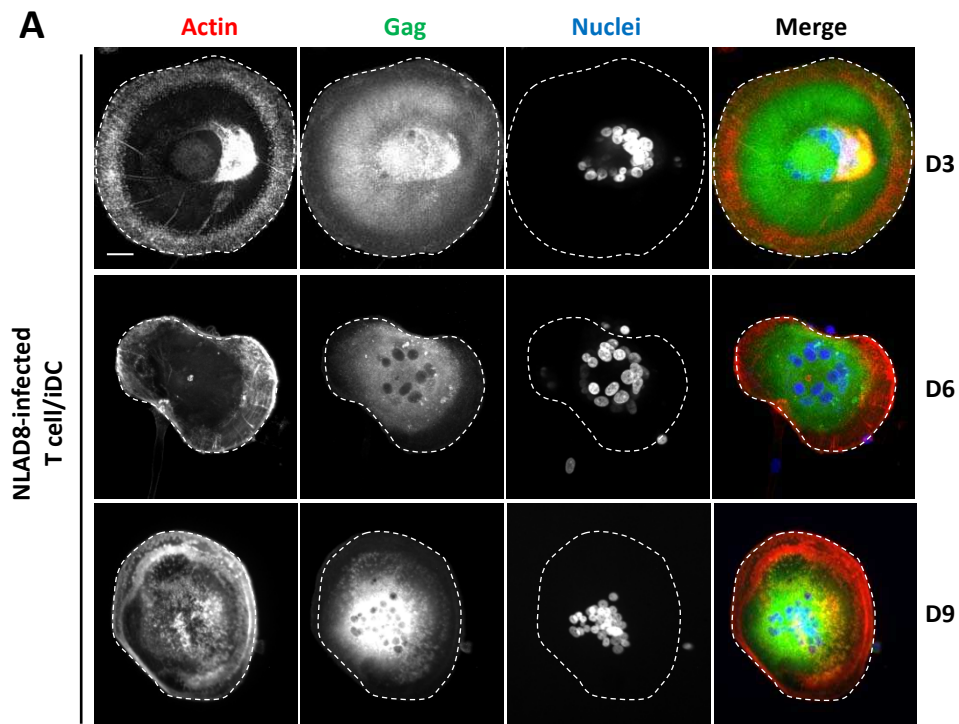
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A**B****C**

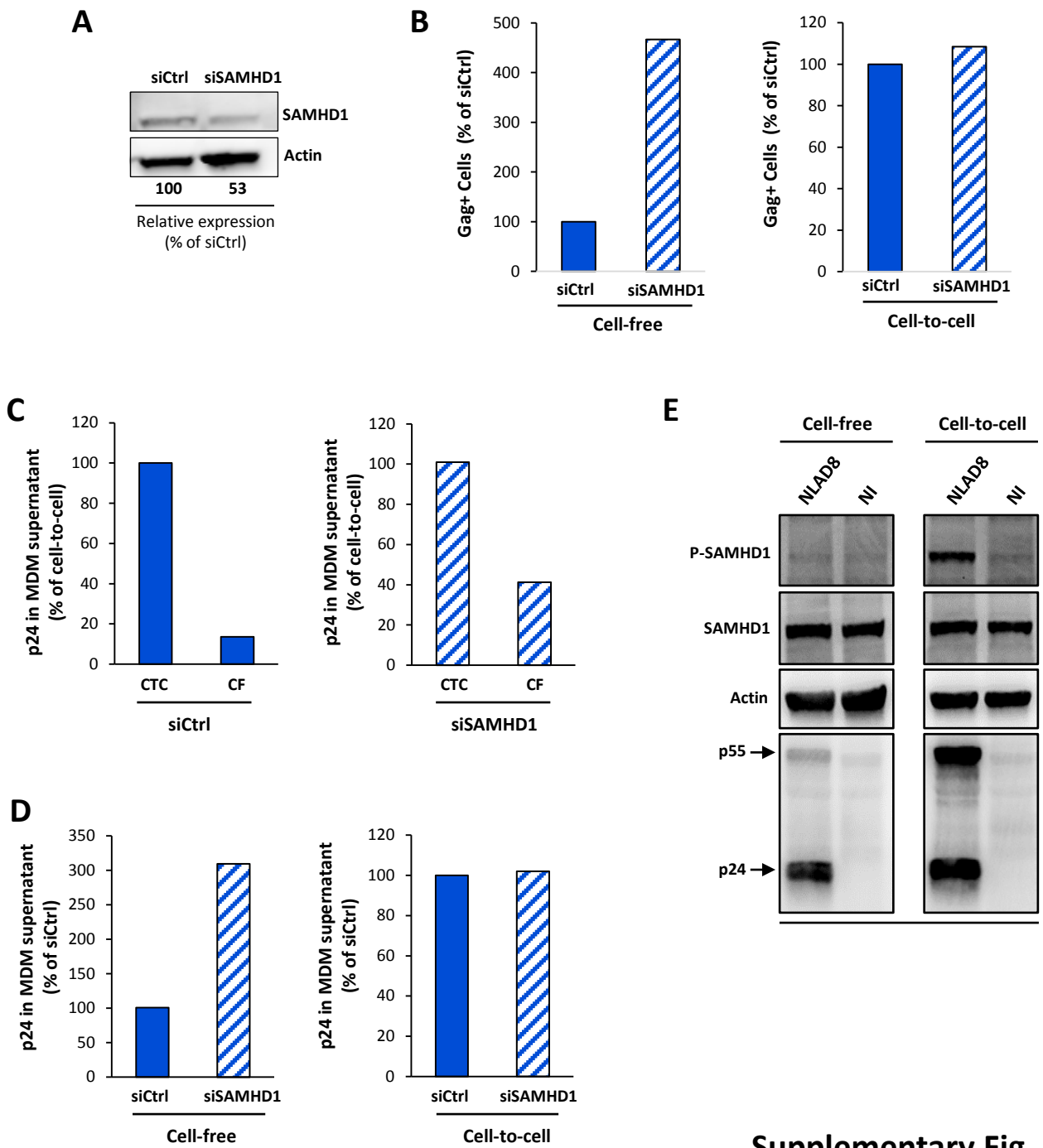




Supplementary Fig. S5



Supplementary Fig. S6



Supplementary Fig. S7

DISCUSSION

Myeloid cells, including monocytes, dendritic cells (DCs), macrophages as well as osteoclasts (OCs) are emerging as target cells of HIV-1 involved in i) sexual transmission, ii) virus dissemination in different tissues, including brain and bones and iii) establishment of persistent virus reservoirs. While these myeloid cells are poorly infected by cell-free viruses, because of the low levels of expression of viral entry receptors and high levels of expression of restriction factors such as SAMHD1 and APOBEC3A (Berger et al., 2011b; Hrecka et al., 2011; Laguette et al., 2011; Peng et al., 2007), the goal of the PhD work presented here, was to characterize the mechanisms of cell-to-cell transfer of HIV-1 from infected CD4⁺ T cells and then virus spreading in these myeloid target cells.

The main results of this work reveals that HIV-1 uses a specific and common cell-to-cell fusion mechanism for initial virus transfer and then dissemination from infected T lymphocytes to the target cells of the myeloid lineage, including immature DCs (iDCs), OCs and macrophages, but not monocytes and mature DCs. The establishment of contacts with infected T cells leads first to heterotypic cell-fusion for the fast and massive transfer of viral material in macrophage, OC and iDC targets. This process subsequently triggers homotypic cell-fusion with non-infected neighboring macrophages (or OCs, or iDCs) for intercellular virus dissemination. Both cell fusion steps are mediated by viral envelope-receptor/coreceptor interactions, and are highly efficient for macrophage-tropic CCR5- and CXCR4-using viruses, dual-tropic R5X4 viruses, and to a lesser extent for non-macrophage-tropic CCR5-using viral strains, including transmitted/founder viruses. Interestingly, these two cell-to-cell fusion processes can overcome the restriction imposed by SAMHD1 in these cell-types and allow a very efficient virus spreading in myeloid cells, resulting in the formation of highly virus-productive multinucleated giant cells (MGCs) that could survive for a long time to produce fully infectious viruses. Together, our *in vitro* results reveal the cellular mechanism for SAMHD1-independent cell-to-cell spreading of HIV-1 in myeloid cell targets through the formation of infected MGCs which have been largely documented *in vivo* in lymphoid and non-lymphoid tissues, including CNS, of HIV-1-infected patients and SIV-infected macaques.

1-Efficient HIV-1 cell-to-cell transfer from infected T cells to macrophages

During my PhD works, I have initially participated in the project in our lab regarding the analysis of HIV-1 cell-to-cell transfer from infected T cells to monocyte-derived macrophages (MDMs). In the first article (Bracq et al., 2017), we have described a novel model for virus cell-to-cell transfer from infected T cells to MDMs and virus spreading between MDMs. Initial viral transfer and subsequent virus spreading are mediated by a two-step cell-fusion process. In the first step, infected T cells establish contacts, initially discharge viral material to MDMs, and then fuse with MDM targets, with virus accumulation in intracytoplasmic compartments and virus assembly and budding at the cell surface of the newly formed Gag⁺ lymphocyte-macrophage fused cells (LMFCs), evidenced by the massive and rapid transfer of Gag⁺ material and the presence specific T cells markers (CD2, CD3 and Lck) in the cytoplasm and at the plasma membrane of the MDM targets. These LMFC then acquire the ability to fuse with surrounding uninfected MDMs, leading to the formation of Gag⁺ MGCs. This route of infection may be a major determinant for virus dissemination and the formation of macrophage virus reservoirs in host tissues during HIV-1 infection.

1.1-Macrophages are important cellular targets of HIV-1

Besides CD4⁺ T lymphocytes, macrophages are also important cellular targets of HIV-1 and play crucial roles in the pathophysiology of infection (Costiniuk and Jenabian, 2014; Gohda et al., 2015; Lee et al., 1999; Stevenson, 2015). The presence of infected macrophages has been evidenced *in vivo* in HIV-1-infected patients and SIV-infected macaques, as well as in "Humanized" mice where macrophages can sustain HIV-1 infection (Frank et al., 1996; Honeycutt et al., 2016). In addition to latently infected CD4⁺ T cells, infected macrophages also participate in virus dissemination and establishment of persistent virus reservoirs in numerous host tissues, including lymph nodes, spleen, lungs, genital and digestive tracts, and the central nervous system (CNS) (Costiniuk and Jenabian, 2014; Fischer-Smith et al., 2008). Virus access to the CNS is indeed mainly related to the migration of infected perivascular monocytes/macrophages through the blood-brain barrier and can result in a massive infiltration of macrophages and microglial cells, often detected as multinucleated giant cells (MGCs) and is

responsible for neurological disorders in patients (Burdo et al., 2013; Fischer-Smith et al., 2008; Spudich and Gonzalez-Scarano, 2012; Williams et al., 2012). More recently, Ganor et al. reported that human urethral macrophages constitute an important HIV-1 reservoir and may contribute to sexual transmission of HIV (Ganor et al., 2019).

In vitro, macrophages can derive from blood monocytes, which express both CD4 receptor and the CCR5 and CXCR4 co-receptors required for virus entry, can be productively infected by CCR5-using macrophage tropic viruses (Carter and Ehrlich, 2008; Sattentau and Stevenson, 2016). However, almost all the data reported so far regarding macrophage infection and analysis of the different steps of the virus life cycle in this cell type had been performed using cell-free virus particles. However, HIV-1 cell-free infection of macrophages is largely inefficient, in part due to low level expression of viral entry receptors, including CD4 and CCR5, as well as high expression of host cell restriction factors.

While cell-to-cell transmission of HIV-1 between T cells have been well documented (Bracq et al., 2018, and Sattentau, 2010, for reviews), at least *in vitro*, and is several orders of magnitude more efficient than cell-free infection, there was a paucity in knowledge of the mechanisms that control infection and dissemination in macrophages by HIV-1 cell-to-cell transfer. So far, only one study regarding the possibility of productive infection of macrophages via selective capture of healthy or rather dying HIV-1-infected CD4⁺ T lymphocytes was reported in the literature (Baxter et al., 2014). Hence, we decided to investigate how HIV-1 is transferred from infected T cells to macrophages through cell-to-cell contacts for virus dissemination and productive infection of macrophage targets.

1.2-Macrophage infection through cell-to-cell fusion from HIV-1-infected T cells is dependent on viral envelope-receptor interactions

In the study by Bracq et al. (2017), we revealed the mechanisms involved in a rapid and massive HIV-1 cell-to-cell transfer from infected T cells to MDMs and the subsequent virus spreading between MDMs by a two-step cell fusion process leading to the productive infection of MDM targets. We found that the initial viral transfer from infected T cells to MDMs was inhibited by neutralizing monoclonal antibodies targeting the HIV-1 surface gp120 envelope glycoprotein as well as by the Leu3a antibody targeting the CD4 receptor, showing that early interactions between infected virus-donor T cells and recipient macrophages involve recognition of CD4 by gp120. Initial virus transfer to MDMs is also blocked by the T20 fusion inhibitor targeting the

viral transmembrane gp41 envelope glycoprotein and the Maraviroc CCR5 antagonist. These results confirm that virus transfer and T cell fusion with macrophages is mediated by initial viral envelope-receptor and -coreceptor interactions. In addition, we have shown that the second cell fusion step between Gag⁺ lymphocyte macrophage fused cells and neighboring MDMs, required for virus dissemination and virus production by multinucleated giant cells, is also dependent on the viral envelope, since it is inhibited by T20 and maraviroc. While our data clearly show that both cell fusion processes for virus transfer and dissemination in macrophages are dependent on the viral envelope, we cannot exclude the possibility that other mechanisms related to the propensity of macrophages to mediate homotypic cell fusion could also participate in the cell fusion processes for virus transfer and dissemination. It would be interesting to investigate the potential role in HIV-1-mediated T cell-MDM and MDM-MDM fusions of some effector proteins known to be involved in programming of macrophages into a fusion-competent state and then in cell fusion for the formation of multinucleated giant macrophages(Helming and Gordon, 2009).

1.3-Macrophage infection by viral cell-to-cell transfer through phagocytosis and cell-to-cell fusion

Our results describe a new model for HIV-1 infection of macrophages through viral cell-to-cell transfer and dissemination by a two-step cell fusion process. In contrast, Baxter and colleagues from the group of Quentin Sattentau have reported that macrophages can be efficiently infected by HIV-1 through viral cell-to-cell transfer from infected T cells (Baxter et al., 2014). They have established a model in which macrophages can selectively engulf HIV-1-infected T cells for efficient HIV-1 cell-to-cell spread and productive infection of macrophages. While the engulfment (or phagocytosis) mechanism of infected T cells was not yet defined and is independent of viral envelope-receptor interactions, they suggest that the subsequent productive infection of MDMs following phagocytosis of infected T cells was largely dependent on viral envelope-receptor interactions and requires cytoskeletal rearrangements. Furthermore, MDM infection through this cell-to-cell route was highly permissive for CCR5-using macrophage-tropic and non-macrophage-tropic viruses, including transmitted/founder viruses, but was restrictive for CXCR4-using T-tropic viruses. The discrepancy between this study (Baxter et al., 2014) and our study by Bracq et al. (2017) may result from distinct systems used to investigate the cell biology of viral cell-to-cell transfer from infected T cells to macrophages. We hypothesize that both models could be involved in HIV-1 cell-to-cell infection of macrophages (see below).

1.31 Different experimental system may observe different aspects of HIV-1 cell-to-cell transfer toward macrophage targets

The differences between our cell-to-cell fusion model and the selective capture/phagocytosis model proposed by Baxter et al. may relate to the different systems used (Baxter et al., 2014; Bracq et al., 2017). First, viral strains and infection of CD4⁺ T cells were different in these studies. It has been shown that HIV-1 infection could induce T cell death through apoptosis and other mechanisms (Cooper et al., 2013; Doitsh et al., 2010), whereas macrophages avidly uptake dead and dying cells (Devitt and Marshall, 2011; Gordon and Pluddemann, 2018). In the Baxter et al. paper, infection of autologous CD4⁺ T cells was performed using HIV-1_{IIIIB} or HIV-1_{BAL}-infected T cells for 7 days, which may generate a significant proportion of apoptotic T

cells (Baxter et al., 2014). CD4⁺ T cells were 10%–20% Gag⁺ before co-culture with MDMs and were largely apoptotic as mentioned by the authors (Baxter et al., 2014). In agreement, Baxter et al. observed that HIV-1 infected dead/dying T cells are significantly captured by macrophages compared to the infected/healthy CD4⁺ T cells. However, in our study, in order to prevent HIV-induced death of infected T cells, infection of autologous CD4⁺ T cells or Jurkat CD4⁺ T cells were performed using HIV-1_{NLAD8}, HIV-1_{Yu2}, or HIV-1_{NL4.3} for only 36 hours to achieve >50% Gag⁺ T cells, but only less than 2% of infected T cells were apoptotic at this time (Bracq et al., 2017). Since macrophages are professional phagocytes specialized in phagocytosis of apoptotic cells (Lemke, 2019), the presence of HIV-1 infected/dying T cells could indeed favour the selective capture and phagocytosis of infected T cells instead of infected T cell-to-macrophage fusion we observed with non-apoptotic HIV-1 infected T cells.

Both studies have performed live-cell imaging experiments using GFP- or mCherry-tagged HIV-1 viral strains to visualize HIV-1 cell-to-cell transfer from HIV-1 infected CD4⁺ T cells to MDMs. Indeed, we could visualize extremely rare event of capture/phagocytosis of infected T cells by macrophages using GFP-tagged CCR5- and CXCR4-using viral strains. However, we have never observe phagocytosis of infected/dying T cells by macrophages using untagged-wild type viruses. Symeonides et al have reported that HIV-1_{NL4.3} Gag-iGFP could induce the formation of small T cell syncytia that can transfer viral particles to neighboring target CD4 cells through transient cell-to-cell contacts (Symeonides et al., 2015). Similarly, we have observed large T cell syncytia formation after HIV-1_{JRFL} Gag-iGFP infection of Jurkat T cells (unpublished observation). Thus, the utilization of GFP- or mCherry tagged viruses may artificially increases the incidence of HIV-1-induced syncytia formation as well as phagocytosis of infected CD4⁺ T cells by macrophages, and the results obtained using GFP- or mCherry tagged viruses should be examined and interpreted carefully.

1.32 HIV infected T cells uptake by macrophages is independent of viral envelope-receptor interactions

HIV-1 cell-free infection of macrophages is rate limited by fluid uptake and is largely inefficient, in part due to low level expression of viral entry receptors, including CD4 and CCR5 (Carter et al., 2011; Lee et al., 1999; Marechal et al., 2001). HIV-1 viral particles can also be captured by macrophages irrespective of viral envelope-receptor interactions, but most of virions appear to be degraded in macropinosomes, and only

few virus-to-cell fusion events takes place at the limiting membrane of macropinosomes and is dependent on viral envelope-receptor interactions (Carter et al., 2011; Marechal et al., 2001). Similarly, Baxter et al. (2014) observed that macrophages can capture HIV-1 infected T cells by phagocytosis in a viral envelope-independent process and irrespective of the virus co-receptor usage, since CD4⁺ T cells infected by either CCR5-using macrophage-tropic viral strain HIV-1_{BaL} or CXCR4-using non-macrophage-tropic viral strain HIV-1_{IIIIB} can be captured by MDMs, but only the macrophage-tropic viral strain HIV-1_{BaL} could lead to productive infection of macrophages, showing that this mode of macrophage infection is dependent on Env-receptor interactions (Baxter et al., 2014). Baxter et al. hypothesize that selective uptake of HIV-1-infected CD4⁺ T cells by MDMs can lead to either phagosomal elimination of infected T cells or viral dissemination from infected T cells to macrophages. This suggests that uptake of infected T cells by macrophages may result in T cell degradation in the phagolysosomal compartment of macrophages (Baxter et al., 2014), whereas some viruses or infected T cells could escape from the phagosomal compartment prior to degradation, resulting in macrophage infection (Baxter et al., 2014). While Baxter et al. (2014) have shown that macrophages can capture and engulf HIV-1-infected CD4⁺T cells, they did not explore the biology of HIV-1 infection of macrophages following engulfment of HIV-1 infected T cells, and the mechanism of escape of HIV-1-infected T cells from the phagosomal compartment within macrophages remains poorly understood (Baxter et al., 2014). Furthermore, none of the canonical inhibitors of phagocytosis of apoptotic/dead cells or the Env-receptor inhibitors could significantly and reproducibly block HIV-1 infected CD4⁺T cell uptake by macrophages, indicating the specific uptake of HIV-1 infected CD4⁺ T cells may require other molecules which specifically recognize and capture infected T cells (Baxter et al., 2014). Since Baxter et al. observed that a small percentage of engulfed CD4⁺ T cells remained intact within MDMs for up to 6 days and MDMs infection following phagocytosis of HIV-infected CD4⁺ T cells is dependent on viral envelope-receptor interactions, we can hypothesize that cell-to-cell fusion may occur at the limiting membrane of phagosomal compartment within macrophages after capture and engulfment of HIV-1 infected CD4⁺ T cells. Thus, macrophage infection through HIV-1 cell-to-cell transfer from infected T cells may involve both phagocytosis of infected T cells and cell-to-cell fusion. Together, we hypothesize that HIV-1-infected T cells can fuse directly with macrophages at the plasma membrane, and could also be captured by macrophages and subsequent cell-to-cell fusion may occurs at the limiting membrane of phagosomal compartment in macrophages, resulting in productive infection of macrophages.

1.33 Phagocytosis and cell-to-cell fusion share several common features

Macrophages can rapidly recognize and uptake apoptotic cells, through recognition of “eat-me” signals on the cell surface of dying cells. Phosphatidylserine (PtdSer) is one of the most potent “eat-me” signal displayed on the apoptotic cell surface and can be recognized by macrophage receptors before subsequent phagocytosis (Elliott and Ravichandran, 2016; Gordon and Pluddemann, 2018; Henson, 2017; Lemke, 2019). Similarly, it has also been shown that macrophages can also display localized areas of PtdSer at the plasma membrane, and the recognition of PtdSer exposure is necessary for efficient fusion between macrophages (Helming and Gordon, 2009). Interestingly, several surface molecules involved in macrophage phagocytosis are also required for macrophage fusion. Although PtdSer exposure on living macrophages is distinct from the apoptosis, PtdSer recognition via the scavenger receptor CD36 is involved in both phagocytosis of apoptotic cells and macrophage fusion (Greenberg et al., 2006; Helming et al., 2009). For both processes, cells must adhere to each other and bring their membranes into close contact (Lemke, 2019; Pereira et al., 2018). The integrin $\alpha\beta3$ is involved in cell-to-cell contacts, and has been shown to be important for macrophage fusion and osteoclast formation (McHugh et al., 2000). Similarly, integrin $\alpha\beta3$ plays important role in recognition of apoptotic cells and subsequent phagocytosis (Akakura et al., 2004; Hanayama et al., 2002). In addition, several factors involved in cytoskeletal rearrangements such as DOCK180 and the small GTPase Rac1 have also been implicated in macrophage fusion and macrophage phagocytosis of apoptotic cells (Albert et al., 2000; Helming and Gordon, 2009; Nakaya et al., 2008). Baxter et al. have found that both engulfment of HIV infected T cells by macrophages and subsequent T-cell-mediated macrophage infection were significantly inhibited in the presence of Jasplakinolide, an actin-specific reagent that inhibits cytoskeletal rearrangements (Baxter et al., 2014; Holzinger, 2009). Similarly, we have observed, using actin-binding drugs, such as Latrunculin A, Cytochalasins D, and Jasplakinolide, that F-actin cytoskeletal rearrangements are required for the first heterotypic cell-fusion between HIV-1-infected T cell and MDMs as well as for subsequent fusion of LMFCs with the surrounding non-infected macrophages (see additional Fig. S1A).

Even if phagocytosis of apoptotic cells by macrophages and cell-to-cell fusion between macrophages share several common features, it is clear that they are functionally and morphologically distinct processes. Helming et al hypothesize that prevention of macrophage phagocytosis may facilitate successful macrophage fusion

(Helming and Gordon, 2009; Helming et al., 2009). It has been shown that the cell surface integrin-associated protein CD47 could work as a so-called "don't eat-me signal", and was able to bind the signal regulatory protein- α (SIRP α) expressed on the cell surface of macrophages to inhibit macrophage phagocytosis (Brown and Frazier, 2001; Lemke, 2019; Oldenburg et al., 2000). However, the interaction between CD47 and SIRP α is necessary for efficient cell-cell fusion during osteoclast multinucleation and formation of MGCs from mononucleated macrophages (Lundberg et al., 2007; Vignery, 2005). CD47/SIRP α interaction inhibits macrophage phagocytosis, but is essential for macrophage fusion (Oldenburg et al., 2001). Thus CD47/SIRP α interaction could differentially regulate the distinct processes of phagocytosis of apoptotic cells and cell fusion.

1.34 Evidence of myeloid cells phagocytosis of infected T cell *in vivo*

More recently, two studies from the group of Jason Brechley have reported that tissue myeloid cells from the spleen of SIV-infected macaques, including tissue macrophages, monocytes, and distinct subsets of dendritic cells, contained viral DNA as well as rearranged TCR DNA, and claimed that infection of these myeloid cells could be attributed to phagocytosis of SIV-infected CD4⁺ T cells, independently of Vpx expression (Calantone et al., 2014; DiNapoli et al., 2017). The viral accessory protein Vpx expressed by certain SIVs and HIV-2 and is able to counteract SAMHD1 restriction in myeloid cells, for efficient cell-free infection of myeloid cells (Laguetta et al., 2011). Interestingly, Vpx expression has no meaningful impact on the frequencies of myeloid cells containing viral DNA *in vivo* in different tissues from macaques infected with SIV either expressing Vpx or lacking Vpx (Calantone et al., 2014), suggesting HIV-1 cell-to-cell transfer could be more relevant *in vivo* and may be responsible for the extensive infection of myeloid cells *in vivo* in SIV-infected macaques. However, in a recent review, Mario Stevenson suggested that cellular DNA of apoptotic cells rather undergoes nuclease digestion prior to phagocytosis by myeloid cells, which could cleave both viral DNA and cellular DNA into small fragments rendering thus difficult for PCR amplifying of intact rearranged TCR DNA or HIV-1 proviral DNA in myeloid cells (Stevenson, 2015). In accordance with the model we proposed in our work, we could also suggest that the presence of viral DNA and rearranged TCR DNA in myeloid cells of SIV-infected macaques may also result from cell-to-cell fusion of HIV-1 infected T cells with myeloid targets.

2-Efficient HIV-1 cell-to-cell transfer from infected T cells to myeloid cells

In the second article (Xie et al., 2019, submitted), we show that HIV-1 uses similar cell-fusion mechanisms for virus transfer from infected T lymphocytes to OCs, immature DCs (iDCs) and macrophages, and subsequent dissemination in these target cells. The establishment of contacts with infected T cells leads to heterotypic cell fusion for the fast and massive transfer of viral material in OC and iDC targets, but not in undifferentiated monocytes and mature DCs (mDCs). This process subsequently triggers homotypic fusion with non-infected neighboring OCs and iDCs for intercellular virus dissemination. Both cell fusion steps are mediated by viral envelope-receptor interactions, and are highly efficient for macrophage-tropic CCR5- and CXCR4-using viruses, dual-tropic R5X4 viruses, and to a lesser extent for non-macrophage-tropic R5 viruses. This two-step cell-fusion mechanism bypasses the SAMHD1-related restriction to HIV-1 replication in these myeloid cells observed with cell-free viruses and may be responsible for virus spreading in these cell targets *in vivo*, as observed in host lymphoid and non-lymphoid tissues, including the central nervous system and bones (Bednar et al., 2015; Gray et al., 2016; Tan and Sattentau, 2013; Watters et al., 2013). In agreement, it was reported that myeloid cells from spleen and lymph nodes of SIV-infected macaques contain T cell markers and viral RNA and DNA originating from infected T cells, irrespective of Vpx expression (Calantone et al., 2014; DiNapoli et al., 2017).

2.1 HIV-1 cell-to-cell transfer escapes SAMHD1 restriction in myeloid cells

Many studies indicate that myeloid cells are poorly infected by cell-free viruses, because virus replication is limited by SAMHD1 at the level of reverse transcription (Hrecka et al., 2011; Laguette et al., 2011; Mlcochova et al., 2018; St Gelais and Wu, 2011). Our findings show that this restriction to virus replication and dissemination in these myeloid cells could be overcome by the two-step cell-fusion process allowing virus spreading in iDCs and macrophages. Interestingly, Puigdomenech et al. previously suggested that HIV-1 cell-to-cell transmission from infected T cells to DCs was restricted by SAMHD1 expression (Puigdomenech et al., 2013). Even if similar experimental system and viral strains were used in both studies, the discrepancy with our findings can be explained by the gating strategy used to analyse virus dissemination

by flow cytometry, since the Gag⁺ iDC-derived MGCs formed through cell-fusion during co-culture with infected T cells were certainly excluded from the analysis performed by Puigdomenech and colleagues (Puigdomenech et al., 2013). In addition, they did not perform fluorescence microscopy analysis to visualize and study MGC formation through cell-fusion of infected T cells with iDC targets. Since we show that the cell-to-cell transfer studied here from infected virus-donor T cells is very efficient for virus dissemination in iDCs, OCs and MDMs (Bracq et al., 2017; Raynaud-Messina et al., 2018), our data also show that this cell-to-cell mechanism of virus spreading overcomes SAMHD1 restriction and supports high levels of virus production by iDC-, OC- and macrophage-derived MGCs. Interestingly, Su and colleagues have also reported that interaction of DCs with CD4⁺ T cells resulted in the significant down-regulation of SAMHD1 expression in DCs (Su et al., 2014), which may renders these cells more permissive to HIV-1 infection (Hertoghs et al., 2015; Martin-Gayo et al., 2015).

While the physiological formation of OCs results from cell-to-cell fusion of monocyte/macrophages (Helming and Gordon, 2009), we show that multinucleated OCs are also able to fuse with infected T cells for virus dissemination in these cell targets. Interestingly, it has been shown that DCs were able to fuse together and to acquire new phenotypical properties upon inflammatory conditions (Laperine et al., 2016) in granulomatous diseases (Yasui et al., 2011) or proliferating disorders such as Langerhans cell histiocytosis (da Costa et al., 2005). In agreement with our results, heterotypic fusion of DCs with HIV-1-infected T cells was already suggested 20 years ago by the group of R.M. Steinman (Frankel et al., 1996; Pope et al., 1994). These studies reported the presence of HIV-1-infected multinucleated syncytia expressing specific markers of both DCs and T cells at the surface of the nasopharyngeal tonsils and adenoid of HIV-1-infected patients (Frankel et al., 1996; Orenstein and Wahl, 1999; Pope et al., 1994), but the mechanisms related to these *in vivo* observations were not further investigated. We demonstrate here that the cell-fusion process for HIV-1 cell-to-cell transfer from T cells to iDCs is likely responsible for the formation of the multinucleated infected DCs observed in infected patients.

In summary, the novel and very efficient two-step cell-fusion mechanism we report for virus cell-to-cell spreading is specific to iDC, OC and macrophage target cells of HIV-1. First, infected T cells fuse with these myeloid cells for massive viral transfer, followed by a second step of homotypic fusion with uninfected myeloid cells for virus dissemination. Both steps are dependent on the viral envelope and receptor interactions and seem restricted to macrophage-tropic viral strains. While HIV-1 infection of these myeloid cells by cell-free viruses is known to be strongly restricted (Laguette et al., 2011; St Gelais and Wu, 2011), this new mechanism of virus spreading in myeloid cells by cell-fusion bypasses the restriction imposed by SAMHD1 and leads to the formation

of highly virus-productive MGCs reminiscent of the infected MGCs originating from myeloid cells observed *in vivo* in infected patients and SIV-infected macaques. These *in vivo* observations support the importance of the mechanisms revealed here, which contribute to a better understanding of virus cell-to-cell dissemination in myeloid cells and their roles in HIV-1 pathogenesis, including cell associated virus sexual transmission, virus spreading, and establishment of long-lived viral tissue reservoirs.

3-The role of co-receptor usage and cell tropism for HIV-1 cell-to-cell spreading in myeloid cells

HIV-1 entry phenotype, namely the co-receptor usage and cell tropism for HIV-1, was defined by *in vitro* cell-free infection of transformed cell lines as well as primary cells expressing the CD4 receptor and a co-receptor, either CCR5 or CXCR4. This definition by cell-free infection has revealed three different types of HIV-1 variants: (1) X4 T cell-tropic viruses, (2) R5 non-macrophage-tropic viruses, and (3) M-tropic viruses.

3.1 Myeloid differentiation and susceptibility to HIV induced cell-to-cell fusion is associated with viral entry receptors expression

Myeloid cells, including monocytes, macrophages, dendritic cells and osteoclasts, are relatively refractory to HIV-1 cell-free infection due to low levels of expression of viral entry receptors and high levels of expression of host cell restriction factors, such as SAMHD1 and APOBEC3 proteins (Berger et al., 2011b; Laguette et al., 2011; Lee et al., 1999; Peng et al., 2007). Our data show that interactions between the viral envelope and the CD4 receptor and CCR5 co-receptor are required for the cell-fusion processes leading to formation of HIV-1-infected MGCs originating from either macrophages, OCs or iDCs (Bracq et al., 2017; Xie et al., 2019).

At the level of viral entry, infection of myeloid cells by cell-free virus particles is limited by low levels of expression of viral entry receptors, including CD4 receptor, and co-receptor CCR5 and CXCR4. Indeed, monocytes express extremely low level of CD4 receptor and CCR5 co-receptor (Lee et al., 1999), which may render monocytes refractory to HIV-1-induced cell-to-cell fusion. However, the expression of CD4 and CCR5 increases upon monocyte differentiation into macrophages or iDCs (Lee et al.,

1999; Naif et al., 1998; Tuttle et al., 1998), and our study clearly shows cell-to-cell fusion between infected T cells and iDCs or macrophages occurs rapidly and results in highly efficient infection of these myeloid target cells. In vitro, macrophages could further differentiate into osteoclasts (OCs) in the presence of M-CSF and RANKL (Helming and Gordon, 2009). While OCs have been shown to express CD4 and CCR5 and CXCR4 co-receptors at a level similar to that expressed in macrophages (Gohda et al., 2015). Similarly, we have observed that HIV-1-infected CD4⁺ T cells could fuse rapidly with OCs, resulting in the fast and massive transfer of viral material into OC targets, which subsequently triggers homotypic fusion with non-infected neighboring OCs for virus dissemination. In contrast, expression of CCR5 is significantly down-regulated upon DC maturation (Cavrois et al., 2006), and we did not detect cell-fusion for cell-to-cell transfer of HIV-1 from infected T cells to mDCs. Thus, these results suggest that cell-fusion between T cells and iDCs, or macrophages is finely regulated by CD4 and co-receptor cell surface expression. Previous studies using cell-free viruses proposed that the lower sensitivity of monocytes and mDCs to HIV-1 was related to the low expression of the CCR5 co-receptor at the cell surface of these cell types (Cavrois et al., 2006; Tuttle et al., 1998), and monocytes and mDCs indeed express low level of CCR5 compared to iDCs and differentiated macrophages (data not shown). These results suggested that a threshold of CCR5 level could be a major rate-limiting step for HIV-1-induced cell-to-cell fusion of macrophages with infected T cells.

3.2 R5 non-macrophage-tropic viruses can be efficiently transferred from infected T cells to macrophages through cell-to-cell fusion

Since HIV-1 cell-to-cell transfer is more efficient than cell-free infection and likely represents the dominant mode of infection *in vivo* for virus dissemination, we compared entry phenotypes of primary viral isolates, including transmitted/founder (T/F) viruses, for virus dissemination in macrophages and dendritic cells (DCs) by cell-free and cell-to-cell infection in collaboration with the group of Bernard Lagane. They have recently characterized the entry phenotype of these primary strains on macrophages using cell-free virus particles and have shown that envelope glycoproteins from different HIV-1 strains exhibit distinct affinity for the CCR5 co-receptor (Colin et al., 2018).

Our additional unpublished data revealed that by both R5 macrophage-tropic and R5X4 dual-tropic viruses are very efficiently transferred and then disseminate in

macrophages as well as in iDCs by cell-to-cell fusion using either CCR5 or both CCR5 and CXCR4. In contrast, X4 T-tropic viruses fail to disseminate in macrophages and iDCs. Interestingly, CCR5-using virus isolates, including some T/F viruses initially characterized as non-macrophage-tropic, exhibit enhanced macrophage tropism in the context of viral cell-to-cell transfer and are also efficiently transferred and disseminated in macrophages and DCs leading to the formation of productively-infected MGCs (see additional Fig. S11B). Similarly, Baxter and colleagues also reported that macrophages were productively infected by cell-to-cell transfer through engulfment of CD4⁺ T cells infected with T/F viruses (Baxter et al., 2014). Baxter et al hypothesize that the enhanced capacity of T/F viruses to infect macrophages by cell-to-cell transfer from HIV-infected T cells could be explained by the significantly increased multiplicity of infection (MOI) conferred by this mode of viral spread. Indeed, macrophage infection by cell-to-cell transfer from this route could be recapitulated using high cell-free MOI infection by some T/F viruses such as REJO. However, increasing the cell-free MOI could not overcome entry restrictions to other T/F viruses, including CH040 and WITO (Baxter et al., 2014).

3.3 CXCR4 is a functional co-receptor for cell-to-cell infection of macrophages by R5X4 M-tropic virus and X4 M-tropic virus

Most studies have considered macrophage tropism as CCR5-tropic, since most M-tropic viruses use CCR5 efficiently for cell-free virus entry (Joseph and Swanstrom, 2018; Sattentau and Stevenson, 2016). Interestingly, we found that the X4 M-tropic viral strain (X4-6) and the R5X4 dual-tropic strain (89.6) could also be efficiently transferred and then disseminate in macrophages as well as in immature DCs by cell-to-cell fusion using either CXCR4 or both CCR5 and CXCR4, as evidenced by inhibition by specific CCR5 and CXCR4 antagonists (see additional Fig. S4D). In fact, early studies have reported that primary macrophages express CXCR4, as well as CCR5, and support cell-free entry of X4 viruses and R5X4 dual viruses (Simmons et al., 1998; Yi et al., 1999; Yi et al., 1998). Therefore, our findings indicate that the relationship between co-receptor usage and cellular tropism should be revisited in the context of HIV-1 cell-to-cell transfer to reflect the capacity of both CCR5- and CXCR4-using virus strains to spread and replicate efficiently in macrophages and DCs through cell-fusion processes.

3.4 Implication of V3 loop in virus entry and HIV-1 mediated cell-to-cell fusion

	[-----V3 loop-----]	Co-receptor	Cell free Tropism	Cell-to-cell Tropism
R5-25	CTRPNNNTRKGIHI--GPGRAFYTTEIIGDIRQAHCN	CCR5	Non M-tropic	M-tropic(+/-)
R5-34	CTRPNNNTRRSIHM--GPGRALYTTGAIIGNIRQAHCN	CCR5	M-tropic	M-tropic
JRCSF	CTRPSNNTRKSIHI--GPGRAFYTTEIIGDIRQAHCN	CCR5	Non M-tropic	M-tropic(+/-)
JRFL	CTRPNNNTRKSIHI--GPGRAFYTTEIIGDIRQAHCN	CCR5	M-tropic	M-tropic
R5-13	CTRPNNNTRKSIINI--GPGRALYTTGAIIGNIRQAHCN	CCR5	M-tropic	M-tropic
R5-22	CTRPNNNTRKSIHI--GPGRAFYTTEIIVGNIRQAHCN	CCR5	Non M-tropic	M-tropic
NL4.3	CTRPNNNTRKSIIRIQRGPGRAFVTIGKI--GNMRQAHCN	CXCR4	T-tropic	T-tropic
B9.6	CTRPNNNTRRRLSI--GPGRAFYARRNIIGDIRQAHCN	CCR5, CXCR4	Dual tropic	Dual tropic
X4-6	CTRPNNNVKRIISL--SPGRVYTTGIRGDIRKAYCN	CXCR4	M-tropic	M-tropic

	[-----V3 loop-----]	Co-receptor	Cell free Tropism	Cell-to-cell Tropism
CH058	CTRPNNNTRKSIITI--GPGRAFYATGDIIGDIRQAHCN	CCR5	Non M-tropic	Non M-tropic
THRO	CTRPNNNTSKSIHM--GPGGAFFATGRIIGDIRKAYCT	CCR5	Non M-tropic	M-tropic
REJO	CTRPNNNTRKSIHI--APGRAFYATGEIIGDIRKAYCT	CCR5	Non M-tropic	M-tropic
RHPA	CTRHNNNTRKSIINI--GPGRAFYATGKIIGDIRQAHCN	CCR5	Non M-tropic	Non M-tropic
NL4.3	CTRPNNNTRKSIIRIQRGPGRAFVTIGKI--GNMRQAHCN	CXCR4	T-tropic	T-tropic

Figure 16: Co-receptor usage and viral tropism in cell-free and cell-to-cell infection

Cell-free HIV infection of macrophages is inefficient, in part due to the low cell surface expression of viral entry receptors. While HIV-1 cell-to-cell transfer could overcome entry restrictions in myeloid cells in a viral envelope-dependent manner.

The role of V3 loop in co-receptor usage and viral tropism has been largely documented in the context of cell free infection (see section 3.5). Early studies have shown that the V3 loop of viral envelope is the primary determinant of M-tropism vs. T-tropism as well as co-receptor usage (Carrillo and Ratner, 1996; Chesebro et al., 1992; Hung et al., 1999b; O'Brien et al., 1990). For example, CXCR4-using T-tropic strains replicates poorly in primary macrophages, whereas the substitution of 20 amino acid residues from the V3 region of CCR5-using M-tropic strains confer a switch of T tropism to macrophage tropism (Hwang et al., 1991). However, the implication of V3 loop in HIV-1 mediated cell-to-cell fusion remains poorly investigated. Interestingly, earlier study has shown that a V3 loop mutation significantly impaired the efficiency of cell-free infection of macrophages by HIV-1, while the same mutation has no effect on cell-to-cell fusion between macrophage and HIV-1 chronically infected T cells (Simmons et al., 1995). Thus, it would be interesting to investigate the role of V3 loop in the cell-to-cell fusion process we described for HIV-1 transfer and dissemination in myeloid cells.

Interestingly, the amino acid alignments of the V3 loop of different viral isolates indicates that the NL4-3 prototype strain harbours a unique QR insertion in the V3 tip as well as a missing isoleucine (I) at position 26 of the V3 loop and has been shown to

be important for the X4 phenotype of NL4-3 prototype strain (Bagnarelli et al., 2003). It has also been shown that the deletion of QR residues in the V3 loop of NL4-3 prototype strain is required for interaction with co-receptor CCR5 (Hung et al., 1999b; Xiang et al., 2013). This notorious and distinctive QR motif insertion in the V3 loop of HIV envelope were used as a flag to identify LAI or IIIB derived contaminations (Chang et al., 1993; Wain-Hobson et al., 1991), while the laboratory-adapted HIV strain NL4-3 was derived from the first two HIV-1 isolate Lai/LAV (HIV-1 Bru, isolated at the Pasteur Institute) and Lai/IIIB (HTLV-IIIB). Robert C. Gallo hypothesized that the first HIV-1 isolate with a very high capacity for growth could readily outcompete other HIV-1 isolates in culture, which has contaminated the cell cultures in several laboratories (Gallo and Montagnier, 2003). However, T-cell line adapted (TCLA) strains adapt to grow in transformed T cell lines and their biological properties have changed, for example, they lose the ability to grow on primary macrophages compared with primary HIV strain. TCLA HIV strains do not fully recapitulate the biological properties of primary isolates. Unfortunately, the majority of studies on HIV research has been carried on using T-cell line adapted (TCLA) strain NL4-3, which may generate conflicting results at odds with the earlier observations with primary isolates.

Simmons et al have reported that macrophages are more sensitive to cell-to-cell fusion than virus-to-cell membrane fusion induced by the viral Env (Simmons et al., 1995). Although both virion-to-cell membrane fusion (cell-free infection) and cell-to-cell fusion of HIV-1 are dependent on the Env-receptor interactions. The physical requirement for cell-to-cell fusion might differ from virus-to-cell membrane fusion (cell-free infection). Cell-free virus particle is a well organised structure, with the viral envelope glycoprotein (Env) spikes the exterior of virus particles (Sundquist and Krausslich, 2012). In contrast, newly synthesised viral envelope could diffuse freely on the cell surface of infected cells and may concentrated at the internal face of cell membrane (Chen, 2012; Sundquist and Krausslich, 2012). Thus, the structural distinctions between the infected cell and virion membrane may impact macrophage infection.

CONCLUSIONS

In this PhD work presented here, we have characterized novel mechanisms of cell-to-cell transfer of HIV-1 from infected CD4+ T cells to myeloid cells. We propose a model on cell-to-cell fusion for HIV-1 spreading in myeloid target cells. HIV infected T cells are able to fuse with either macrophage or osteoclast, and even dendritic cells, leading to formation of multinucleated giant cells, which were evidenced *in vivo* in different tissue of HIV-1 infected patients. However, monocytes and CD4+ T cells are refractory to HIV-1 induced cell-to-cell fusion.

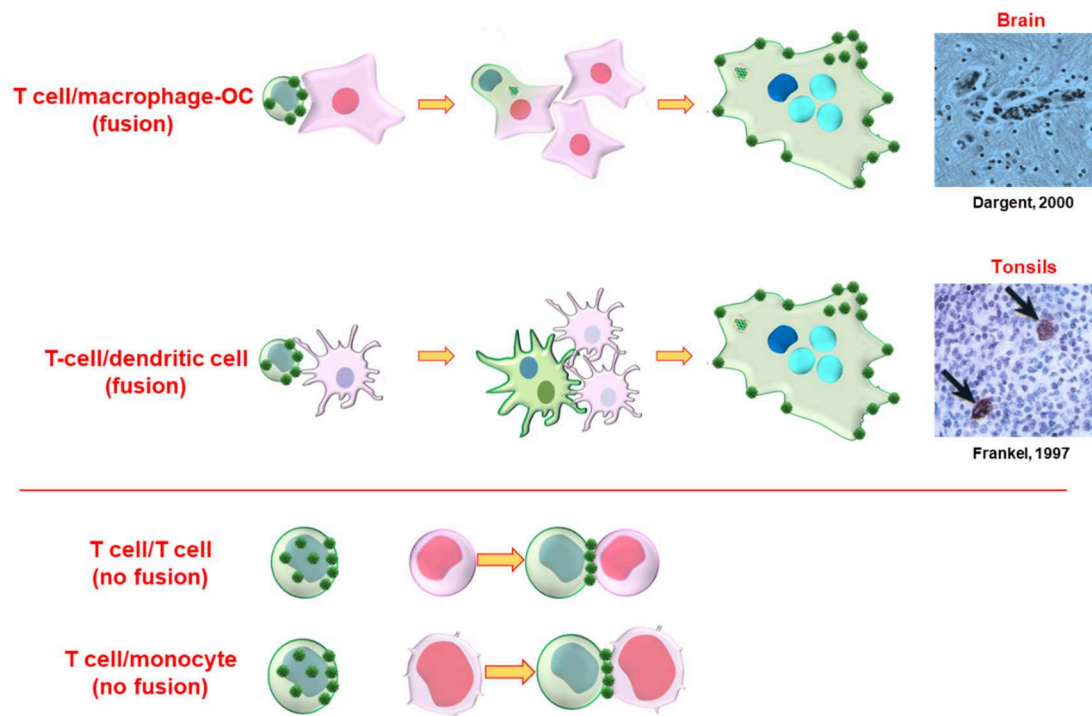


Figure 17: Cell-to-cell fusion for HIV-1 spreading in myeloid target cells

HIV-1 uses a specific and common cell-to-cell fusion mechanism for virus transfer and dissemination from infected T lymphocytes to the target cells of the myeloid lineage, including immature DCs (iDCs), OCs and macrophages, but not monocytes and mature DCs. This two-step cell-fusion process results in the formation of highly virus-productive multinucleated giant cells observed *in vivo* in lymphoid and non-lymphoid tissues of HIV-1-infected patients.

In the first article, we revealed a very efficient mechanism involved in cell-to-cell transfer from infected T cells to macrophages and subsequent virus spreading between macrophages by a two-step cell fusion process. Infected T cells first establish contacts and fuse with macrophage targets. The newly formed lymphocyte-macrophage fused cells then acquire the ability to fuse with surrounding uninfected macrophages, leading to the formation of infected multinucleated giant cells that can survive for a long time and can produce large amount of infectious viral particles, as evidenced *in vivo* in lymphoid organs and the central nervous system.

In the second article, we demonstrate that HIV-1 uses a common and specific cell-to-cell fusion mechanism for massive virus transfer from infected T lymphocytes and dissemination in myeloid cell targets, including immature dendritic cells, macrophages, as well as osteoclasts, but not monocytes and mature dendritic cells. We show that the establishment of contacts with infected T cells leads to heterotypic cell-fusion for the fast and massive transfer of viral material in myeloid cells, which subsequently triggers homotypic fusion with noninfected neighboring osteoclasts and dendritic cells for virus dissemination. Both cell fusion steps are mediated by viral envelope-receptor interactions, and are highly efficient for macrophage-tropic CCR5- and CXCR4-using viruses, dual-tropic R5X4 viruses, and to a lesser extent for non-macrophage-tropic R5 viruses, including transmitted/founder viruses. Interestingly, this cell-to-cell infection process by cell-fusion bypasses the restriction imposed by the SAMHD1 host cell restriction factor for replication of HIV-1 when these myeloid cells are infected by cell-free viruses.

Furthermore, this cell-to-cell infection process by cell-fusion also overcome the restriction of limiting CD4 and CCR5 for efficient macrophage infection by R5 non-M-tropic viruses, including transmitted/founder viruses. This two-step cell-fusion process indeed results in the formation of highly virus-productive multinucleated giant cells. Together, these results describe a new and very efficient mechanism for cell-to-cell virus spreading and high productive infection of the HIV-1 myeloid cell targets. These important results reveal the cellular mechanisms involved in the formation of the infected multinucleated giant cells observed *in vivo* in lymphoid and non-lymphoid tissues of HIV-1-infected patients. Since myeloid cells are now emerging as important target cells of HIV-1, these results thus contribute to a better understanding of the role of these myeloid cells in pathogenesis, including cell-associated virus sexual transmission, cell-to-cell virus spreading, and establishment of long-lived viral tissue reservoirs.

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APPENDIX



Mechanisms for Cell-to-Cell Transmission of HIV-1

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While HIV-1 infection of target cells with cell-free viral particles has been largely documented, intercellular transmission through direct cell-to-cell contact may be a predominant mode of propagation in host. To spread, HIV-1 infects cells of the immune system and takes advantage of their specific particularities and functions. Subversion of intercellular communication allows to improve HIV-1 replication through a multiplicity of intercellular structures and membrane protrusions, like tunneling nanotubes, filopodia, or lamellipodia-like structures involved in the formation of the virological synapse. Other features of immune cells, like the immunological synapse or the phagocytosis of infected cells are hijacked by HIV-1 and used as gateways to infect target cells. Finally, HIV-1 reuses its fusogenic capacity to provoke fusion between infected donor cells and target cells, and to form infected syncytia with high capacity of viral production and improved capacities of motility or survival. All these modes of cell-to-cell transfer are now considered as viral mechanisms to escape immune system and antiretroviral therapies, and could be involved in the establishment of persistent virus reservoirs in different host tissues.

Keywords: HIV-1, cell-to-cell transfer, macrophages, dendritic cells, T cells

CELL-FREE AND CELL-TO-CELL INFECTION

The worldwide pandemic of HIV-1 infection is a global and major public health problem, and sexual transmission is the major route of infection of the newly HIV-1-infected adults. Even though the use of antiretroviral therapies led to a net decrease of morbidity and mortality of HIV-1-infected patients, a better understanding of the cellular mechanisms involved in the sexual transmission and early dissemination of the virus is still required in order to rationally design more specific and potent strategies for HIV-1 prevention. A research priority for HIV-1 eradication is then the elucidation of the events involved in the mucosal viral transmission through the mucosa of the male and female rectal and genital tracts. While both cell-free and cell-associated HIV-1 are present in the semen, vaginal secretions, and anal mucus, and are thought to contribute to the virus sexual transmission, the cell-associated HIV-1 mucosal transmission has been largely understudied. Cell-to-cell transmission of HIV-1 involves various cell types of the immune system, including T lymphocytes, macrophages, and dendritic cells (DCs). Whereas the infection of T lymphocytes *via* cell-to-cell transfer was broadly investigated *in vitro*, there is a paucity in knowledge of the mechanisms that control infection and virus dissemination in macrophages and DCs by cell-to-cell transfer. Yet, macrophages and DCs play crucial roles in the physiopathology of infection, with macrophages being involved in the establishment of persistent virus reservoirs in different host tissues

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while DCs participate in the early stages of virus transmission and dissemination after primo-infection at the level of genital and rectal mucosa. Specific therapies blocking HIV-1 mucosal transmission to these target cells should therefore be designed.

While HIV-1 infection of target cells with cell-free viral particles has been largely documented, pioneer studies from early 1990s showed that HIV-1 dissemination was largely increased through the establishment of direct cell-to-cell contacts between infected donor CD4+ T cells and target T cells [for review: Ref. (1)]. At least *in vitro*, cell-to-cell transfer of HIV-1 between T cells leads to a massive and very efficient infection that may be 100–1,000-fold more efficient than infection carried out by cell-free viral particles (1–5). The efficiency of cell-to-cell infection between CD4+ T cells has been related to a high-multiplicity of infection at the site of the cell–cell contact, probably leading to the integration, and accelerated viral gene expression of multiple proviruses in the target cell (6–10). While some reports suggest that cell-to-cell infection could be the main route of HIV-1 infection *in vivo* (10, 11), the specific contribution of cell-free and cell-to-cell infection by HIV-1 in infected hosts is still a matter of debate. Using multiphoton intravital microscopy in HIV-1-infected “humanized” mice, Murooka et al. showed that HIV-1-infected T cells establish interaction with surrounding cells and can even form syncytia with other lymph node-resident cells. The potency of infected T cells in lymph nodes to migrate may facilitate virus cell-to-cell transmission and spreading *in vivo* (12). Interestingly, exposure of human or macaque mucosal explants to HIV-1- or SIV-infected cells, allows more efficient viral transmission and infection than cell-free viruses (13, 14), suggesting the potency of HIV-1- or SIV-infected T cells to transmit viruses and propagate infection in host tissues. The high efficiency of cell-to-cell infection was also proposed to be a mechanism for HIV-1 to escape to antiretroviral therapy and neutralizing antibodies (15) but these results are still controversial and will be discussed below (4, 6, 16).

Different modes of infection through different cellular structures enabling close contacts between virus-donor cells and recipient target cells have been described over the past years for cell-to-cell transmission of HIV-1 *in vitro*. Intercellular transfer of viral material has been described mainly through establishment of the infectious or virological synapses, but also using membrane protrusions such as filopodia and tunneling nanotubes (TNTs), and cell fusion or cell engulfment processes. The implication of these different structures and processes for cell-to-cell transfer and dissemination of HIV will be discussed in this review.

NANOTUBES, FILOPODIA

The role of membrane protrusions in intercellular communications has been widely explored [for review: Ref. (17)]. A large diversity of membrane protrusions have been described, both *in vitro* (18, 19) and *in vivo* (20–22), and play important roles in the transmission of information between cells from different physiological systems, such as neurons (18, 23, 24), myeloid cells (25–29), or T cells (30).

Among the described membrane protrusions, two different types of nanotubes have been reported, corresponding to

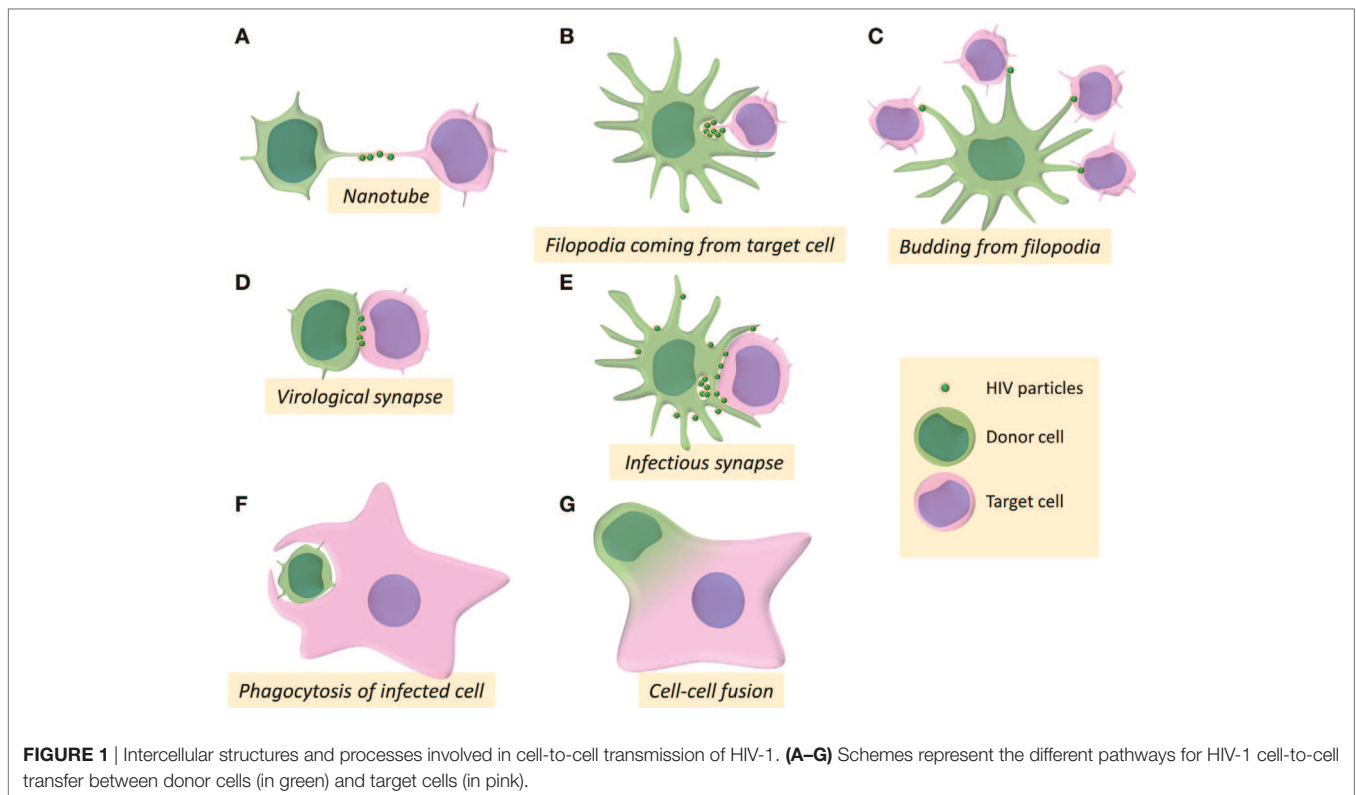
close-ended nanotubes and open-ended nanotubes (also known as TNTs) (27, 31, 32). Intercellular communications involving TNTs were first observed in 2004 as F-actin-containing membrane extensions able to connect distant cells during minutes to hours (18). TNTs are fragile and dynamic structures extended up to 100 μm in length with diameters ranging from 50 to 200 nm, and are not attached to the substratum (18, 30). They can mediate and facilitate the transfer, between several cell types, of cytoplasmic, and plasma membrane molecules, Ca^{2+} (29, 33), cargos including vesicles derived from various organelles such as early endosomes, endoplasmic reticulum, Golgi complex, and lysosomes (24, 33, 34), and even bigger cellular organelles like mitochondria and endosome-related structures (18, 32), but also pathogens such as bacteria (28).

Several studies showed that HIV-1 utilizes TNT networks to move from one cell to another leading to virus cell-to-cell transfer (25, 30, 34, 35) (**Figure 1A**). The frequency of TNT formation is not affected by HIV-1 in T cells but these structures could allow rapid spread of virus between T cells (30). Virus particles can thus be transferred by surfing along the surface of TNTs between T cells (30). Virus dissemination through TNTs was also reported between macrophages, in which HIV-1 particles can be transferred through intracellular vesicles derived from the endosomal reticulum or the Golgi apparatus (34, 35). Furthermore, in macrophages, HIV-1 increases the number of these intercellular structures to infect new cells (25). The HIV-1 Nef auxiliary protein has been reported to be responsible for the formation of TNTs in the THP-1 macrophage-like cell line (36) as well as in primary monocyte-derived macrophages, in which Nef alters the localization of the scaffolding protein M-Sec (37), which is a key regulator of TNT formation by a still undefined mechanism (26).

Another route of viral cell-to-cell transmission through membrane extension involving formation of filopodia has been first described for transmission of the retroviral murine leukemia virus (MLV) (19). Filopodia are F-actin-rich thin plasma membrane extensions that are involved in several cellular functions, such as chemo-migration, adhesion to the extracellular matrix, or formation of cell–cell contacts [for review: Ref. (38)]. In DCs, after engagement of the lectin DC-SIGN, HIV-1 mediates the activation of the small GTPase CDC42 and the remodeling of actin cytoskeleton to promote filopodia extension that allows virus transmission to neighboring CD4+ T cells (39) (**Figure 1B**). By budding at the tip of filopodia in DCs, HIV-1 could be able to tether concomitantly several neighboring CD4+ T cells, leading to viral transfer and infection of the target T cells (40) (**Figure 1C**).

THE VIROLOGICAL SYNAPSES

The formation of the so-called virological synapse is the major and well-established route for viral cell-to-cell transmission, and was first described in the context of human T-lympho-tropic virus infection as a close and organized cell-to-cell contact structure between an infected donor cell and a target cell, enabling the transfer of viral material between the two cells (41) (**Figure 1D**). The virological synapse has been named after its homologies with



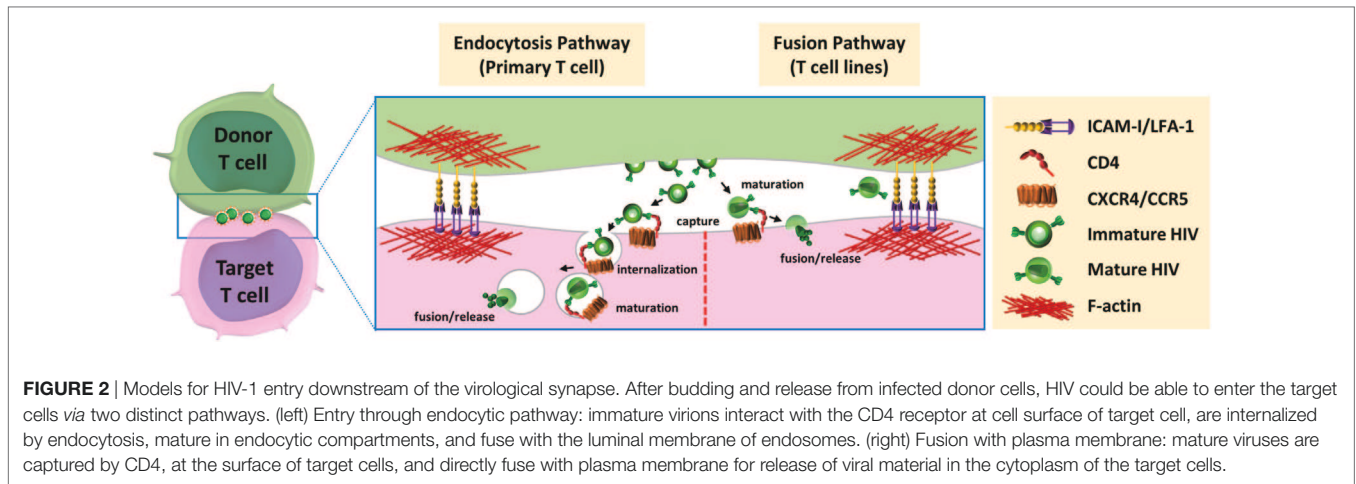
the immunological synapse formed between antigen-presenting cells (APCs) and T cells for antigen presentation. During the formation of the immunological synapse, binding of the T-cell receptor (TCR) to the MHC-peptide complex expressed at the surface of APCs ensures T-cell activation by transducing signals that cause transcriptional upregulation of numerous genes, polarized secretion of cytokines or cytotoxic granules, and cell-proliferation (42, 43). Microtubules and actin cytoskeleton, together with adhesion molecules (LFA-1 and its ligand ICAM-1), participate in the stabilization of the immunological synapse.

Whilst virus cell-to-cell transmission was defined, from the early 1990s, to be more efficient than cell-free virus infection (1, 44–46), the concept of “virological synapse” between HIV-1-infected donor cells and target cells was defined by the group of Quentin Sattentau as a “cytoskeleton-dependent, stable adhesive junction, across which virus is transmitted by directed transfer” (47). The virological synapse shares several common features with the immunological synapse. Indeed, formation of both virological and immunological synapses involves the recruitment of receptors and cell adhesion molecules to an adhesive interface in an actin-dependent manner (48, 49). We focus here on the structure of the virological synapse established between a donor infected CD4+ T-cell and a CD4+ T-cell target which has been the best documented (48, 50, 51). The specific synapses formed between DCs or macrophages and target CD4+ T cells will be described below.

The virological synapse is a dynamic structure initiated by the recognition of the target T-cell surface receptor CD4 by the viral surface envelope glycoprotein gp120 expressed at the

surface of the infected donor T cell (**Figure 2**). This interaction allows the recruitment of the viral Gag polyprotein precursor to the intercellular interface (52) and triggers the recruitment of co-receptors, CXCR4 or CCR5, adhesion molecules LFA-1, ICAM-1, and other cell surface proteins such as tetraspanins to the site of intercellular contact, for stabilization of the virological synapse and efficient viral transfer (48, 52, 53). The interaction between HIV-1 envelope glycoprotein and the receptor CD4 is the major determinant for virus transfer across the virological synapse (54). In addition to CD4, the implication of the co-receptors CCR5 and CXCR4 has been investigated by several groups. Some initial studies suggested that the formation of the virological synapse could be independent of the co-receptor usage since the inhibition of these co-receptors did not affect the number of cellular conjugates between cells (55) or HIV-1 transfer (4, 56). However, other reports showed that expression of these co-receptors was necessary for productive infection of the target cells downstream of the formation of the virological synapse (57–60). Therefore, the use of the co-receptor could be dispensable for the formation of the virological synapse but could be required for efficient infection of the target cell after viral transfer through the virological synapse.

Similarly, the role of the adhesion molecules LFA-1 and ICAM-1 in the formation of the virological synapse was debated. Initially, interaction between LFA-1 and ICAM-1 has been proposed to stabilize the virological synapse for efficient viral transfer. Jolly et al. first showed that antibodies against LFA-1 and ICAM-1 were able to partially block viral transfer through the virological synapse (48, 52). However, the percentages of inhibition were



very different depending on the antibody used (40–90% inhibition using LFA-1 antibodies and 30% inhibition with ICAM-1 antibodies). The role of adhesion molecules for the formation of the virological synapse and virus transfer was then confirmed, showing a significant threefold inhibition in virus transfer using a Jurkat T-cell line lacking the α subunit of LFA-1 (61). However, a third group obtained opposite results showing that virus transfer through the virological synapse between T cells did not require LFA-1 binding to ICAM-1 (55). Using antibodies blocking adhesion molecules such as LFA-1, ICAM-1, and ICAM-3 in MOLT or primary T cells, or 293 T cells lacking LFA-1, no inhibition of viral transfer through the virological synapse was observed in this study. The different assays used to analyze viral transfer and viral production could explain this divergence. In these studies, antibodies targeting different epitopes were used to block adhesion molecules and could have differential effects on virological synapse formation and HIV-1 transfer. The discrepancy in the results obtained by several groups could also be explained by the different cell models used in these experiments. Indeed, the level of adhesion molecule expression (i.e., ICAMs and Integrins) largely differs from T-cell lines to primary T cells (52, 55). To conclude, while the recruitment of adhesion molecules to the virological synapse is well established (52, 53), their specific role and requirement for cell-to-cell transmission of HIV-1 remains to be determined.

Finally, specific rearrangements of the cytoskeleton are required for the formation of the virological synapse and efficient viral transfer. Engagement of the CD4 receptor by the gp120 viral envelope glycoprotein triggers actin cytoskeleton remodeling and microtubule polarization toward the virological synapse (51). These cytoskeleton modifications are needed for polarization of the Gag precursor and envelope glycoproteins to the site of cell–cell contact (61, 62). By comparison, engagement of the TCR by MHC-antigen complex expressed at the surface of APC induces a polarization of the T cell toward the immunological synapse that triggers a cascade of intracellular signals leading to cytoskeleton remodeling, cytokine gene expression, proliferation, and execution of the T-cell effector functions. Similarly, polarization of the microtubule-organizing center (MTOC) of infected

donor T cells toward the virological synapse has been observed in 30–60% of the conjugated formed with target T cells (54, 63, 64). Viral envelope glycoproteins are also reoriented to the virological synapse through their interaction with an intracellular compartment associated with the polarized MTOC. This suggests an active role of the microtubule network in the recruitment of the viral envelope to the virological synapse (4, 53, 54, 59, 63). Electron microscopy analyses with tomography reconstruction revealed the polarization of organelles such as mitochondria, lysosomes at the site of cell–cell contact in around 75% of the virological synapse formed. The colocalization of viral material with secretory lysosomal compartments and the relocation of these vesicular compartments toward the cell–cell interface indicates that HIV-1 could take advantage of intracellular traffic and secretory pathways to disseminate through the virological synapse (63). Some molecules involved in T-cell signaling also participate in cell-to-cell transmission of HIV-1. Accordingly, polarization of the cells can be mediated by ICAM-1/LFA-1 signaling, which in addition to its effect on stabilizing virological synapse formation, induces a ZAP70-dependant signaling pathway for cytoskeleton remodeling, T cells polarization, and efficient HIV-1 transfer at the virological synapse (53, 64). In target T cells, CD4 engagement by viral gp120 induces phosphorylation of the T-cell specific Src kinase Lck allowing the recruitment of several TCR signaling molecules such as ZAP70, LAT, SLP76, or PLC γ in their active phosphorylated form. This unusual signaling does not trigger regular T-cell activation nor proliferation, but could induce a local depletion of F-actin at the center of the virological synapse that could facilitate viral transfer to target cells (54).

The first observation of the virological synapse by electron microscopy by Jolly et al. showed that mature HIV-1 particles were clustered in the synaptic space (48), but they did not show evidence of endocytosis of viral particles by target cells, suggesting that mature viruses released from the donor T cells at the virological synapse space could fuse directly with the plasma membrane of the target T cells (48). Another group also observed HIV-1 particles in the synaptic space by electron microscopy, but suggested that these particles could be internalized by target

cells into trypsin-resistant large intracellular vesicles containing several virus particles. This internalization of viruses could occur using an actin-dependent mechanism through lamellipodia-like structures (56). Several groups confirmed that HIV-1 can be internalized by endocytosis at the virological synapse. Therefore, immature HIV-1 particles have been shown to be transferred across the virological synapse through dynamin- and clathrin-dependent endocytosis leading to productive infection of the target T cells (59, 65–67). After internalization, the HIV-1 particles were found in intracellular compartments that colocalized with the early-endosomal marker EEA1 but not with the lysosomal-associated membrane protein LAMP1, indicating that viruses are internalized in endosomal compartments but are not addressed to lysosomal degradation (68). In accordance with these results, Dale et al. demonstrated that after endocytosis of immature virions by the target T cells, the cleavage of the Gag polyprotein precursor by the viral protease induced the maturation of the viral particle in some endosomal compartments (57). This cleavage restored the membrane fusion activity of the viral envelope and led to viral-cell membrane fusion into these endosomal compartments.

However, the group of Quentin Sattentau, who first described the formation of the virological synapse for HIV-1 cell-to-cell transfer, failed to observe endocytosis of viral particles at the virological synapse using confocal microscopy, electron microscopy, or electron microscopy coupled with tomography (47, 60). Puigdomenech et al. hypothesized that these different results could be explained by the different experimental systems used and the use of primary T cells or immortalized T-cell lines (69). They suggest that these differences may be associated with the different kinetics of the fusion events observed between primary CD4⁺ T cells and T-cell lines. Delayed fusion at the plasma membrane of the target cell may increase virus endocytosis in primary cells (**Figure 2**, left). In contrast, a rapid fusion at the cell membrane in T-cell lines may favor HIV-1 transmission without need for endocytosis (**Figure 2**, right). From this hypothesis, these authors suggest that endocytosis of virus particles is the main mechanism used by HIV-1 for cell-to-cell transfer and infection of primary CD4⁺ T cells. Remarkable differences between virological synapses formed with Jurkat cells or primary CD4⁺ T cells have been indeed reported (70). Scanning electron microscopy experiments revealed strong differences in spatial distribution of virions at the intercellular interface and important differences in the architecture of the contacts between T cells. These differences could be due to the increase deformability of primary T cells compared with the immortalized Jurkat cells, and questioned the relevance of cell line models used so far for studying virological synapse formation and cell-to-cell transmission of HIV-1.

It is noteworthy to mention that cell-to-cell transfer of HIV-1 between T cells is associated to an increase mortality of the target cells, when compared with cell-free infection of T cells. First, the massive entry of viral particles into target cells across the virological synapse has been shown to be responsible for caspase-1-mediated pyroptosis of target T cells (71). In addition, while the formation of the virological synapse is triggered by gp120/

CD4 interaction, it has been reported that upon cell-to-cell contact, the gp41 transmembrane viral glycoprotein was able to trigger hemifusion but not fusion events responsible for rapid CD4⁺ T-cell death (72, 73).

Viral Transfer and Resistance to Neutralizing Antibodies and Antiretroviral Drugs

Like some other viruses (Herpesviruses, poxviruses, and Hepatitis C virus), it has been proposed that HIV-1 could escape, at least partially, to neutralization by specific antibodies targeting the viral envelope when it is transferred across the virological synapse. Failure of inhibition of HIV-1 cell-to-cell infection by neutralizing antibodies was first suggested in 1995 in a study showing that antibodies against the glycan V3-loop of gp120 were unable to block cell-to-cell viral transfer (74). However, after characterization of the virological synapse for HIV-1 cell-to-cell transfer in 2004, several different groups tried to elucidate the mechanisms of neutralizing antibody escape in the virological synapse-mediated viral transfer. There is a general agreement that the potency of neutralizing antibodies is reduced during cell-to-cell transmission compared with cell-free infection and that only a subset of neutralizing antibodies can efficiently inhibit cell-to-cell transmission (47, 56, 75–77). For example, it was reported that several specific anti-gp120 antibodies targeting the CD4 binding site lost considerable potency (10- to 100-fold decrease) when HIV-1 was transferred by cell-to-cell transmission (75). Though some antibodies are still able to block cell-to-cell viral transmission at high concentration, VRC01, which is one of the most potent antibody for inhibition of cell-free infection, is particularly ineffective for blocking cell-to-cell viral transfer (75). While most anti-gp120-directed antibodies, and in particular those directed against the gp120 CD4 binding site displayed a reduced activity during viral cell-to-cell transmission, the same group reported that the T20 fusion inhibitor targeting the gp41 transmembrane glycoprotein as well as neutralizing antibodies directed against gp41 maintained their activity and were thus able to block both cell-free and cell-to-cell infection with the same efficiency. However, other groups showed that some anti-gp41 neutralizing antibodies failed to inhibit cell-to-cell transmission (4, 56, 77). Globally, the efficiency of neutralizing antibodies for neutralization of the virological synapse-mediated viral transfer is variable and some epitopes of the viral envelope glycoproteins seem more susceptible than others to neutralization of the viral cell-to-cell transfer between T cells.

Similarly, the activity of the T20 peptide entry inhibitor of the Env-mediated membrane fusion on HIV-1 transmission through the virological synapse is still a matter of debate, and a lot of contradictory results have been published regarding the effect of this inhibitor. Whereas it was initially reported that T20 was unable to block virological synapse-mediated viral transfer using flow cytometry analysis (4), Martin et al. then showed that cell-free and cell-to-cell infection across the virological synapse were equivalently susceptible to T20, using qPCR for detection of infection in the target T cells

(60). These different results can be explained by the different experimental approaches used since the first study was looking for viral transfer of viral material when the other study analyzed *de novo* synthesized viral DNA after viral transfer. We can hypothesize that the T20 fusion inhibitor does not affect viral transfer across the virological synapse, but inhibits HIV-1 infection in the target cell after the viral transfer mediated through the virological synapse. The fusion inhibitors T20 and C34, targeting gp41, could also have no effect on capture and endocytosis of virus particles for viral cell-to-cell transfer, but would rather block subsequent viral fusion in the endosomal compartments leading to inhibition of productive infection (65).

While it is usually accepted that HIV-1 could escape or is less sensitive to the inhibitory activity of antiviral drugs used in clinic when T cells are infected by virus transfer through the virological synapse, only a few studies have reported rational analyses of the effects of antiretroviral drugs such as anti-protease or anti-reverse transcriptase inhibitors on viral cell-to-cell transfer. Some studies indeed reported that HIV-1 virological synapse-mediated infection and cell-free infection were similarly inhibited by anti-protease drugs (15, 78). Regarding reverse transcriptase inhibitors, it seems that non-nucleoside-analog inhibitors of the reverse transcriptase could block virological synapse-mediated infection of T-cell targets (15, 78), whereas nucleoside-analog reverse transcriptase inhibitors (NRTI) were unable to do it. However, it was also reported that cell-to-cell infection was blocked efficiently when these NRTIs were used in combination even if the level of inhibition is lower than for cell-free infection (15, 78, 79). Usage of HIV-1 protease inhibitors Lopinavir and Darunavir has been reported as an efficient way to inhibit cell-to-cell transmission as efficiently as cell-free infection (79). This effect of protease inhibitors compared with reverse transcriptase inhibitors is suspected to be due to their ability to target immature virions and blocking their maturation in fully infectious viruses.

In conclusion, it seems from the data reported in the literature that the idea that the cell-to-cell viral transfer through the virological synapse can escape from neutralizing antibodies and antiretroviral drugs is not so evident and is probably dependent on the inhibitors used. Further systematic investigations with standardized assays to evaluate and compare activities of neutralizing antibodies and antiretroviral drugs in both cell-free and cell-to-cell infections are absolutely needed to address this important point.

HETEROGENEITY OF THE VIROLOGICAL SYNAPSES

HIV-1 transmission through the virological synapse established between an infected donor T cell and a recipient target T cell have been extensively studied. Nonetheless, DCs and macrophages can also transmit HIV-1 to target CD4+ T cells through the formation of a related virological synapse (80–82). If virus cell-to-cell transfer between DCs and T cells, as well as between macrophages and T cells, presents some similarities with the

virological synapse observed between T cells, they also showed some specific differences.

The DC Infectious Synapse

Regarding DCs as virus-donor cells, two types of cell-to-cell viral transfer and infection of T-cell targets have been proposed: in *cis*-infection, DCs are productively infected and can then transfer viruses to CD4+ T cells, whereas in *trans*-infection, DCs are able to capture HIV-1 independently of CD4 and then transfer viruses to CD4+ T cells through the formation of the so-called “infectious synapse” (39, 83–85) (Figure 1E). HIV-1 *cis*-infection and *trans*-infection of CD4+ T target cells from DCs have been proposed to be mediated by distinct processes (86). However, because DCs highly express antiviral cellular restriction factors (APOBEC3G, TRIM5 α , BST-2, and SAMHD-1), HIV-1 replicates poorly in this cell type. That is probably the reason why most of the studies of cell-to-cell transmission of HIV-1 from infected DCs to T cells developed *cis*-infection models of DCs using massive quantities of VSV-G-pseudotyped HIV-1 particles to infect DCs *in vitro*, thus questioning the validity of these models of *cis*-infection.

Regarding the *trans*-infection cell-to-cell process from DCs, several studies showed that DCs can capture HIV-1 through binding of the viral envelope glycoproteins to the mannose specific C-type lectin receptor (DC-SIGN) and store viruses into tetraspanin-enriched compartments, in continuity with the plasma membrane without viral replication in recipient DCs (58, 83, 84, 87). More recently, it has been reported that the immunoglobulin(I)-type lectin Siglec-1 (or CD169) can also bind to HIV-1 particles carrying sialyl-lactose gangliosides on their envelope (88–90). After capture, viruses remain at the cell surface or are internalized in vesicular containing compartments (see below). Then, DCs are able to transfer these viruses to CD4+ T cells independently of viral replication through the formation of an “infectious” synapse (91–94), thus decreasing the efficiency of broadly neutralizing antibodies on HIV-1 infection of target T cells (95).

While the formation of the virological synapse depends on the interaction between the viral gp120 envelope glycoprotein and the CD4 receptor, the infectious synapse formed during *trans*-infection does not rely upon CD4/gp120 interaction since Rodriguez-Plata et al. showed that the number of cell-to-cell conjugates formed between DCs and CD4+ T cells was not increased in the presence of HIV-1 (85). However, they also reported that the formation of the infectious synapse was decreased by 60% when the interaction between the adhesion molecules ICAM-1 and LFA-1 was disrupted. Furthermore, recognition of MHC-superantigen complexes presented at the surface of DCs by the TCR significantly enhanced virus transfer across the infectious synapse (85, 91). These authors suggest that the formation of the infectious synapse is not triggered by the virus but is more related to a hijacking of the immunological synapse by HIV-1. However, their experiments do not prove formally the formation of a canonical immunological synapse in their system as TCR or signaling molecules recruitment at the interface was not verified. Moreover, a previous study failed to detect any MHC-II or TCR recruitment at

the interface between DCs carrying viruses and target CD4+ T cells, thus excluding the identification of this structure as a *bone fide* immunological synapse (87).

While the formation of the infectious synapse does not rely on CD4/gp120 interaction, this interaction is still required for efficient productive infection of CD4+ T cells following formation of cell-to-cell conjugates with DCs carrying HIV-1. After the formation of conjugates, the CD4 receptor and CXCR4 or CCR5 co-receptors are recruited at the site of the cell–cell contact (96). Furthermore, intercellular transfer to CD4+ T cells of virus particles captured by DCs can be blocked using high concentrations of broadly neutralizing antibodies targeting the gp120 and gp41 envelope glycoproteins (97).

Rearrangements of the actin cytoskeleton also play a key role in HIV-1 transfer across the infectious synapse. A recent screening shows that tetraspanin 7 and dynamin-2 control nucleation and cortical stabilization of actin to maintain viruses onto dendrites for efficient cell-to-cell transfer from DCs to CD4+ T cells when cells are cocultured, and simultaneously infected with HIV-1 (98). Furthermore, it has been shown that large sheet-like membrane structures derived from DCs carrying HIV-1 wrap around T cells leading to a large interface at the infectious synapse (58). Within this interface, filopodia extensions from T cells are able to interact with the virus-containing compartments (VCCs) in continuity with the plasma membrane of DCs for efficient cell-to-cell transmission (58). Together, these results show that in DCs, HIV-1 can be efficiently transferred by hijacking the immunological synapse without productive infection of DCs.

As specialized DCs present in cervico-vaginal and rectal mucosa, Langerhans cells have been proposed to be the first targets of HIV-1 after sexual exposure. However, these cells were proposed to capture and degrade captured virions through expression of the DC-SIGN-related lectin Langerin at their surface, avoiding virus transmission from Langerhans cells to CD4+ T cells (99). More recently, in an *ex vivo* human tissue explant model, Ballweber et al. showed that vaginal Langerhans cells were able to transmit HIV-1 to CD4+ T cells, without being productively infected, rendering HIV-1 particles insensitive to inhibition by reverse transcriptase inhibitors in this context (100).

Of note, some studies suggest that the expression of Siglec-1 in macrophages mediates the internalization of HIV-1 particles in a VCC. As for DCs-to-T-cell transmission, the presence of these storage compartments in macrophages allows for the transfer of virions to CD4+ T cells (3, 101). Interestingly, the role of Siglec-1 for viral capture by macrophages has been evidenced *in vivo* in lymph node from HIV-1 or MLV infected mice (90). By showing subsequent transfer of MLV from macrophages to B cells, Sewald et al. proposed that this mechanism could be transposed to HIV-1 macrophage-to-T-cell transmission (90).

HIV-1 Transfer through Conjugates Formed with Macrophages

Compared with the cell-to-cell transmission mechanisms of HIV-1 involving T cells or DCs as infected donor cells, little

is known about the formation of conjugates between HIV-1-infected macrophages and target CD4+ T cells. As evidenced by the presence of infected macrophages in different tissues in infected patients, macrophages are cellular targets of HIV-1 and probably play an important role in HIV-1 pathogenesis (102–108). Like other infected cells, they participate in cell-to-cell transmission of HIV-1 and are essential for HIV-1 spread in tissues of infected patients. One major feature of HIV-1-infected macrophages is related to the assembly and budding steps of the new viral particles. While in T cells viral assembly takes place at the plasma membrane, *de novo* formed viral particles accumulate both at the plasma membrane and in tetraspanin-enriched compartments called VCCs in productively infected macrophages (109–112). These VCCs, which do not exhibit the same characteristics than endosomes or multivesicular bodies, but are characterized by the presence of the tetraspanins CD63 and CD81 (113–116), and are connected to the extracellular space by thin channels in continuity with the plasma membrane (110, 117, 118). Viruses thus assemble in macrophages in a protected compartment and are then released in the extracellular environment.

Infected macrophages can efficiently transfer viruses to uninfected CD4+ T cells across the formation of a virological synapse but the mechanisms involved in the formation of this macrophage/T-cell virological synapse are not completely characterized (3, 119). During the formation of the conjugates between infected macrophages and target T cells, VCCs could rapidly move to the virological synapse (82) through an actin cytoskeleton-dependent mechanism (81). Similarly to the virological synapse formed between T cells, the virological synapse involving virus-donor macrophages leads to the recruitment of CD4, CCR5, LFA-1, and ICAM-1, as well as the viral Gag precursor and envelope glycoproteins at the site of cell–cell contact (81, 119). In contrast to the virological synapse formed between T cells, which is dependent of CD4, the formation of conjugates between infected macrophages and CD4+ T cells appears to be independent of gp120 (82), while the viral transfer is dependent of gp120/CD4 and LFA-1/ICAM-1 interactions (81). Through the formation of the virological synapse, infected macrophages can transfer a high multiplicity of HIV-1 to CD4+ T cells, promoting reduced viral sensitivity to reverse transcriptase inhibitors as well as to a panel of neutralizing antibodies (81, 82). While the virological synapse between infected macrophage and T-cell targets show several differences with the virological synapse between T cells (82, 119, 120), no similar structure has been described so far, to our knowledge, between infected T cells and uninfected macrophages for virus cell-to-cell transfer in these HIV-1 target cells.

ENGULFMENT OF INFECTED T CELLS BY MACROPHAGES AND ENTOSIS

Despite the fact that HIV-1 infection of T cells by cell-to-cell transfer has been largely documented, cell-to-cell infection of macrophages remains poorly investigated. Recently, the

group of Quentin Sattentau pointed out for the first time a new mechanism of specific cell-to-cell transfer of HIV-1 from infected CD4+ T cells to macrophage targets (121) (**Figure 1F**). The authors showed that macrophages could engulf HIV-1-infected T cells leading to productive infection of the macrophage targets. The engulfment of infected healthy but rather dying T cells was significantly higher compared with uninfected healthy T cells, indicating that the cell death and infection of virus-donor T cells might independently promotes T-cell engulfment by macrophages. This preferential uptake of infected dying T cells was independent of the interaction between gp120 and the CD4 receptor but was dependent of remodeling of the actin cytoskeleton. Since this engulfment/uptake of infected T cells by macrophages was dependent of actin remodeling and was not inhibited by amiloride, an inhibitor of macropinocytosis, the authors suggested that this engulfment process likely results from phagocytosis mechanisms.

While the uptake of infected T cell by macrophages was independent of the Env/CD4 interaction, the productive infection of macrophages following uptake of T cells would be dependent of this interaction, and also depended of the tropism of the viruses. The uptake of infected T cells was evidenced by a concentrated localization of the viral Gag precursor in the macrophage target observed in association with the CD3 T-cell specific marker. The engulfment of T cells infected with CCR5-tropic viruses led to productive infection of the macrophage targets able to produce infectious virus particles in the cell-culture supernatant. In contrast, after engulfment of T cells infected with CXCR4-tropic viruses, no productive infection of the macrophage targets was observed (121). From these data, it appears evident that macrophages can be productively infected by phagocytosis of HIV-1-infected T cells, but the mechanisms for virus entry in macrophages from intracellular phagocytosed T cells remains to be defined. Nonetheless, the dependency of co-receptor usage by HIV-1 particles for productive infection could indicate a fusion of viral particles with the phagosome membrane.

Phagocytosis of SIV-infected T cells by myeloid cells *in vivo* was also suggested by the group of Jason Brenchley in SIV-infected macaque models (122, 123). Their studies point out the presence of viral RNA and DNA originating from T cells in myeloid cells from spleen and lymph nodes of infected monkeys, but do not formally prove that this presence of specific markers was related to a phagocytosis mechanism of SIV-infected T cells by myeloid cells, since no *per se* phagocytosis experiment was carried out in these studies.

Of note, another type of engulfment of HIV-1-infected CD4+ T cells by epithelial cells *via* an entosis mechanism has been observed. Entosis refers to the invasion of non-apoptotic cells into another live cell and then induces the formation of cell-in-cell structures (124, 125). Using infected cells from an immortalized T-cell line, Ni et al. reported that epithelial cells could internalize infected T cells *in vitro*, as well as *in vivo* in sigmoid colon explants from HIV-1-infected patients showing the formation of cell-in-cell structures (126). Through this entosis process, the epithelial cells could be productively infected, as

evidenced by the diffuse intracytoplasmic staining of the viral capsid protein.

CELL-CELL FUSION

Formation of T-Cell Syncytia

Cell-to-cell fusion between infected CD4+ T cells, or between infected and uninfected CD4+ T cells, has been initially proposed to be another mechanism for HIV-1 infection and dissemination between T cells (127–131). Early studies suggested that infected T cells could fuse with uninfected T cells to form giant syncytia, 5–100 times bigger than individual cells (131, 132). In this context, cell-to-cell fusion occurs through actin cytoskeleton rearrangements, is dependent of LFA-1/ICAM-1 interaction and is mediated through interaction between envelope glycoproteins expressed at the cell surface of infected cells and CD4 expressed on the target cells (127, 129, 131, 133). Yet, these T-cell syncytia, initially observed only *in vitro* with infected immortalized T-cell lines, have been shown to die rapidly by a mitochondrion-dependent apoptosis mechanism (134, 135). The formation of HIV-1 T cell syncytia leads to activation of the serine-threonine kinase, mTOR, which mediates phosphorylation of the transcription factor p53. Thus, p53 induces upregulation of the pro-apoptotic regulator Bax leading to mitochondrial permeabilization and release of pro-apoptotic mitochondrial proteins (136, 137). Several groups thus proposed that this cytopathic effect related to the formation of T-cell syncytia could be the mechanism of the CD4+ T cells loss observed during infection in HIV-1-infected patients (127, 128, 132).

However, formation of T-cell syncytia has been a controversial subject since other studies did not observed formation of T-cell syncytia using HIV-1-infected primary CD4+ T cells (3, 4, 11). Because no evidence of the formation of such T-cell syncytia was reported *in vivo*, it has been suggested that these giant syncytia could be *in vitro* artifacts only observed with immortalized cell lines and restricted to CXCR4-viruses (138). Viral strains were indeed initially classified as syncytia-inducing or non-syncytia inducing (NSI) strains, referring to their capacity to induce syncytia *in vitro*, and then to their ability to use either CXCR4 or CCR5 for virus entry, respectively (139). However, cells infected with NSI viruses can readily form syncytia with CCR5-positive cells, and a new classification of viral strains based on CXCR4 or CCR5-usage was adopted (140).

Interestingly, small T-cell syncytia, containing no more than five nuclei, have been then observed *in vivo* in lymph nodes from HIV-1-infected patients (141). In addition, some more recent studies using HIV-1-infected “humanized mouse models” reported the presence of motile infected syncytia in lymph nodes, smaller than those observed *in vitro* (12). These motile small T-cell syncytia can establish tethering interactions with uninfected T cells that may facilitate cell-to-cell transmission through the formation of a virological synapse (12, 142). Interestingly, these interactions between infected small syncytia and uninfected T cells do not lead to cell-to-cell fusion suggesting that this mechanism of cell fusion is finely regulated (12).

Inhibition of T-Cell Fusion at the Virological Synapse

The numerous studies describing the formation of conjugates between infected and uninfected T cells for viral transfer across the virological synapse do not present observations of cell–cell fusion between the virus-donor T cells expressing the viral envelope glycoproteins and the target T cells expressing the CD4 receptor and CXCR4 and CCR5 co-receptors, confirming that this process is finely regulated during the formation of the virological synapse between T cells.

Since HIV-1 is a highly fusogenic virus that is preferentially transmitted by direct contact between two cells, the mechanisms inhibiting the fusion between an infected cell and a target cell deserves to be questioned. Indeed, in most of the T cell-to-T-cell transmission of HIV-1, a very few syncytia are observed compared with the formation of virological synapses (3, 4, 11). Moreover, large syncytia formed with T cells were characterized as rapid-dying cells (134, 136), which might indicate that HIV-1 has developed some mechanisms to inhibit the formation of these large T-cell syncytia to survive in infected host. As usually, it was proposed that HIV-1 took advantage of the intracellular systems displayed by its host, and used molecular mechanism to inhibit cell–cell fusion.

For example, the CD81 tetraspanin membrane protein is recruited at the site of cell–cell contact during HIV-1 cell-to-cell transmission and was proposed to be involved in the regulation of fusion (143). CD81 knockdown or inhibition with a specific antibody dramatically increased the capacity of infected T cells to fuse with surrounding non-infected cells, indicating that CD81 is a negative regulator of T cell–T cell fusion during virological synapse formation. CD81 expression at the virological synapse seems to be regulated by the actin/plasma membrane connector, ezrin. Accordingly, reduction of ezrin expression in T cells decreased the level of CD81 localized at virological synapses, thus increasing cell–cell fusion between infected T cells (144). Other tetraspanin proteins such as CD9, CD63, or CD82, have been also studied for their role in HIV-1-mediated cell–cell fusion (144, 145). In addition, it has been shown that CD63 may interact with the CXCR4 co-receptor and induces downregulation of CXCR4 expression at the cell surface, resulting in the inhibition of HIV-1 infection (146, 147). Like CD81, these molecules may act as regulators of cell fusion, but most of the experiments have been performed using HeLa or HEK293T non-lymphoid cell lines. Therefore, experiments need to be performed in more relevant cell models to precisely define the molecular requirement of these tetraspanins in the regulation of syncytia formation between HIV-1-infected T cells.

Presence of Multinucleated Giant Cells (MGCs) in Infected Tissues

The mechanism of T-cell syncytia formation has been largely documented *in vitro* and discussed for its *in vivo* relevance. Nonetheless, processes of cell–cell fusion for HIV-1 infection and dissemination are not restricted to T cells. Some studies showed that infected multinucleated macrophages, as well

as multinucleated DCs, could be found in different tissues *in vivo* in HIV-1-infected patients (148, 149). The presence of HIV-1-infected multinucleated syncytia expressing specific DC markers was found at the surface of the nasopharyngeal tonsils of HIV-1-infected patients and reported 20 years ago (148). The same group reported that infected T cells could fuse *in vitro* with skin-derived DCs (94), thus leading to multinucleated cell formation between T cells and DCs. Similarly, multinucleated giant HIV-1-infected macrophages have been found *in vivo* during infection in several different tissues, including lymph nodes, spleen, lungs, genital, and digestive tracts, and the central nervous system (CNS) (102–106, 108, 148, 150–153). While several groups showed the presence of infected multinucleated macrophages in tissues, and more specifically in the brain of HIV-1-infected patients and SIV-infected monkeys, the cellular and molecular mechanisms related to their formation remained poorly investigated, with only one *in vitro* study demonstrating a role for the HIV-1 auxiliary protein Nef in the formation of multinucleated macrophages (154).

We recently reported *in vitro* original results and proposed a model for the formation of HIV-1-infected MGCs related to a two-step cell–cell fusion process for HIV-1 cell-to-cell transfer from infected T cells and viral dissemination in macrophage targets (155) (**Figure 1G**). First, a heterotypic fusion occurs between HIV-1-infected T cells and macrophage targets for virus transfer. Then the newly formed lymphocyte/macrophage infected fused cells acquire the ability to fuse with surrounding uninfected macrophages for virus dissemination. As evidenced by cell imaging analyses, the first step is related to the establishment of contacts with infected T cells leading to the fusion of the infected T cells with macrophages. This cell fusion, dependent of the viral envelope/CD4 receptor interaction and restricted to CCR5-tropic viral strains, has been evidenced by a massive and fast transfer of Gag + material, as well as the presence of specific T cells markers (CD3, CD2, and Lck) in the cytoplasm and at the plasma membrane of the macrophage targets. The newly formed lymphocyte/macrophage fused cells (LMFCs) are then able to fuse with surrounding uninfected macrophages. These two sequential cell fusion processes are dependent on the viral envelope and lead to the formation of highly virus-productive MGCs that could survive for a long time in host tissues to produce infectious virus particles as shown *in vivo* in lymphoid organs and the CNS of HIV-1-infected patients and SIV-infected macaques (103, 106, 108, 152, 156). Similarly, the first step related to the initial T cell-to-macrophage fusion agrees with results showing that myeloid cells from lymphoid tissues of SIV-infected macaques contain T-cell markers and viral RNA and DNA originating from infected T cells (122, 123). This route of infection may be a major determinant *in vivo* for virus dissemination and establishment of macrophage virus reservoirs in host tissues.

CONCLUSION

Since the HIV-1 discovery, a lot of works have been accomplished to decipher its life cycle, in order to counteract virus

transmission and dissemination. Counteraction of host defenses is a major feature of HIV-1, since it is able to infect a large panel of immune cells and to largely decrease their effector functions. During the past years, several entry pathways for virus dissemination have been discovered but still need further investigations. While HIV-1 was initially suspected to enter its target cells only through fusion of viral particles with host T-cell membranes, it appears more and more evident that it can use a large diversity of cellular features to infect target cells, especially through direct cell-to-cell transmission by close contacts between an infected virus-donor cell and a target cell. Cell-to-cell transfer of HIV-1 indeed allows a massive release of viral material toward the target cell inducing a strong increase of viral infectivity, compared with infection with cell-free viruses. Depending on the infected donor cells as well as the target cell lineages, HIV-1 can take advantage of their specific physiology and features to infect its host. In T cell-to-T-cell transmission, HIV-1 provokes the formation of contacts, through Env-CD4 interactions, to allow a massive transfer of viruses between the two cells, thus improving its ability to enter the host cell. This intercellular structure, called virological synapse, is strongly similar to another but physiological one, the immunological synapse, indicating the HIV-1 ability to hijack T-cell pathways to spread. During trans-infection from DCs to target T cells, HIV-1 is even able to re-use at its advantage the interaction between APCs and T cells to cross the intercellular space for infection of target T cells. Through the interface formed between donor and target cells, filopodia coming from target T cells penetrate some VCCs in donor DCs to capture virions.

Therefore, intercellular communication pathways seem to be a road of choice for HIV-1 spreading. By increasing the frequency of filopodia formation in host infected cells, HIV-1 also enhances the surface of contacts of infected cells with the environment, thus improving its efficiency to be transferred to a non-infected neighboring target cell. In addition, HIV-1 can also create real bridges between cells, the so-called TNTs, which resemble to highways for virus transmission and dissemination. Moreover, when infected T cells are engulfed by specialized-phagocytic cells such as macrophages, instead of being destroyed, HIV-1 is still able to infect the phagocyte.

Finally, HIV-1 uses its own ability to fuse with host cell membranes to provoke fusion between an infected donor cell and a target cell. By this way, HIV-1 is totally hidden from extracellular milieu since the sharing of the cytosol between the two cells appears to be sufficient for viral material dissemination and productive infection the new-formed fused cell able to produce new fully infectious virus particles. While fusion between T cells has been observed since a long time but was controverted as an *in vitro* artifact, more recent studies have pointed out the presence of small T-cell syncytia *in vivo* in lymph nodes from infected patients and proposed that these syncytia are fully able to transmit viral particles by cell-to-cell transfer to other cell targets. Multinucleated macrophages or DCs have also been observed *in vivo* in different tissues from HIV-1-infected

patients, indicating the cell-to-cell fusogenicity of HIV-1 for different cell types. We have recently discovered a new mechanism of cell fusion between HIV-1-infected T cells and target macrophages. Heterotypic fusion between these cells appears, at least *in vitro*, to be a massive and very fast process allowing the formation of LMFCs that acquire the ability to fuse rapidly with neighboring non-infected macrophages, thus forming MGCs. The new-formed MGCs can survive for a long period *in vitro* and are highly productive of fully infectious viral particles. This important process could explain the presence of MGCs observed in several tissues of infected patients. Because of their long-time survival capacity, infected MGCs could participate in virus dissemination and establishment of persistent virus reservoirs in host tissues.

To conclude, while cell-associated HIV-1 transmission is believed to contribute to sexual transmission or viral spreading as well as to the formation of latent virus reservoirs, only few studies address the importance of cell-to-cell transfer of HIV-1 *in vivo*, and further investigations are needed to decipher the role of all the intercellular structures and processes described *in vitro* for pathogenesis in HIV-1-infected patients.

AUTHOR CONTRIBUTIONS

All authors listed, have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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