CELLULAR CHOLESTEROL REGULATION OF HIV-1 TRAFFICKING DURING MACROPHAGE-MEDIATED *TRANS* INFECTION

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ABSTRACT

Professional antigen presenting cells (APC: myeloid dendritic cells (DC) and macrophages (MΦ); B lymphocytes) mediate highly efficient HIV-1 infection of $CD4^+$ T cells, termed *trans* infection, that could contribute to HIV-1 pathogenesis. We have previously shown that lower cholesterol content in DC and B lymphocytes is associated with a lack of HIV-1 *trans* infection in HIV-1 infected nonprogressors (NP). Here we assessed whether HIV-1 *trans* infection mediated by another major APC, MΦ, is deficient in NP due to altered cholesterol metabolism. When comparing healthy HIV-1 seronegatives (SN), rapid progressors (PR), and NP, we found that monocyte-derived MΦ from NP did not mediate HIV-1 *trans* infection of autologous $CD4^+$ T cells, in contrast to efficient *trans* infection mediated by SN and PR MΦ. MΦ *trans* infection efficiency was directly associated with the number of DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)-expressing MΦ. Significantly fewer NP MΦ expressed DC-SIGN. Unesterified (free) cholesterol in MΦ cell membranes was significantly lower in NP than PR, as well as virus internalization in early endosomes. Furthermore, simvastatin (SIMV), decreased the subpopulation of DC-$SIGN^+$ MΦ, as well as MΦ *cis* and *trans* infection. Notably, SIMV decreased cell membrane cholesterol and led to lipid raft dissociation, effectively mimicking the incompetent APC *trans* infection environment characteristic of NP. Our data support that DC-SIGN and membrane cholesterol are central to MΦ *trans* infection, and a lack of these limits HIV-1 disease progression. Lastly, we

identified differentially expressed miRNA in NP and PR plasma which may contribute to the altered cholesterol phenotype we have identified in NP APC. Understanding the mechanisms of HIV-1 *trans* infection has great public health importance. For example, targeting the ability of MΦ to drive HIV-1 dissemination in *trans* could significantly enhance HIV-1 therapeutic strategies.

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PREFACE

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1.0 IMPORTANCE AND RATIONALE

Globally, over 35 million people are currently living with human immunodeficiency virus-1 (HIV-1), and despite several decades of research, the development of a successful prophylactic vaccine against or successful cure for HIV-1 infection remains elusive. The development and implementation of combination antiretroviral therapy (cART), which can effectively lower HIV-1 viral load to undetectable levels, has greatly decreased the morbidity and mortality associated with HIV-1 infection. However, some individuals battle persistently low CD4/CD8 ratios (1) and virus rebound despite cART (2). Unsuccessful treatment has also been attributed to delayed diagnoses and late onset of therapy (3). Even in the event of cART-mediated viral suppression, there is a reservoir of HIV-1 infected $CD4^+$ lymphocytes ($CD4^+$ T cells) in the circulation and in tissue (4) that successfully evades immune surveillance, and contributes to incomplete viral clearance or eradication despite treatment (5-8).

Many host, microbe, and environmental factors act concurrently to establish the unique microenvironment found within each HIV-1 positive individual and determine their associated prognosis. The complexity of HIV-1 pathogenesis has complicated the development of a successful cure or vaccine, demanding the identification of novel mechanisms for treatment and prevention. Without cART, fewer than 5% of seropositive individuals can control HIV-1 infection and prevent progression to AIDS (9). Collectively referred to as nonprogressors (NP) this group of individuals represents an enigma in the field of HIV-1. Protective factors, such as the Δ32 deletion in the HIV-1 coreceptor CCR5 gene (10-12) or the presence of specific HLA alleles (13), have been associated with a lack of or lower susceptibility to HIV-1 infection, as well as protection from disease progression. Interestingly, some NP in the Multicenter AIDS

Cohort Study (MACS) lack Δ32 homozygosity or heterozygosity as well as known protective HLA types, and we believe these NP serve as the ideal natural model within which novel mechanisms for controlling HIV-associated disease can be unveiled.

The presence of a latent HIV reservoir within $CD4^+$ T cells in the circulation and in tissue (4) that successfully evades immune surveillance is thought to be a barrier preventing virus clearance despite cART. *Trans* infection, i.e. transfer of virus from cell-to-cell, of $CD4^+$ T cells is believed to be a crucial event contributing to viral persistence during cART (14, 15). $CD4^+$ T cell *trans* infection mediated by professional antigen presenting cells (APC), i.e., dendritic cells (DC), macrophages, and B lymphocytes (B cells), yields much higher virus replication in T cells compared to both direct *cis* infection of T cells and T cell-to-T cell *trans* infection (16). Transfer of virus during direct contact from one cell to another through the infectious synapse represents a mechanism to evade immune responses, particularly in lymphoid tissue, thereby aiding the maintenance of an infected $CD4^+$ latent HIV-1 reservoir. As APC-to-T cell *trans* infection is an important method of massive virus replication, it is an essential form of HIV-1 dissemination to be studied. Additionally, elucidation of *trans* infection mechanisms will provide novel targets for prophylactic and therapeutic medicine, as well as potentially help in identifying and eliminating the viral reservoir.

We have recently demonstrated that DC and B cells of NP do not *trans* infect autologous or heterologous $CD4^+$ T cells. In addition, we have found an association with decreased DC and B cell total cholesterol content and their inability to *trans* infect (17). This was the first time *trans* infection was linked to HIV-1 pathogenesis and disease progression. Cholesterol-rich macrophages, known as foam cells, are heavily involved in cardiac plaque development (18, 19), a common risk factor of cardiovascular disease, which is a known comorbidity in HIV-1 infected

individuals (20). Although monocyte-derived macrophages (MΦ) are known to *trans* infect $CD4^+$ T cells (21) with HIV, their cholesterol content and *trans* infection ability has not been studied in association with disease progression. While past research has focused on the impact of virion envelope cholesterol content on HIV-1 infection and pathogenesis (22, 23), the role of cellular cholesterol in MΦ – mediated HIV-1 *trans* infection is unknown. Understanding the role of cholesterol in HIV-1 *trans* infection will provide insight into novel mechanisms of HIV-1 pathogenesis and disease control.

2.0 INTRODUCTION

2.1 HIV-1 PUBLIC HEALTH

HIV Pandemic

It is believed that the current HIV pandemic originated in Central Africa in the early 1920s (24). However, HIV was not identified as the causative agent of Acquired Immune Deficiency Syndrome (AIDS) until 1983 (25). The arrival of HIV and the AIDS epidemic to the United States in 1980 and the subsequent death of thousands of people generated a demand for research and a cure by the western world. Yet, despite several decades of research, the development of a successful prophylactic vaccine against or successful cure for HIV infection remains elusive. The discovery of antiretroviral drugs in the 1990s and implementation of cART in the mid-1990s, has since increased the quality of life and life expectancy of many seropositive individuals (26). Yet cART drug resistance is on the rise. Today HIV remains one of the most fatal infectious diseases, second only to tuberculosis, worldwide (27). With over 36 million people living with HIV globally, 1.8 million new infections occur each year, and approximately one million HIV-related deaths annually (28), the need for novel therapeutics is outstanding.

The origin of the HIV pandemic in humans can be traced back to inter- or cross- species transmission of Simian immunodeficiency virus from non-human primates to humans in Africa (29). Multiple instances of inter-species transfer of simian immunodeficiency virus is believed to explain the generation of two types of HIV, HIV-1 and HIV-2, within which multiple groups of genetically different HIV have formed, groups A – H for HIV-2 and M, N, O, and P for HIV-1, within which some groups are even further categorized into subtypes (29). These groups vary

in many ways including, infectivity, pathogenicity, and geographical prevalence (29, 30). The most common type of HIV-1 infection in North America, South America, Europe, and Australia is HIV-1 group M subtype B, while subtype C is the most common type identified in Africa and Asia (31). Although HIV-1 and -2 share both virological features and infection requirements, such as receptors for virus entry, replication cycle, and mechanisms of pathogenicity (32), HIV-1 strains are more infectious, as well as cause more severe disease and faster disease progression compared to HIV-2 strains (32, 33). Therefore, HIV-1 is more widely studied and is henceforth the focus of the work presented.

HIV-1 transmission, replication, and restriction

HIV-1 is a blood-borne pathogen that is transmitted by the exchange of infected bodily fluids. HIV-1 can be transmitted sexually via the urogenital or gastrointestinal tract, as well as through contact with infectious blood, most commonly via contaminated needles during intravenous drug use (34). Transmission can also occur from infected mother to child if the child is exposed to infectious maternal blood or breast milk intrapartum or during breast feeding (35).

Following transmission, HIV-1 productive infection of $CD4^+$ T cells is the primary method by which virus replication occurs and new progeny virions are formed. The ability of cell free virus to enter target host cells, replicate, and produce new infectious virions is known as productive *cis* infection. *Cis* infection is a complex pathway that requires specific cellular conditions. The HIV-1 envelope glycoprotein gp120 binds to the CD4 on the host cells, causing a conformational change that allows the binding of gp41 to the host co-receptor, either CCR5 or CXCR4. The gp41 protein inserts into the host cell PM, mediating the fusion of the virus envelop and cell PM. Once the genome containing nucleocapsid enters the host cell, a viral enzyme,

reverse transcriptase, reverse transcribes the single-stranded RNA viral genome into double-stranded DNA. The viral DNA is then transported into the cell nucleus and integrated into the host genome facilitated by the viral enzyme integrase. Once integrated, upon cellular activation, the viral DNA is transcribed into mRNA that serves as viral genome for packaging into new progeny virions or is translated into viral proteins. The viral proteins are either transported to and expressed in the host PM, i.e. envelope glycoproteins, or packaged with the viral genome into newly formed viral nucleocapsids. New virions bud from the host cell encompassed by an envelope derived from the host cell PM (36).

Despite the hijacking of host cell machinery by HIV-1, human immune cells deploy multiple defense mechanisms, commonly known as restriction factors, which limit of block infection. These host factors target various steps in the HIV-1 *cis* infection pathway. For example, APOBEC3G hinders reverse transcription and replication of the HIV-1 genome (37). Additionally, TRIM5α causes rapid un-coating of the nucleocapsid (38), and tetherin blocks the release of new mature virions budding from the cell surface (39). Interestingly, certain HIV-1 host restriction factors are expressed in particular cell types, and their expression can be constitutive or induced, for example by interferon (IFN) during innate immune responses. SAMHD1 is highly expressed in myeloid-derived cells, such as dendritic cells (DC), and is considered responsible for the low infection and replication efficiency of HIV-1 in DC (40). Although $CD4^+$ T cells express APOBEC3G, they also contain an intrinsic factor that inhibits APOBEC3G activity, which contributes to the less effective ability of $CD4^+$ T cells to block HIV-1 reverse transcription and replication leading to their high susceptibility to infection.

HIV-1 also contains counter mechanisms to bypass or block host restriction factors. Intracellularly, HIV-1 protein vif facilitates the degradation of APOBEC3G (41), while HIV-1

non-virion associated env, nef, and vpu can effectively sequester tetherin through competitive binding on the cell surface allowing budding of intact virions (42). Despite tissue-resident MΦ expression of SAMHD1, when such cells are in a G1 state HIV-1 can bypass the restriction function of SAMHD1 and replicate efficiently (43). Additionally, several HIV-1 proteins act to decrease detectability of the virus and infected cells by the host immune system. For example, the viral protein nef down-regulates MHC Class 1 expression to decrease viral antigen presentation and subsequent natural killer cell and cytotoxic $CD8^+$ T cell responses (44). Also, the $CD4^+$ T cell response can be altered by nef through the down-regulation of CD4, altering T cell receptor functions (45). Lastly, HIV-1 can evade HIV-1 specific adaptive immune responses such as antibody neutralization and $CD8^+$ T cell cytotoxicity due to high genomic mutation rates, resulting in epitope escape and antigenic variation. Nef also can be excreted in exosomes and act in a paracrine fashion on neighboring cells (46). When the virus wins this tug-of-war, it establishes a primary infection and causes disease.

HIV-1 disease and therapeutics

Following transmission and primary infection, HIV-1-associated disease is divided into three phases, acute infection, chronic clinical latency, and AIDS. During acute infection, robust viral replication increases resulting in peak plasma viremia levels mirrored by a dramatic decrease in blood $CD4^+$ T cell counts. Concurrently, immune activation in response to the infection occurs and infected individuals develop common influenza-like symptoms, such as a fever and body aches, often leading to misdiagnose, or remain asymptomatic. After a several weeks, individuals enter the chronic phase of HIV-1 infection during which an effective anti-HIV-1 immune response lowers viral load and a recovery of $CD4^+$ T cells is observed. However,

due to the establishment of an undetectable latent viral reservoir, consisting of inactive, non-replicating infected cells, the virus is not fully cleared from the body. Without cART, the chronic phase lasts anywhere from six months to seven years after which an irreversible increase in viral load and decrease of $CD4^+$ T cell count below 200 cells/ mm^3 triggers the onset of AIDS and ultimately death (47, 48). However, with proper access and adherence to cART, the development of AIDS can be delayed for decades, if not a lifetime (26).

cART helps control viral load and immune activation, thereby decreasing virus transmission, as well as HIV-1-associated disease, comorbidities, and mortality (49). Antiretroviral drugs target several steps of the HIV-1 replication cycle, including virus binding, reverse transcription, integration, and viral protease function. Combination therapies utilizing nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, and integrase strand transfer inhibitors lead to greater virus suppression and control compared to previously used single drug therapy (49).

While cART can effectively treat chronic infection with proper access and drug adherence, it does not eliminate latently infected cells. Once integration has occurs, viral DNA resembles host DNA and is therefore not easily distinguishable for excision and elimination from infected cells. There is a latent reservoir of HIV-1 infected $CD4^+$ T cells in the circulation and in tissue (4) that successfully evades immune surveillance, even in the presence of cART, and is believed to contribute to incomplete viral clearance or eradication though treatment. The inability of the host immune system to eliminate the HIV-1 reservoir despite decades of research and therapeutic discoveries is the primary reason HIV-1 causes lifelong chronic infection for which we lack a sterilizing cure.

Natural HIV-1 Disease Control

Despite the success of cART, resistance to antiretroviral drugs is becoming more prevalent globally, and while not epidemiologically recorded in the United States of America, it is being monitored in much of South and Central America, as well as several African and Asian countries by the World Health Organization (50). The rise of antiretroviral drug resistance has demanded a need for novel therapeutic targets and strategies for HIV-1 control and elimination. Without combination antiretroviral therapy (cART) fewer than 5% of seropositive individuals can naturally control HIV-1 infection and prevent progression to AIDS (9). These few individuals consist of viremic controllers (VC; 50 – 2,000 plasma HIV-1 RNA copies/ml), elite controllers (EC; consistent undetectable RNA), and long term nonprogressors (LTNP; CD4+ counts > 500/mm3 and varying levels of detectable RNA) which we collectively refer to as nonprogressors (NP). NP represent an enigma in the field of HIV-1 and serve as a natural model of HIV-1 disease suppression wherein we can define mechanisms preventing disease progression.

Several virological, immunological, and genetic factors have been found to be associated with HIV-1 disease progression. Early case reports suggested that infection with a weaker or less virulent virus leads to less pathogenesis and slower progression (51-53). However, other studies have produced confounding results (54, 55), suggesting that some, but not all, cases of nonprogression are a result of the specific infecting virus. Nonprogression is also associated with the production of anti - HIV-1 antibodies (56), strong CTL responses (57), and the production of a specific cytokine profile(58, 59). Additionally, the presence of certain single nucleotide polymorphisms (SNPs) in MHC class I and III genes are associated with nonprogression (60),

and the delta 32 mutation in the CCR5 and the presence of HLA B57 genotype are associated with nonprogression (61).

However, these characteristics do not explain all cases of nonprogression. It is important to note that in the MACS, elite and viremic controllers are characterized based on their plasma viral load at particular points after seroconversion, while long term nonprogressors are alternatively characterized solely based on their $CD4^+$ T cell count over time, regardless of viral load. Due to the heterogeneity of the NP classifications, it is likely that there is more than one genetic or biological feature responsible for the NP phenotype. Illuminating the magnitude and mechanisms of cholesterol involvement in HIV-1 trans-infection will further our understanding of HIV-1 persistence and disease progression. Revealing novel events essential for HIV-1 disease control an elimination likely lies within studying differences between groups of HIV-1 infected individuals with different virological and disease progression patterns.

2.2 HIV-1 *TRANS* INFECTION

2.2.1 What is *Trans* Infection?

Trans infection, i.e. the transfer of virus from one cell to another cell is believed to aid in virus escape from immune surveillance, as well as facilitate highly efficient infection of CD4+ T cells and contribute to HIV-1 persistence during cART. HIV-1 *trans* infection has been shown to occur from $CD4^+$ T cells to other $CD4^+$ T cells, $CD4^+$ T cells to APC (16), as well as APC to $CD4^+$ T cells. CD4+ T cell trans infection mediated by professional antigen presenting cells (APC), i.e., DC, MΦ, and B lymphocytes (B cells), yields much higher virus replication in $CD4^+$ T cells compared to both direct, cis infection of $CD4^+$ T cells and $CD4^+$ T cell-to-T cell *trans* infection (16). This may be particularly pertinent to APC interactions with $CD4^+$ T cells in

lymphoid tissue, a prominent location of HIV-1 replication. During antigen presentation by APC, the close proximity between the APC and $CD4^+$ T cells is necessary for MHC – T cell receptor within the immunological synapse, the space between the two cells. During *trans* infection, HIV-1 can be passed from the APC to the T cell with greater ease due to the close proximity of the cells. This passage of virus from APC-to- T cell is proposed to facilitate pathogen transmission under the radar of humoral immune factors such as neutralizing antibodies (62) and is hypothesized to contribute to HIV-1 persistence during cART. We have recently demonstrated that DC and B cells of NP do not *trans* infect autologous or heterologous $CD4^+$ T cells (17), linking *trans* infection to HIV-1 pathogenesis and disease progression for the first time.

2.2.2 *Trans* Infection Mechanisms.

Cis infection

Trans infection of $CD4^+$ T cells mediated by DC has been shown to occur following *de novo cis* infection of DC. At least 48 hour after infection, progeny virions budding from the DC utilize the small space between the DC and $CD4^+$ T cells during cell-to-cell interactions as a virological synapse where they infect the T cells (63). However, HIV-1 replication in DC varies based on the maturation state of the DC. Due to the effectiveness of HIV-1 host restriction factors expressed by DC, the amount of *trans* infection that occurs following DC *cis* infection is likely minimal *in vivo*. Langerhans cells have also been shown to *trans* infect $CD4^+$ memory T cells following *cis* infection (64). Due to Langerhans cells residing in skin and mucosal membranes, HIV-1 *trans* infection mediated by Langerhans cells could facilitate HIV-1 penetration and dissemination from peripheral sites of infection. Unlike DC, MΦ are highly susceptible to infection with M-tropic HIV-1 (65). Although MΦ have been shown to pass HIV-

1 to $CD4^+$ T cell following MΦ *cis* infection, this mechanism of *trans* infection is minimal (66). Studies have demonstrated that blocking HIV-1 fusion with the PM decreases both DC and LC *trans* infection, but not entirely (64, 67). This suggests that *trans* infection following *cis* infection is not the only mechanism by which virus is transferred from APC to $CD4^+$ T cells. Indeed, following exposure to HIV-1, APC have been shown to *trans* infect $CD4^+$ T cell in the absence of *cis* infection(16). Furthermore, B cells, which lack the ability to support HIV-1 *cis* infection, are known to *trans* infect $CD4^+$ T cells very efficiently (68).

Endocytosis.

APC have been found to internalize intact virions independent of CD4/co-receptor interactions or fusion. DC, LC, MΦ, and B cells can also mediate HIV-1 *trans* infection in multiple ways following receptor-mediated endocytosis. DC and B cells have been shown to take up HIV-1 through DC-SIGN-mediated endocytosis (68, 69). The virus is then passaged through the intracellular endosomal network within until it is regurgitated or exocytosed, alone or in association with exosomes, into the immunological / infectious synapse where it efficiently infects $CD4^+$ T cells (70, 71). CD169 (siglec-1) mediates the capture of retroviruses by macrophages lining the sinus region of LN and its passage to B cells within LN follicles (72). Despite this, virus transfer does not result in virus replication in the B cell, and it is feasible that upon activation, B cells could subsequently *trans* infect $CD4^+$ T cells, including T helper follicular cells (Thf), which are a primary population of cells within which virus production occurs (73). Although proteins such as DC-SIGN (68, 74) and siglec-1 (75) have been found to mediate HIV-1 transfer from DC and B cells to $CD4^+$ T cells, such a factor has not been identified for MΦ.

Infected $CD4^+$ T cells can pass HIV-1 to neighboring uninfected $CD4^+$ T cells through connected membrane nanotubes *in vitro* (76). Nanotubes are extensions of the plasma membrane which have the ability to fuse with PMs from other cells, effectively connecting the cytoplasm of two cells. These connections facilitate the passage of molecules, proteins, and even pathogens (77). DC also generate nanotubes and upon stimulation with CD40L and IFNγ, the network of nanotubes formed by DC is significantly enhanced (78). In addition to exocytosis of internalized HIV-1, the transfer of internalized HIV-1 from DC to $CD4^+$ T cells through nanotubes, or larger microtubules is an additional proposed mechanism of *trans* infection. HIV-1 infection has also been shown to induce nanotubes in MΦ through which virus could potentially be passaged (79).

Extracellular Hitchhiking.

HIV-1 can attach to the surface of several cell types in multiple ways including interacting with CD4 (80), DC-SIGN (81, 82), mannose binding C-type lectin receptors (83-86), and heparan sulfate proteoglycans expression (87-90). Through these interactions, HIV-1 can alternatively remain on the surface of cells, often within extracellular invaginations of the PM, known as crypts, common to MΦ and DC (16). This allows the virus to better evade antibody binding until the APC interacts with other target cells, during which the virus can detach from the APC and more easily infect target $CD4^+$ T cells or other APC in close proximity (16). This has been demonstrated for several cell types including spermatozoa (87), basophils (91), mucosal mast cells (92), dendritic cells (93), and B cells (94-96).

Transfer of virus in *trans* during direct contact from one cell to another through the infectious synapse represents a possible way to evade host immune detection, such as antibody-mediated opsonization and cytotoxic T cell recognition. This undetected spread of virus likely

aids in HIV-1 persistence and reservoir maintenance. Although $CD4^+$ T cell-to-T cell *trans* infection may account for some virus spread during HIV-1 latency, the superior robustness of APC – mediated $CD4^+$ T cell *trans* suggests it plays a larger role in HIV-1 pathogenesis and disease.

2.2.3 *Trans* infection Relevance to HIV-1 Disease.

Trans infection is challenging to study *in vivo* due to the advanced technical requirements for visualizing and measuring molecular interactions between HIV-1 and cells in a living animal model. However, the potential impact of HIV-1 *trans* infection on HIV-1 – associated disease is considerably significant. If the features of HIV-1 *trans* infection determined by *in vitro* studies are representative of natural HIV-1 infection, *trans* infection is likely implicated in multiple stages of acute and chronic infection.

Virus Dissemination and Enhanced Replication.

The earliest stages of HIV-1 infection (i.e. virus exposure, establishment of the primary infection, and virus dissemination) are heavily targeted by prophylactic and therapeutic measures. Many cell types in the periphery of the urogenital and gastrointestinal tracts mediate virus infection and dissemination after sexual exposure through a variety of non-specific and cell-specific interactions previously described. Infected semen is the primary source of HIV-1 transmission and spermatozoa carrying bound HIV-1 can efficiently *trans* infect DC, MΦ, and $CD4^+$ T cells (87), three cell types that are found in vaginal and colorectal submucosa and support HIV-1 replication. However, $CD4^+$ T cell and macrophage concentrations are low in the lower and upper vagina relative to DC (97). APC are the primary cell type found in the female

cervix (98), and upon disruption of the mucosal layer Langerhans cells are the first cells infected with HIV-1 following sexual exposure. Other APC within the local tissue infected with or non-productively associated with through binding or endocytosis of virus as they interact with various cell types in the peripheral tissue aiding in the establishment of primary infection. Upon infection or antigen detection and migration to secondary lymphoid tissues, they can serve as sentinels into the body. It has been visually observed that MΦ pass virus in *trans* to B cells within LN (72). Additionally, due to their ability to enter the central nervous system, HIV-1 harboring MΦ mediate dissemination of virus into the brain and have been implicated in HIV - associated neurocognitive disorder (HAND).

It is feasible that MΦ and DC entering the LN, as well as B cells, can pass HIV-1 efficiently in *trans* to highly abundant $CD4^+$ T cells leading to an amplification of virus. Robust virus replication in $CD4^+$ T cells is a hallmark of acute HIV-1 infection causing a sharp spike in viremia prior to control of virus and the chronic stage of disease (48). Overtime, chronic immune activation as a result of HIV-1 infection can lead to tissue and organ damage affecting the liver, heart, and nervous system (30).

Maintenance of the HIV-1 Reservoir.

HIV-1 *trans* infection is believed to be a critical factor contributing to viral persistence during cART (14, 15). In addition to contributing to virus dissemination, robust replication, and subsequent long-term HIV-1 – associated comorbidities such as chronic immune activation and HAND, low levels of HIV-1 *trans* infection may be a mechanism by which the viral reservoir is maintained. HIV-1 *trans* infection from DC to $CD4^+$ T cells has recently been shown to occur despite the presence of certain antiretroviral drugs, such as tenofovir and raltegravir (99). Low

levels of virus transfer to nearby susceptible cells could facilitate the growth of the population of productively or latently infected cells under the radar of the immune system. In this capacity, *trans* infection could be a relevant mechanism of virus spread and replication considered for targeting during kick and kill studies and novel therapeutic development.

HIV-1 Disease Progression.

We have previously shown that an inability of DC and B cell to *trans* infect autologous $CD4^+$ T cells with HIV-1 is associated with non-progression. We further identified a positive association between APC cholesterol content and *trans* infection ability (17). This was the first time *trans* infection was linked to HIV-1 disease progression. However, the mechanisms by which APC cellular cholesterol influences their *trans* infection ability and the ways in which HIV-1 *trans* infection contributes to disease progression remain unknown. A better understanding of the factors controlling *trans* infection in HIV-1 infected individuals may provide insight about therapeutic targets for disease control.

2.2.4 DC-SIGN and *Trans* Infection.

Many of the factors controlling *trans* infection ability or efficiency by a particular cell are related to the various mechanisms of *trans* infection described above. Since the first step in all three of the *trans* infection pathways (*cis*-mediated, endocytosis, and extracellular hitchhiking) require virus binding, the presence or absence of surface proteins and molecules known to mediate virus attachment, greatly dictate the ability of a cell to capture virus for eventual *trans* infection (16). HIV-1 CCR5 co-receptor blocking, which interrogates virus binding and the *cis* route of *trans* infection, decreases *trans* infection (64). This suggests that HIV-1 attachment to

APC via direct or indirect facilitators of *cis* infection is important for subsequent *trans* infection. DC-SIGN is known to mediate *cis* infection of cells by HIV-1 (81), as well as other viruses, including dengue virus (100) and ebola virus (101). The absence or blocking of DC-SIGN, known to mediate HIV-1 binding and endocytosis, can prevent *trans* infection of HIV-1 from DC to $CD4^+$ T cells (74). Although DC-SIGN has historically been considered a DC specific marker, IL-4 and CD40L activation of B cells induces DC-SIGN expression in approximately 10% of B cells (68), and it is essential for B cell *trans* infection of HIV-1 (68). *In vivo*, DC-SIGN is constitutively expressed on MΦ in lymph nodes (102), as well as adult lung and uterine tissue (103). *In vitro*, macrophage-stimulating colony factor (M-CSF) differentiation (104) or granulocyte-macrophage colony-stimulating factor (GM-CSF) differentiation and IL-13 stimulation (105) of MΦ also induce DC-SIGN expression. Although MΦ expression of DC-SIGN is well documented, the role of DC-SIGN in MΦ-mediated HIV-1 *trans* infection in the context of disease progression has not been determined.

2.3 CHOLESTEROL AND HIV-1

2.3.1 Cell Function.

Cholesterol is a naturally occurring lipid that is an essential component of all mammalian cells (106). It is required for cell survival and plays a role in many structural and biological functions of the cell (107, 108). Cholesterol is the primary component of PM lipid rafts, along with sphingolipids and various other proteins (109, 110). Lipid rafts serve as regions of stability in the membrane thereby influencing membrane fluidity, trafficking, protein sorting, as well as

cell – to – cell interactions and signaling (111-113). Cholesterol metabolism and cellular storage play a well- documented role in cardiovascular disease (114). Cholesterol loaded cells, such as macrophages, are primary components of arterial plaques and atherosclerosis, the main cause of cardiovascular disease (115). In addition, alternations in cholesterol are implicated in several forms of cancer (116), as well as diabetes and Alzheimer's disease (117). Additionally, many viruses have come to both indirectly and directly take advantage of the presence of cholesterol in host target cells. Determining the ways in which cellular cholesterol impacts disease viral infections and subsequent outcomes requires an understanding of how cholesterol homeostasis, trafficking and compartmentalization occurs within cells.

2.3.2 Cellular Cholesterol Homeostasis.

Cellular cholesterol metabolism relies on an intricate balance of cholesterol uptake, efflux, and intracellular synthesis. Cholesterol enters cells as part of low density lipoprotein (LDL) complexes. LDL and oxidized LDL are endocytosed into early endosomes mediated by their respective receptors, the LDL receptor (LDLR) and CD36 (118, 119). Within endosomes, oxidized LDL is reduced by cholesterol esterase into unesterified free cholesterol (FC) and oxysterols. The majority of FC is incorporated in cellular membranes, primarily the PM (120). When intracellular cholesterol levels rise, oxysterols act as a ligand for nuclear receptors, such as PPARg, LXRs, and RXRs, which when bound initiate transcription of cholesterol transporter proteins ABCA1 and ABCG1 which can actively transport cholesterol to the PM for efflux of excess FC out of the cell to acceptor high density lipoprotein (HDL) complexes (121). FC can also passively diffuse from the PM to acceptors (122). Alternatively, when intracellular levels of cholesterol are low, absence of FC in the endoplasmic reticulum activates sterol regulatory

element-binding proteins (SREBPs) which increases LDLR production and expression on the cell surface to aid in cholesterol uptake (123).

FC is commonly converted to esterified cholesterol, or cholesterol esters, in order to be more effectively packaged into lipoprotein complexes. Once cholesterol is effluxed to an acceptor, such as Apolipoprotein rich HDL and LDL complexes, it is transported to other parts of the body. HDL then facilitates cholesterol excretion through reverse cholesterol transport (RCT). The removal of cholesterol from plaque imbedded cells by HDL through RCT is an important natural mechanism of reducing atherosclerosis (124, 125). Once RCT is accepted from cells, it is transported by HDL through the blood to the liver from which it is excreted through the urinary tract directly as cholesterol or after conversion to bile acid (126).

Alternatively, cholesterol can be synthesized *de novo* within in cells by the mevalonate pathway. More specifically, cholesterol is generated from acetyl CoA mediated by the rate limiting HMG-CoA reductase, followed by a series of 19 enzymatic reactions (127). Cholesterol synthesis is regulated by the presence of absence of sterols detected in the cells. In the absence of FC in the endoplasmic reticulum, activated SREBPs also initiate intracellular *de novo* cholesterol synthesis by inducing HMG CoA reductase synthesis (123).

2.3.3 Cholesterol and HIV-1 Infection.

Cellular cholesterol is required for HIV-1 *cis* (128, 129) and *trans* infection (130) and is therefore likely essential for HIV-1 pathogenesis. In addition, cholesterol has been implicated in mechanisms of infection for many other viruses (131-140). Cholesterol within APC and target cell membranes, as well as the virions, has been shown to play a crucial role in HIV-1 binding, fusion, and entry of target cells (128, 129, 141, 142) as well as HIV-1 endocytosis by APC.

The role of cholesterol in HIV-1 *cis* infection has been thoroughly studied. The concentration of cholesterol in cells targeted by HIV-1, as well as the envelope of the virus affects *cis* infection efficiency. Cholesterol content in cell PMs is important for HIV-1 binding. CXCR4 and CCR5 coreceptor functional integrity requires cholesterol in membrane lipid rafts (143, 144), and cell membrane cholesterol depletion leads to an increase in resistance to both R5- and X4- tropic HIV-1 infection (129). Furthermore, sufficient cholesterol and lipid rafting is important for the function of endocytosis mediating receptors. For example, DC-SIGN localizes in cholesterol-rich lipid rafts on the cell surface (50) and relies on rafting to for efficient internalization of ligands (50, 145). Additionally, cholesterol in the virus envelope is required for fusion with the target cell membrane and subsequent infection (128). In fact it has been shown that HIV-1 virions prefer to bud from PM areas rich in lipid rafts (22).

Glycosphingolipid content of the cell membrane from which the HIV-1 virion is derived dictates the ability of immature and mature DC to *trans* infect the virus (146). In addition to independently mediating HIV-1 binding (142), cholesterol is implicated in HIV-1 uptake by APC and *trans* infection. Increased cholesterol efflux through the activation of PPARg and LXR significantly decreases DC *trans* infection of HIV-1 to $CD4^+$ T cells (130). Previously, our lab identified a relationship between APC cholesterol, HIV-1 *trans* infection, and disease progression. More specifically, significantly lower DC and B cell cholesterol content was associated with a complete lack of HIV-1 *trans* infection by NP DC and B cells (17). While extensive work has characterized the association of cholesterol with binding, entry, and budding of HIV-1 particles from target $CD4^+$ T cells, few studies have analyzed the mechanisms by which cellular cholesterol content controls the course of HIV-1 passage through APC in the setting of *trans* infection. Analyzing virus-APC interactions in NP compared to PR and SN will

provide insight into disease progression of these individuals. More importantly, it will help identify essential components of *trans* infection that might serve as therapeutic targets for preventing HIV-1 progression to AIDS, elimination of the latent HIV-1 reservoir, or ultimately virus clearance.

2.3.4 Statin Alterations of HIV-1 Infection.

Several studies and clinical trials have analyzed the potential uses of statins as an HIV-1 therapy. Statins have been assessed for their ability to lower chronic inflammation and immune activation associated with HIV-1 infection (147-149), combat cART-associated dyslipidemia and cardiovascular disease risk (150, 151), as well as decrease overall mortality in HIV-1 seropositive individuals (152). To date, these studies collectively suggest that the use of statins does not significantly augment current HIV-1 treatment regimes. However, a recent study found that individuals currently taking statins have fewer documented cases of viral rebound after therapeutic viral suppression (153). Importantly, these studies were all conducted on groups of HIV-1 infected individuals on cART and a more recent study has shown that cART drugs significantly alter statin pharmacokinetics, including SIMV (154). In cART naïve individuals, statin treatment reduces viral load, which rebounds after statin interruption (155). Since cell-to-cell transmission reduces the susceptibility of HIV-1 to cART (14) there is a need for novel therapeutics that target HIV-1 *trans* infection in addition to cART. However, further re-evaluation of the effect of statins is required to determine their potential as an anti-HIV-1 therapeutic.

2.4 MACROPHAGES AND HIV-1

2.4.1 Multifunctional MΦ.

MΦ are immune cells with great functional diversity. In addition to aiding in immune surveillance and responses they are important for the removal of necrotic cell debris, wound healing, and lipid metabolism (156). Coincidentally, MΦ are implicated in the pathophysiology of noninfectious diseases such as ischemic heart disease (157), multiple sclerosis (158), diabetes (159), kidney disease (160), asthma (161), among others (162). In addition to their involvement in noninfectious disease, they are targeted by several viruses (163-168), including HIV-1 (65). Secondary to the $CD4^+$ T cell, MΦ are the primary cells infected by HIV-1 and are involved in HIV-1 pathogenesis and disease progression. Due to their central location at the intersection of HIV-1 infection, cholesterol metabolism, and the anti-HIV-1 immune response, MΦ serve as an avenue by which the interaction of these factors can be studied.

2.4.2 Front Line Defense and Antigen Presentation.

MΦ are prominent and efficient phagocytic and antigen presenting cells. Following differentiation from monocytes, a small percentage of MΦ remain in the blood and circulate through the body, while the majority translocate into peripheral tissue where they continually survey the extracellular environment through pinocytosis. They also engulf larger constituents of the extracellular space, such as protein complexes, exosomes, and even pathogens, through phagocytosis (169). Additionally, they mediate active receptor mediated endocytosis of a number biological complexes, as well as pathogens (170-172). Their expression of pathogen recognition

receptors, such as DC-SIGN and Toll-like receptors, allows for efficient detection of intact pathogens on the surface and within endocytosed vesicles, as well as in the cytoplasm following infection.

Following pathogen detection, MΦ aid in the recruitment of other immune cells to the site of infection through the induction of an innate proinflammatory response. Intracellular pathogens are then processed into peptide antigens for presentation by class I and class II MHC molecules as MΦ migrate out of the tissue and through the blood and lymph system to lymphoid organs rich in lymphocytes. MΦ then return to the peripheral tissue were they reside and continue surveillance (173).

Some MΦ reside in the outer sinus of lymphoid organs on guard for entering cell-free or cell-associated pathogens, and retroviruses traveling to lymph nodes can be picked up by macrophages and passed in *trans* to B cells in follicles (72), which may aid in the entry to HIV-1 into lymph node T cell zones, and ultimately the enhancement of HIV-1 replication. However, unlike their antigen presenting counterparts, the DC and the B cells, MΦ are crucial for systemic processes other than antigen presentation and mounting an effective immune response, such as wound healing and cholesterol metabolism.

2.4.3 Wound Healing and Cholesterol Metabolism.

MΦ which have internalized excessive lipoproteins, known as cholesterol-rich foam cells, are commonly found in arterial plaques and are heavily involved in cardiac plaque development (18, 19). More specifically, oxidized LDL (ox-LDL) induces plaque endothelial cell production of inflammatory mediators which results in an accumulation of MΦ. An increase in cholesterol uptake paired with a decrease in cholesterol efflux leads to MΦ lipid – overloading and foam

cells embedding in the growing plaque. Various autocrine and paracrine factors influence foam cell formation (174). MΦ ER stress up regulates CD36 expression and subsequent foam cell formation (175), and extracellular factors such as ox-LDL and the cytokine thymic stromal lymphopoietin promote MΦ lipid accumulation by increasing CD36 mediated cholesterol uptake and decreasing ABCA-1 mediated cholesterol efflux (176, 177). Anti-atherosclerotic therapies designed to decrease foam cell formation target these factors and their mechanisms (178). However, HIV-1 has mechanisms that decrease cholesterol efflux from infected cells to maintain higher cholesterol content which increases cell susceptibility to infection and is beneficial for progeny virions, as previously discussed. More specifically, the HIV-1 encoded protein nef alters MΦ ability to maintain cellular cholesterol homeostasis by binding ABCA1 and thereby decreasing RCT (179).

2.4.4 MΦ and HIV-1.

MΦ are the primary targets for CCR5 coreceptor using, or R5-tropic HIV-1 strains (180). In response, MΦ target virus for degradation, induce IFN-mediated immune responses, and express HIV-1 restriction factors, such as APOBEC3G and SAMHD1 (*see* ***"HIV-1 restriction factors and therapies"***), which limit virus replication as previously discussed. However, HIV-1 have developed mechanisms to negatively regulate such MΦ antiviral measures. HIV-1 encoded vpr protein can block MΦ degradation of the newly synthesized viral env protein (181). MΦ comprise up to 10% of HIV-1 infected cells in infected individuals (182), and although MΦ were one of the first cells types shown to *trans* infect $CD4^+$ T cells with HIV-1 (183), their cholesterol content and *trans* infection ability has not been studied in association with HIV-1 disease

progression. Due to their intimate interactions with T cells, the role of MΦ in *trans* infection may be of paramount importance for HIV-1 replication, persistence, and pathogenesis.

2.5 A ROLE FOR MIRNA IN HIV-1 DISEASE PROGRESSION

2.5.1 miRNA – Health and Disease.

MicroRNA (miRNA) are endogenous small non-coding RNA sequences, commonly between 21 and 25 nucleotides long, that act to silence and in some cases enhance the expression of genes by interfering with protein expression post transcriptionally. Mature miRNA are generated from longer per-miRNA transcribed and processed in the nucleus into 70nt with a stem loop structure. Once processed into mature miRNA, they are translocated to the cytoplasm where they interact with mRNA prior to translation along the ER. miRNA most commonly bind to the 3' untranslated region (UTR) of the target mRNA, without altering coding regions and repress subsequent translation (184), known as RNA interference (RNAi). Perfect complementary binding of miRNA causes cleavage of the target mRNA by the RNA-induced silencing complex (RISC) associated with the miRNA (185). When a miRNA does not have sufficient complementary binding, translation of the mRNA is repressed without cleavage (186).

MiRNA encoded by human cells play important roles in the regulation of gene expression. They influence the differentiation, function, and survival of many cell types including B cells (187), T cells (188), endothelial cells (189), natural killer T cells (190), and macrophages (191), among others (192). miRNA are also linked to human pathologies, such as cancer (193) and neurological disease (194-196). Despite the initial excitement surrounding the discovery of miRNA for their therapeutic potential, the development of gene expression altering

miRNA treatments has been limited, primarily due to unwanted off-target effects (197). However, novel research is progressing the design and use of miRNA for therapy (198).

Due to their relatively short sequence, mRNA target sequences of many miRNA are found in multiple locations within the transcriptome. This allows miRNA to serve multiple biological functions. For example, the human miRNA miR-122 targets the NOD2 gene and is implicated in Crohn's disease (199), but also down regulates VEGFC gene expression which has positive implications for bladder cancer (200). In addition to their regulation of gene expression for normal cell function and homeostasis (192), some human miRNA aid in fighting infections and decreasing pathogen burden.

2.5.2 MiRNA and Cholesterol Metabolism.

MiRNA are also implicated in cardiovascular disease and atherosclerosis. The posttranscriptional regulation of expression of proteins involved in cholesterol metabolism by miRNA is a key element in cholesterol homeostasis (201). MiRNA are involved in cholesterol sensing, efflux, and uptake by cells.

Multiple miRNA have been found to target SREBP expression, a protein essential for intracellular cholesterol sensing. Interestingly, they act in a positive feedback loop initiated by SREBP to inactivate SREBP suppressors. For example, miR-182 lowers protein expression of FBXW7 (202) and miR-24 targets INSIG1 (203), two proteins involved in SREBP functional suppression. This leads to an increase in SREBP cholesterol sensing ability.

The two SREBP genes, SREBF1 and SREBF2, which encode proteins SREBP1 and SREBP2, contain introns that encode miR-33b and miR-33a, respectively (204). Humans express two copies of miR-33, referred to as miR-33a and miR-33b. The SREBP-1 proteins are primarily

involved in lipogenesis, while SREBP-2 proteins are more involved in cholesterol metabolism (205). Consequently, miR-33a co-regulates cholesterol efflux through the down regulation of ABCA1 expression in human cells thereby decreasing ABCA1-mediated cholesterol efflux (206). MiR-33 expression in MΦ also plays a role in MΦ differentiation, as well as metabolism, which is particularly pertinent in the development of atherosclerosis (207). In fact, anti-miR-33a treatment studies have demonstrated that in the absence of miR-33a, cholesterol efflux from cells increases from human cells, and miR-33a knockout results in decreased arterial plaque progression in mice (208). Additionally, miRNA are now considered viable targets for novel atherosclerosis treatments (209). Other miRNA, such as miR-144 (210) and miR-26 (211), are known to target and down regulate protein synthesis of ABCA1. Alternatively, LXR negatively regulates cholesterol efflux through inducing the synthesis of miR-613 from SREBP-1c, which in turn inhibits expression of LXR, thereby decreasing ABCA1 synthesis and cholesterol efflux (212).

In addition to cholesterol efflux, miRNA have been implicated in the uptake of cholesterol. When miRNA down regulate the activity of SREBPs, expression of cholesterol synthesis and uptake genes, such as HMG CoA reductase and the LDL receptor, controlled by SREBPs are also down regulated due to their regulation by SREBPs (213). Additionally, miRNA can directly target proteins that mediate cholesterol uptake. For example, miR-27a and miR-133a directly target and decrease expression of the LDL receptor (214) and CD36 (215), respectively. Due to their extensive regulation of cholesterol metabolism, miRNA likely play a role in cholesterol homeostasis differences observed in APC of NP and PR.

2.5.3 MiRNA and HIV-1 Infection.

Since the discovery of the therapeutic potential of miRNA, the relationship between miRNA and HIV-1 infection and disease has been studied. Individual miRNA, as well as miRNA profiles are associated with host cell – HIV-1 interactions, susceptibility to HIV-1 infection, the immune response to HIV-1 infection, as well as disease progression. Understanding the role of miRNA in HIV-1 infection and disease is essential for determining if they can be targeted or used in treatment.

MiRNA profiles during HIV-1 infection.

Several studies have demonstrated that host miRNA profiles are altered following HIV-1 infection, however the manner in which is unclear. More specifically, the expression of miRNA involved in the regulation of cell proliferation, apoptosis, and immune activation, is altered in PBMC following HIV-1 infection (216). This suggests that altered miRNA profiles may be involved in immune dysregulation during infection. Additional work further suggests that the down-regulation of miRNA is more abundant than up-regulation in PBMC after HIV-1 infection and that highly abundant miRNA associated with T cell function were down-regulated (217).

However, it is unclear whether changes in miRNA profiles are caused by the host response to infection or the virus directly. Changes in miRNA profiles proceeding HIV-1 infection can be an indirect result of the physiological and immunological response to exposure to the pathogen. Alongside mRNA expression, *ex vivo* activation or inactivation of $CD4^+$ T cells with anti-CD3 or IL-10, respectively, can alter miRNA expression (217). Alternatively, HIV-1 proteins can directly mediate miRNA expression. The presence of HIV-1 nef in cells, as well as excreted in exosomes, is associated with altered miRNA profiles in the cells and exosomes, and

nef containing exosomes are enriched for miRNA that target genes involved in immune activation and inflammation (218). It is possible that regulation of cellular miRNA expression and excretion through exosomes is another mechanism by which HIV-1 regulates the host response in favor of virus survival. This suggests that host miRNA play a role in determining HIV-1 – associated disease.

MiRNA and HIV-1 associated disease.

Since miRNA profiles change in response to HIV-1 infection and studies and HIV-1 can directly influence miRNA expression, it is becoming clearer that the mechanism of gene expression regulation mediated by miRNA is paramount to HIV-1 – host interactions. Additionally, more evidence is mounting for a role of miRNA in host susceptibility to HIV-1 infection, as well as subsequent vial pathogenesis and disease progression.

MiRNA expression is also associated individual susceptibility to HIV-1 transmission. A recent study revealed that HESN peripheral blood mononuclear cells (PBMC) and plasma have unique miRNA expression profiles that differentiate them from other healthy seronegative individuals and HIV-1 seropositive individuals. HESN express significantly higher levels of miRs-29a, 138, 150, 190, and 223 compared to HIV-1 positive individuals. More importantly, HESN express higher levels of miR-29a and miR-223 compared to healthy control (219), suggesting that these two miRNA are implicated in protecting HESN from infection.

Host cell susceptibility to HIV-1 infection varies by cell type. In some cases, this is a result of lack of expression of essential factors for infection, such as the primary receptor and co-receptors. Yet, while MΦ are highly susceptible to infection *in vitro* and *in vivo*, the monocytes from which they are derived are far less susceptible despite their expression of the HIV-1

receptor and co-receptors (220). In one study, circulating monocytes from healthy individuals were found to express high levels of miRNA known to limit HIV-1 replication (220). However, another study found anti- HIV-1 miRNA were up-regulated in MΦ rather than monocytes (221). Due to such contradictory findings, the extent to which miRNA dictate HIV-1 restriction is not clear. Overall, the literature suggests that miRNA play a more general role in HIV-1 infection rather than serve as primary determinants of cell susceptibility (222).

MiRNA have been further implicated in disease progression and the development of HIV-1 associated diseases. In addition to influencing the induction of HIV-1 latency (223), miRNA have been studied for their contribution to natural control of disease. Studies comparing HIV-1 seropositive progressors and nonprogressors or controllers revealed that there are miRNA profile differences in such groups (217, 224). These studies contributed to our understanding of miRNA profiles in individuals based on serostatus and disease state. Houzet *et.al.* assessed PBMC of PR, LTNP, and VC with unknown therapy status, while Reynoso *et.al.* assessed PBMC and plasma from EC and healthy seronegatives. These studies have contributed to our understanding of miRNA profiles in individuals based on serostatus and disease state, however a comprehensive study of therapy naïve NP is required for better insight into the role of miRNA in natural disease control in the absence of cART.

3.0 STATEMENT OF PURPOSE

Globally, over 35 million people are currently living with HIV-1, and cART fewer than 5% of seropositive individuals can naturally control HIV-1 infection and prevent progression to AIDS. NP represent an enigma in the field of HIV-1 and serve as a natural model of HIV-1 disease suppression wherein we can define mechanisms preventing disease progression. *Trans* infection, i.e. transfer of virus from cell-to-cell, of $CD4^+$ T lymphocytes is believed to be a crucial event contributing to HIV-1 persistence during cART. APC - mediated *trans* infection yields much higher virus replication in T cells compared to both *cis* infection of T cells and T cell-to-T cell *trans* infection. This may be particularly pertinent to APC interactions with T cells in lymphoid tissue, a prominent location of HIV-1 replication.

Our lab showed that lower cholesterol content in DC and B cells is associated with a total lack of *trans*-infection in NP (17). In addition to immune surveillance and antigen presentation, MΦ are involved in lipid metabolism (156), yet their role in HIV-1 *trans* infection is unknown**. We hypothesized that unique differences in MΦ cholesterol content contributes to lower association of HIV-1 with MΦ, thereby contributing to the lack of *trans* infection and disease progression characteristic of NP.** I proposed three specific aims to identify cholesterol-associated biological patterns and gene expression determinants of HIV-associated disease progression to further elucidate the effect of cholesterol metabolism on HIV-cell interactions during *cis* and *trans* infection.

4.0 SPECIFIC HYPOTHESES AND AIMS

4.1 AIM 1

Goal: Characterize MΦ cholesterol content and MΦ ability to mediate HIV-1 *trans* infection of CD4+ T cells in SN, NP, and PR

Hypothesis: Lower MΦ cholesterol content is associated with an inability to *trans* infect $CD4^+$ T cells.

Methods Summary: MΦ, DC, and B cells were assessed for total cellular cholesterol, as well as used for *in vitro* studies to investigate APC *cis* and *trans* infection of $CD4^+$ T cells in SN, NP, and PR. To further delineate the mechanism of cholesterol in *trans* infection, cell membrane – associated unesterified (free) cholesterol, and lipid rafting was assessed in MΦ.

Sub-aims

4.1.1 Aim 1a. MΦ, DC, and B cell *trans* infection in SN

4.1.2 Aim 1b. MΦ *trans* infection of NP and PR

4.1.3 Aim 1c. MΦ *cis* infection (high MOI) SN, NP, and PR

4.1.4 Aim 1d. Cholesterol characterization in MΦ of SN, NP, and PR

4.2 AIM 2

Goal: Investigate the role of cell cholesterol in MΦ *trans* infection.

Hypothesis: Alterations in MΦ cholesterol metabolism influence MΦ-mediated *trans* infection efficiency by regulating MΦ – virus interactions.

Methods Summary: SN, NP, and PR MΦ were phenotyped by flow cytometry to assess surface expression of proteins important in HIV-1 *cis* and *trans* infection. Additionally, MΦ cholesterol content was manipulated to assess the importance of cholesterol in MΦ – mediated *cis* and *trans* infection. MΦ binding and internalization of HIV-1 was assessed in NP and PR.

Sub-aims

4.2.1 Aim 2a. MΦ phenotyping for known regulators of *trans* infection

4.2.2 Aim 2b. SIMV effect on MΦ *cis* infection

4.2.3 Aim 2c. SIMV effect on MΦ - mediated *trans* infection

4.2.4 Aim 2d. Effect of altered cholesterol homeostasis on MΦ virus binding and internalization in NP

4.3 AIM 3

Goal: Identify potential mechanisms of differential cholesterol metabolism in NP and PR.

Hypothesis: Regulators of cholesterol metabolism are differentially expressed in NP and PR

Methods Summary: RNA extracted from NP and PR MΦ were assessed for mRNA expression levels corresponding to proteins involved in HIV-1 *trans* infection and cholesterol metabolism pathways including cellular cholesterol uptake, efflux, and synthesis. MiRNA extracted from NP and PR plasma was used to generate and assess miRNA profiles using NanoString technology to

identify miRNA that may be involved in disease progression, *trans* infection, and cholesterol metabolism.

Sub-aims

4.3.1 Aim 3a. Expression levels of proteins associated with nonprogression by PCR

4.3.2 Aim 3b. Expression levels of proteins involved in MΦ cholesterol metabolism by PCR

4.3.3 Aim 3c. MiRNA screening of NP and PR plasma by NanoString

5.0 RESULTS

5.1.1 Aim 1a. MΦ, DC, and B cell *trans* infection in SN

MΦ-mediated HIV-1 *trans* infection enhances autologous $CD4^+$ T cell virus production in SN

To establish our model for assessing HIV-1 *trans* infection mediated by MΦ, we cultured monocyte-derived MΦ from 10 SN recruited from the Multicenter AIDS Cohort Study (MACS) (**Table 1**).

Table 1. Virological and genotypic characterization of SN, NP, and PR.

Subject	Infection duration (yrs)*	Mean $CD4^+$ T cell count ± SE	Mean HIV-1 load ± SE	CCR5Δ32 genotype	HLA class I B locus genotype
SN1	N/A	1200 ± 206	N/A	WT/WT	0801/5501
SN2	N/A	987 ± 142	N/A	WT/WT	0702/1501
SN3	N/A	770 ± 64	N/A	WT/WT	3503/4403
SN4	N/A	924 ± 94	N/A	WT/WT	2705/4403
SN5	N/A	615 ± 40	N/A	WT/WT	3801/3801
SN6	N/A	1198 ± 168	N/A	WT/WT	1402/5802
SN7	N/A	786 ± 101	N/A	WT/WT	4001/5501
SN8	N/A	683 ± 95	N/A	WT/WT	5001/5201
SN9	N/A	924 ± 233	N/A	WT/WT	3501/5501
SN10	N/A	928 ± 71	N/A	WT/WT	2705/4501
NP1	>8	924 ± 62	3,055 ± 566	WT/WT	3910/5301
NP2	>8	743 ± 59	3,242 ± 1,931	WT/WT	3501/8101
NP3	>29	1,259 ± 62	4,764 ± 4,432	WT/WT	1402/4701
NP4	>8	1,007 ± 31	571 ± 90	WT/WT	1501/4201
NP5	>18	759 ± 57	20,893 ± 7,585	Δ32/WT	1501/4402
PR1	7	232 ± 49	396,572 ± 136,552	WT/WT	5001/5701
PR2	>13	415 ± 119	12,507 ± 8019	WT/WT	1401/5101
PR3	>6	335 ± 154	13,524 ± 9606	WT/WT	0801/0801
PR4	>7	424 ± 135	25,643 ± 3,935	WT/WT	4403/5601
PR5	4	598 ± 387	188,576 ± 119,553	WT/WT	0801/3503

*Years HIV-1 seropositive prior to cART therapy while enrolled in the Multicenter AIDS Cohort Study (MACS). N/A = not applicable; > indicates individuals were seropositive upon enrollment into the MACS. Mean $CD4^+$ T cell count and Mean HIV-1 load were calculated from compiled data from all visits while enrolled in the MACS prior to ART.

MΦ were loaded with a low multiplicity of infection (MOI) of HIV-1 R5-tropic Bal (MOI 10^{-3}), followed by co-culture with autologous $CD4^+$ T cells and assessment of HIV-1 p24 core antigen in cell-free supernatant. We chose to load MΦ with an MOI of 10^{-3} because it is suboptimal for efficient *cis* infection of $CD4^+$ T cells, yet it is highly effective in APC-T cell *trans* infection (17). Under these conditions, MΦ-mediated *trans* infection of HIV-1 to autologous $CD4^+$ T cells was detected in eight of 10 SN. The *trans* infection of the eight SN was

demonstrated by detectable HIV-1 p24 by 4-12 days of co-culture (**Fig.1A**). This was comparable to our established models of *trans* infection mediated by activated B cells (**Fig. 1B**) and monocyte-derived DC (**Fig. 1C**) from the same SN. By day 12 of co-culture, MΦ-mediated *trans* infection of $CD4^+$ T cells was significantly greater ($P< 0.05$) than virus production in *cis* infected $CD4^+$ T cells (**Fig. 1D**). Moreover, as seen with MΦ, both B cell- and DC-mediated *trans* infection enhanced overall production of p24 compared to T cell *cis* infection (**Fig. 1E and 1F**).

Notably, none of the APC types mediated detectable *trans* infection in the 2 of the ten SN tested (SN4 (orange) and SN5 (purple); **Figs. 1D, 1E, 1F**). These results were confirmed by repeated testing of APC from these same MACS participants at different clinic visits.

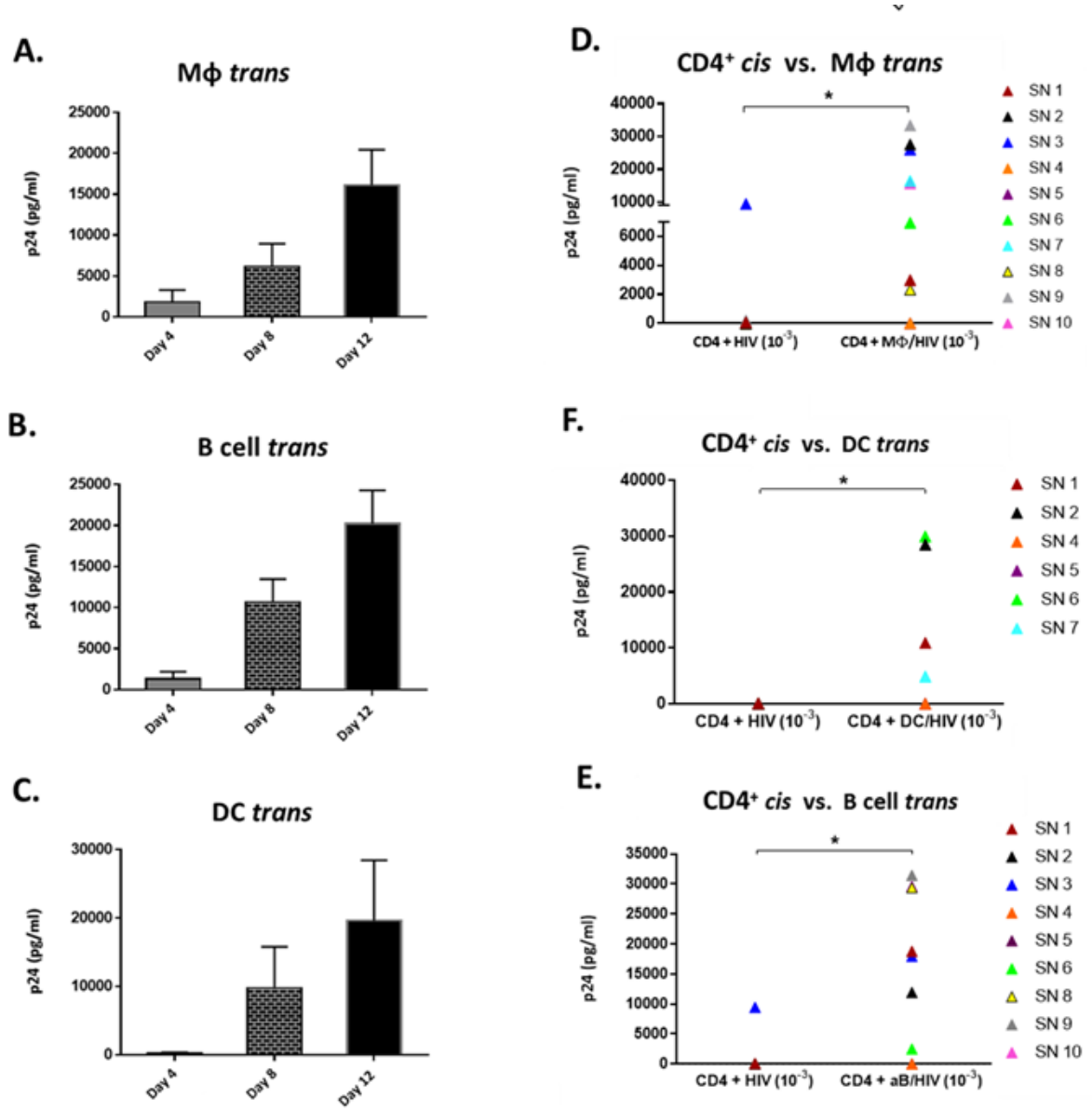

Figure 1. MΦ - mediated *trans* infection enhances virus production in autologous CD4$^+$ T cell in SN.

(A) SN MΦ (n = 8), (B) B cells (n = 7), and (C) DC (n = 6) pulsed with HIV-1 Bal (MOI 10^{-3}) were co-cultured with autologous activated CD4$^+$ T cells for 12 days. Supernatant was assessed for p24 concentration at days 4, 8, and 12. (D) CD4$^+$ T cells pulsed with a higher concentration of HIV-1 Bal (MOI 10^{-1}) and analyzed for *cis* infection were compared to MΦ, (E) B cell, and (F) DC *trans* infection at day 12. * = $p < 0.05$. Histograms are expressed as mean $\pm$ SE.

MΦ *trans* infection efficiency is not due to CD4$^+$ T cell susceptibility to HIV-1 infection.

MΦ loaded with the low concentration of HIV-1 (MOI 10^{-3}) and cultured alone for *cis* infection in parallel to each *trans* infection experiment demonstrated negative or minimal levels of p24 for all SN (**Fig. 2A**). We also did not detect infection in *cis*-exposed CD4$^+$ T cells at the low MOI (10^{-3}) (**Fig. 2B**) with the exception of one participant (SN3). As expected, CD4$^+$ T cells from all SN, including SN4 and SN5, were susceptible to *cis* infection with a high concentration of HIV-1 (MOI 10^{-1}) (**Fig. 2C**). Although SN4 and SN5 in the cohort did not support detectable *trans* infection, no correlation was found between MΦ *trans* infection and autologous CD4$^+$ T cell *cis* infection efficiency (**Appendix C, Supplemental Fig. 1**). Taken together, these data support that both myeloid- and lymphoid-derived APC share a common feature essential for HIV-1 *trans* infection, independent of susceptibility of T cells to *cis* infection.

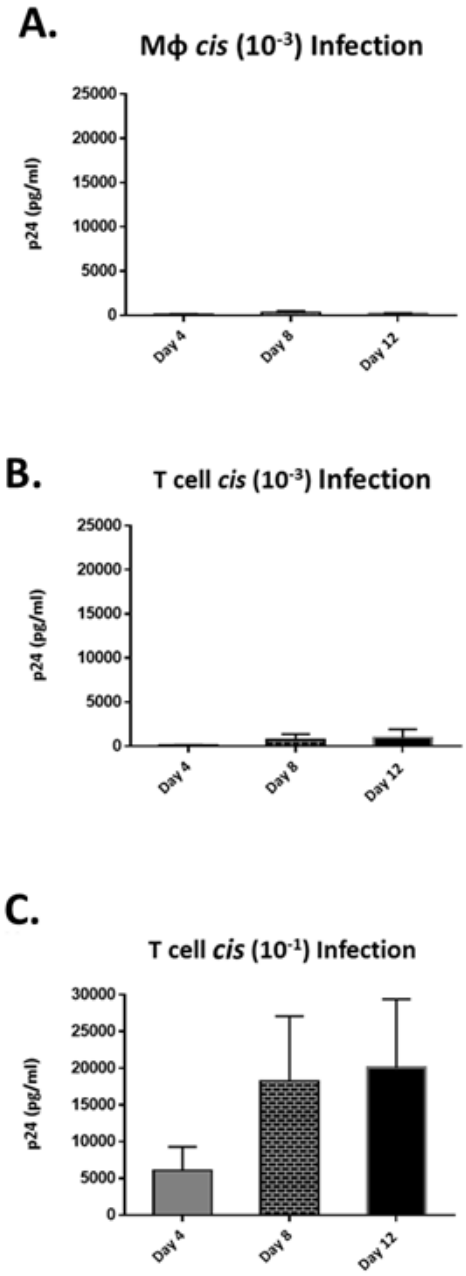

Figure 2. MΦ *trans* infection efficiency is not due to CD4$^+$ T cell susceptibility to HIV-1 infection.

(A) MΦ pulsed with HIV-1 Bal (MOI 10^{-3}) were cultured alone to control for MΦ *cis* infection in the co-cultures (n = 10). (B) CD4$^+$ T cells were pulsed with HIV-1 Bal (MOI 10^{-3}) as a *cis* control and compared to MΦ – T cell co-cultures (n = 10). (C) CD4$^+$ T cells were pulsed with a higher concentration of HIV-1 Bal (MOI 10^{-1}) as a *cis* control to assess susceptibility to infection (n = 10). * = $p < 0.05$. Histograms are expressed as mean $\pm$ SE.

5.1.2 Aim 1b. MΦ *trans* infection of NP and PR

MΦ mediate HIV-1 *trans* infection in PR but not NP

Previous work from our lab demonstrated that DC- and B cell-mediated *trans* infection was defective in NP while being completely functional in PR (17). Therefore, we sought to determine if MΦ-mediated *trans* infection was also altered in NP compared to PR and SN. We assessed *trans* infection efficiency of MΦ from 5 PR, 5 NP, and 10 SN (Table 1). PBMC samples used were acquired while individuals were cART naïve and not taking cholesterol-lowering medication.

MΦ exposed to the low concentration (MOI 10^{-3}) of HIV-1 were co-cultured with autologous $CD4^+$ T cells for 12 days; co-culture supernatants were assessed over time for HIV-1 p24 production. Under these conditions, MΦ-mediated *trans* infection was undetectable in all NP tested throughout the 12 days of co-culture, while MΦ from PR were able to *trans* infect autologous $CD4^+$ T cells. In addition, MΦ *trans* infection in PR was not significantly different from that of SN beyond day 4 (**Fig. 3A**). *Cis* infection controls of $CD4^+$ T cells alone with the low MOI used for *trans* infection experiments were very low or undetectable in all clinical groups (**Fig. 3B**). However, $CD4^+$ T cells from all PR and NP (**Fig. 3C**) were susceptible to *cis* infection with a higher MOI (10^{-1}). Taken together, these data show that MΦ derived from NP do not efficiently transfer HIV-1 to autologous $CD4^+$ T cells, which are capable of supporting HIV-1 $CD4^+$ T *cis* infection, in accordance with our previous DC and B cell data (17).

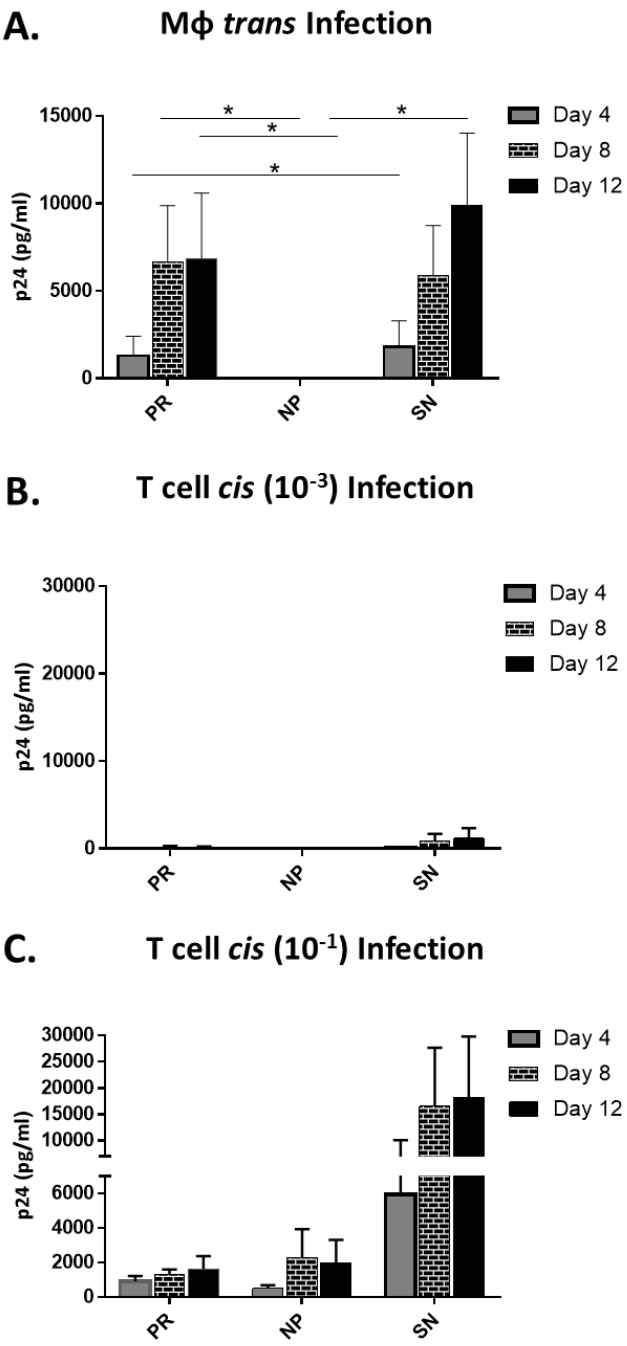

Figure 3. MΦ-mediated *trans* infection is negative in NP compared to PR and SN.

(A) MΦ from PR, NP, and SN were pulsed with HIV-1 Bal (MOI 10^{-3}) and co-cultured with autologous activated CD4 T cells for 12 days. Supernatant was assessed for P24 concentrations at days 4, 8, and 12. (B) CD4 T cells were pulsed with HIV-1 Bal (MOI 10^{-3}) as a *cis* control and compared to MΦ – T cell co-cultures. (C) CD4 T cells were also pulsed with a higher concentration of HIV-1Bal (MOI 10^{-1}) as a *cis* control to assess susceptibility to infection. PR n=5, NP n=5, and SN n=10. * = $p \leq 0.05$. Histograms are expressed as mean $\pm$ SE.

5.1.3 Aim 1c. MΦ *cis* infection (high MOI) SN, NP, and PR

MΦ susceptibility to HIV-1 *cis* infection is associated with HIV-1 disease progression, but not required for HIV-1 *trans* infection

As demonstrated in **Figs. 1A and 2B**, MΦ *cis* infection was not required for MΦ *trans* infection in SN, as 8 or 10 SN *trans* infected despite undetectable MΦ *cis* infection. Next, MΦ susceptibility to HIV-1 *cis* infection was assessed across the three study groups using a higher concentration of virus. MΦ were exposed to HIV-1 Bal (MOI 10^{-1}) and cultured for 12 days. Four of the 10 SN exhibited detectable p24 by day 12 (**Fig. 4A**). However, no correlation was found between MΦ *trans* and *cis* infection efficiency in SN (**Appendix C, Supplemental Fig. 2**). MΦ *cis* infection was only detected in 1 NP (NP2) (**Fig. 4B**). However, it was detected in 4 of 5 PR (PR1, 2, 4, and 5) (**Fig. 4C**). Importantly, when compared at day 12, the magnitude of MΦ positive *cis* infection was significantly higher in PR than NP (**Fig. 4D**). These findings further support a novel divergence of MΦ function among PR and NP and its correlation with disease progression.

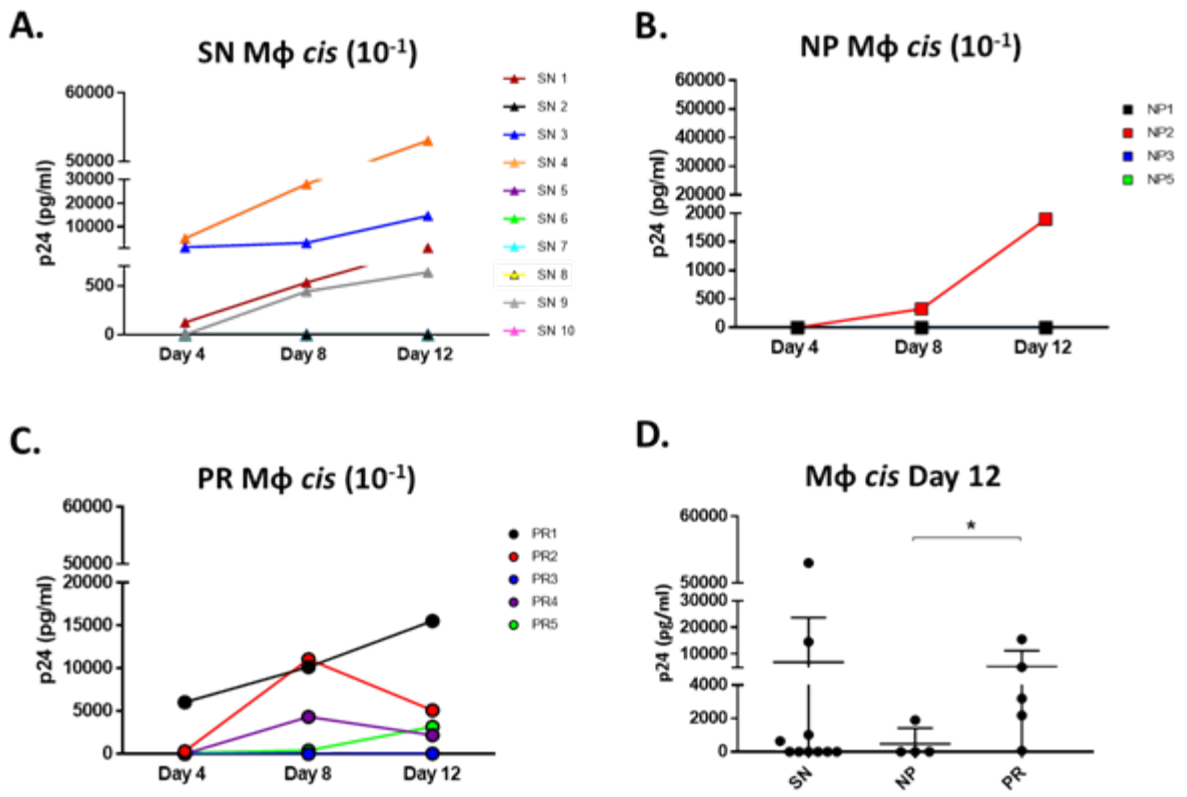

Figure 4. MΦ *cis* infection is significantly higher in PR than NP.

(A) MΦ from SN, (B) NP, and (C) PR pulsed with HIV-1 Bal (MOI 10^{-1}) were cultured alone and analyzed by p24 ELISA at days 4, 8, and 12. (D) P24 content was compared from day 12 supernatant of SN (n = 10), NP (n = 4), and PR (n = 5). *$p \leq 0.05$.

5.1.4 Aim 1d. Cholesterol characterization in MΦ of SN, NP, and PR

NP MΦ have lower total cellular than PR

We have previously shown that alterations in cellular cholesterol metabolism correlate with the inability of APC from NP to efficiently *trans* infect $CD4^+$ T cells (17). Therefore, we sought to determine if cellular cholesterol levels in MΦ were linked to HIV-1 *trans* infection and disease progression by quantifying total esterified and unesterified (free) cholesterol. In SN participants, total cholesterol was highest in MΦ, which was significantly higher than the B and T cells but not significantly different than DC. However, total cholesterol in DC trended lower than in MΦ (**Fig. 5A**). When comparing clinical groups, total cholesterol concentration of MΦ was significantly lower in NP than PR (**Fig. 5B**), suggesting that there is a cholesterol-dependent mechanism of *trans* infection in MΦ, in line with our previous findings in DC and B cells (17).

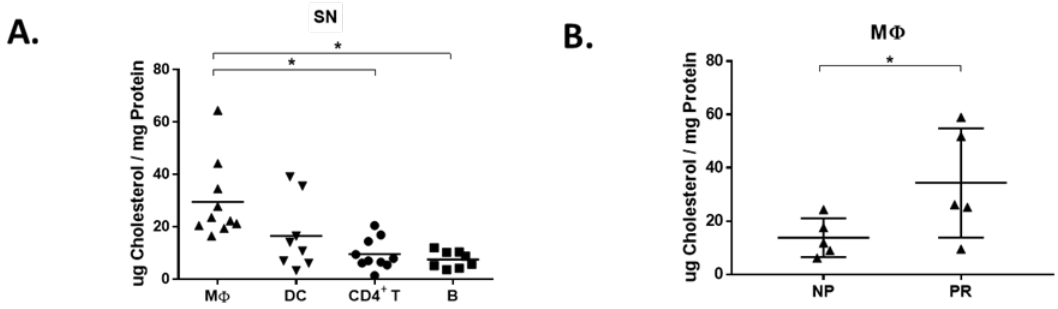

Figure 5. MΦ have lower total cellular than PR.
(A) Total cholesterol measured by Amplex Red of SN MΦ (n = 10), DC (n = 8), activated $CD4^+$ T cells (n = 10), and activated B cells (n = 8). (B) MΦ total cholesterol content was measured in NP and PR by Amplex Red.

NP MΦ have lower cell membrane-associated cholesterol than PR

To further elucidate the effect of decreased MΦ cholesterol in NP, we assessed the level of cell membrane-associated cholesterol as well as lipid rafting in NP and PR MΦ by flow cytometry using filipin III, a naturally occurring fluorescent polyene antibiotic that binds unesterified cholesterol (225), and the lipid raft containing GM1 specific marker cholera toxin subunit B (CTx-B). Membrane-associated cholesterol was significantly lower in NP MΦ compared to that of PR (**Fig. 6A**). Although some PR MΦ had very high levels of lipid raft staining, overall the lipid rafting detection of PR was similar to NP (**Fig. 6B**). Interestingly, despite similar intensities of CTx-B staining in NP and PR, single cell fluorescent images (acquired with the Millipore ImageStream X Mark II flow cytometer) revealed more punctate "rafting" of the CTx-B staining of PR compared to NP MΦ (**Fig. 6C**). This suggests lipid rafting distribution may indeed be different in NP than PR. Together, these data support that the presence of sufficient cholesterol in MΦ, specifically MΦ cell membranes, could be driving MΦ-mediated *trans* infection.

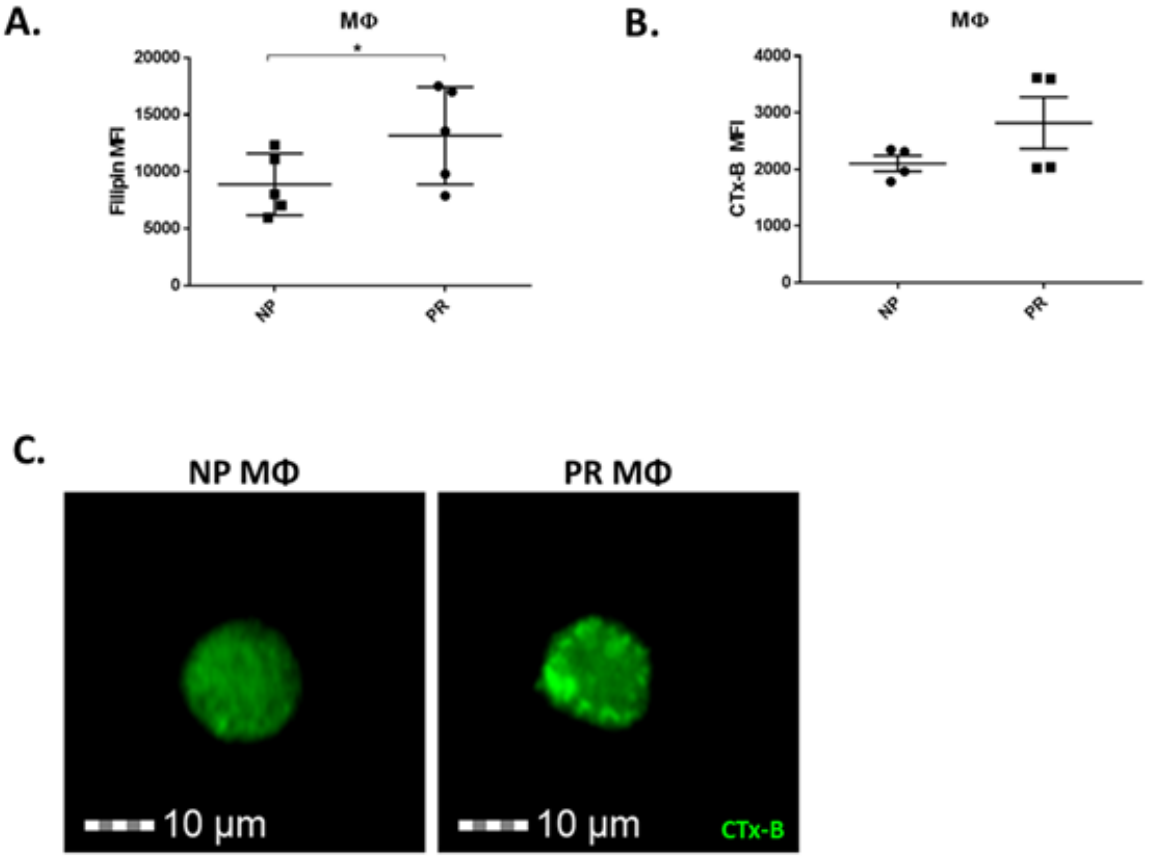

Figure 6. NP MΦ have lower cell membrane-associated cholesterol than PR.

(A) NP and PR MΦ were exposed to fillipin III for cell membrane cholesterol-specific labeling and analyzed by flow cytometry. (B) MΦ were exposed to CTx-B for lipid raft labeling and analyzed by flow cytometry with a Millipore ImageStream. (C) Representative images of NP and PR MΦ labelled with CTx-B analyzed using an ImageStream.

5.1.5 Aim 2a. MΦ phenotyping for known regulators of *trans* infection

The number of DC-SIGN expressing MΦ correlates with *trans* infection efficiency

To identify potential factors influencing MΦ *trans* and *cis* infection efficiency, MΦ were phenotyped by flow cytometry. To assess MΦ differentiation, CD16 expression, which is low on monocytes and high on macrophages (226), was compared on $CD14^+$ monocytes (Day 0) and cultured macrophages (Day 7). A significant increase in CD16 expression levels (mean fluorescence intensity or MFI) was observed in the cultured macrophages (**Fig. 7A and 7B**).

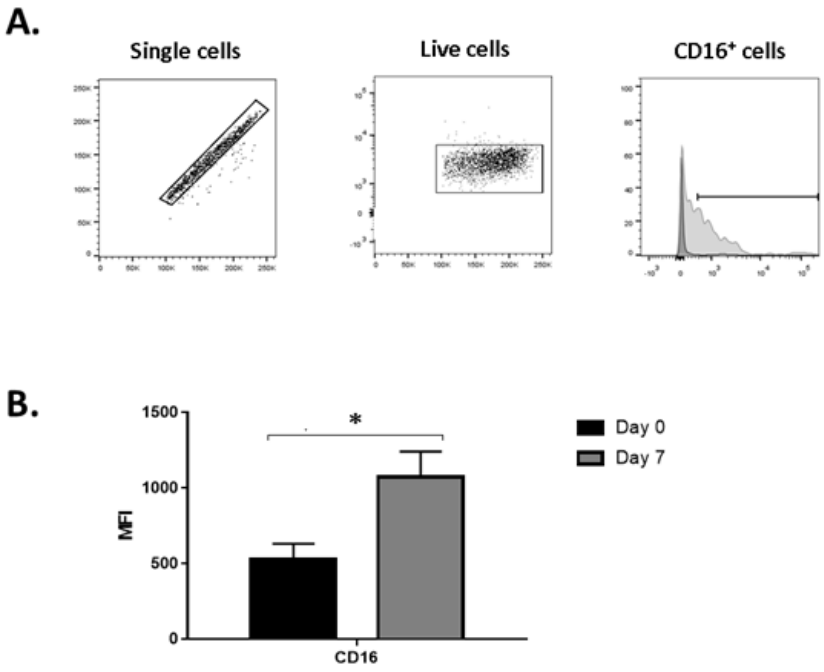

Figure 7. M-CSF stimulates MΦ differentiation from $CD14^+$ monocytes.

(A) Gating strategy and live cell selection for MΦ phenotype by flow cytometry. (B) CD16 surface expression level (MFI) of SN $CD14^+$ monocytes (Day 0) and cultured MΦ (Day 7) was assessed by flow cytometry (n = 9). Histogram is expressed as mean $\pm$ SE.

Trans infection of $CD4^+$ T cells is believed to occur by two different, but not mutually exclusive, pathways following DC or MΦ exposure to virus: **(1)** through rapid virus uptake via endocytosis into vesicles, and **(2)** 48h after *de novo* infection (16). Because MΦ are both susceptible to *cis* infection with R5-topic HIV-1 and capable of *trans* infection, cells were analyzed for classic markers associated with *cis* infection as well as HIV-1 endocytosis. MΦ surface expression of the primary HIV-1 receptor CD4, and co-receptor CCR5 for R5-tropic HIV-1Bal, was analyzed. CD4 expression level was significantly lower in HIV-1 infected NP compared to SN, and similar to MΦ expression in HIV-1 infected PR, while CCR5 expression was similar among the three groups (**Fig. 8A**). The C-type lectin, DC-SIGN facilitates endocytosis-mediated HIV-1 *trans* infection (68, 74). The sialic acid binding adhesion molecule, Siglec-1 has also been implicated in HIV-1 *trans* infection (75). Surface expression of both DC-SIGN and Siglec-1 was also assessed on MΦ. Expression levels of both glycoproteins did not differ among the three groups (**Fig. 8B**). However, a significantly lower percentage of NP MΦ were DC-SIGN positive compared to PR, while no difference was seen in the percentage of Siglec-1 positive cells (**Fig. 8C**). MFI of all four markers was variable within SN (**Fig. 8A and 8B**). Both cholesterol and DC-SIGN staining appeared dimmer in NP compared to SN and PR (**Fig. 9**). Collectively, these data suggest that the lack of MΦ-mediated *trans* infection seen in NP is associated with the expression of DC-SIGN, known to be involved in HIV-1 attachment (74, 227) and endocytosis (69).

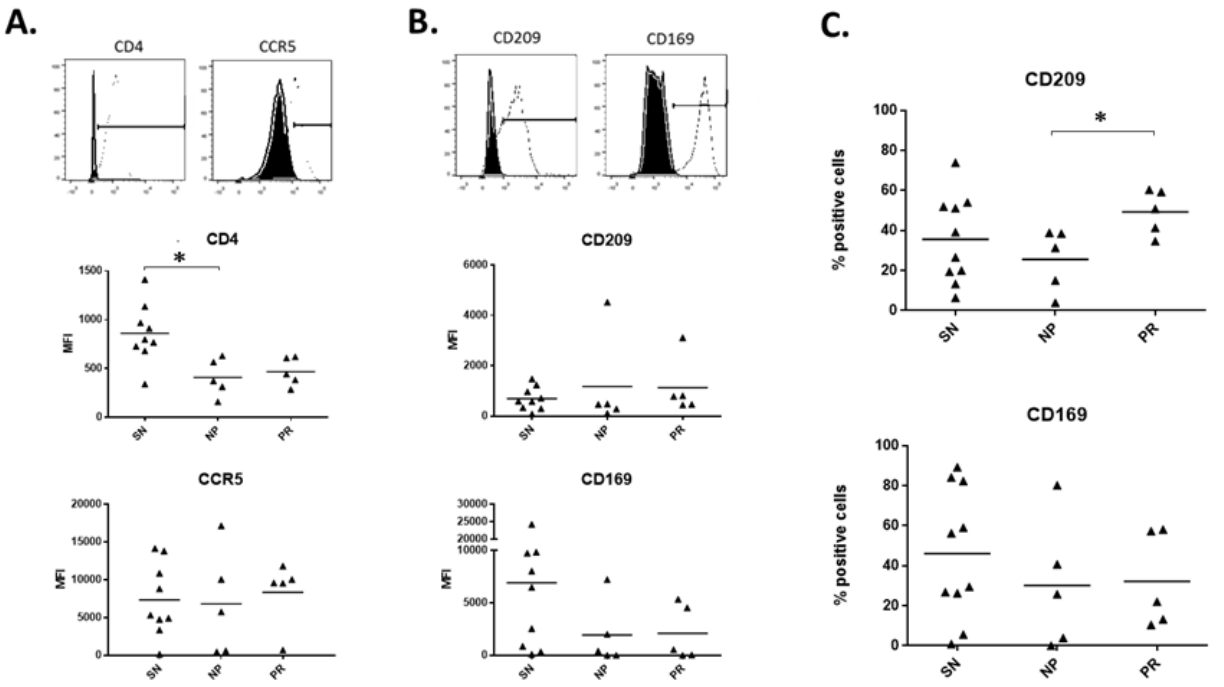

Figure 8. The number of DC-SIGN^{+} MΦ is lower in NP compared to PR.

(A) Representative fluorescence intensity histograms and individual mean fluorescence intensity (MFI) of MΦ measured by flow cytometry at culture day 7 for surface expression of CD4, CCR5, (B) CD209 (DC-SIGN), and CD169 (Siglec-1). (C) Percentage of MΦ displaying surface expression of DC-SIGN and Siglec-1 was analyzed by flow cytometry and compared among SN (n = 9 or 10), NP (n = 5), and PR (n = 5). *$p \leq 0.05$.

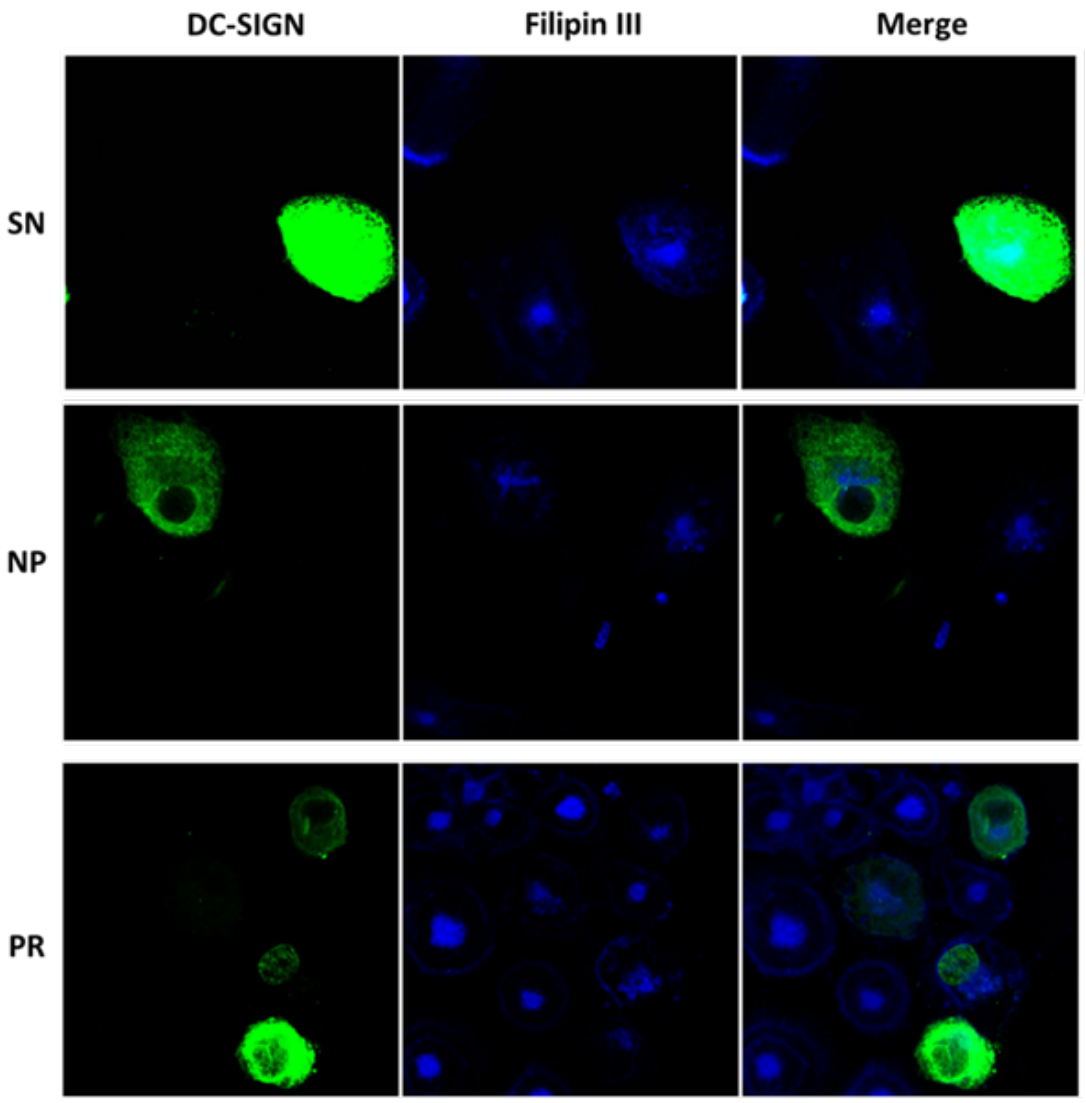

Figure 9. NP MΦ have lower cell membrane-associated cholesterol than PR.

Representative images from SN, NP, and PR MΦ labelled with Filipin III (blue) and DC-SIGN (green) followed by imaging using confocal microscopy.

MΦ *trans* and *cis* infection of HIV-1 are facilitated by DC-SIGN

In addition to observed differences in the number of MΦ expressing DC-SIGN in NP compared to PR, the number of DC-SIGN$^+$ MΦ positively correlated with *trans* infection efficiency in SN (**Fig. 10A**). To investigate further the role of DC-SIGN in HIV-1 *trans* infection, antibody blocking experiments were done as detailed in Materials and Methods. Blocking of DC-SIGN prior to virus exposure reduced MΦ susceptible to *cis* infection by 72% compared to isotype- treated MΦ by day 12 (**Fig. 10B**). In addition, blocking DC-SIGN on MΦ prior to virus exposure and co-culture with autologous CD4$^+$ T cells reduced MΦ *trans* infection by 87% by day 12 (**Fig. 10C**). Blocked MΦ stained negative for DC-SIGN by flow cytometry compared to isotype-treated MΦ, supporting a successful occlusion of DC-SIGN (**Fig. 10D**). These data indicate that a DC-SIGN-dependent mechanism of MΦ-virus engagement is pivotal for both MΦ *cis* and *trans* infection.

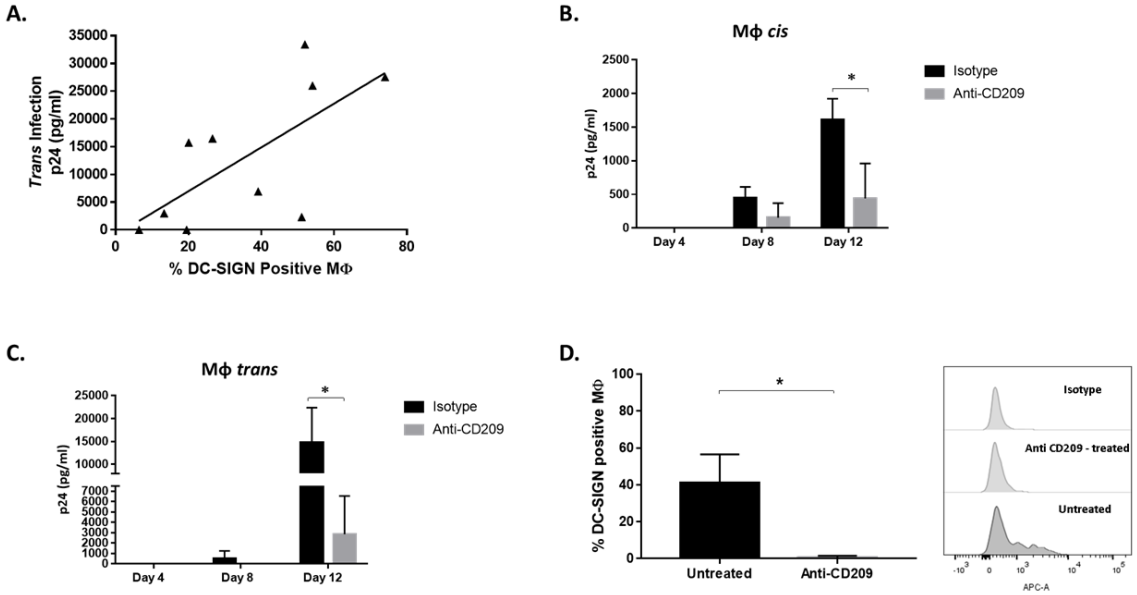

Figure 10. The number of DC-SIGN$^+$ MΦ is positively correlated with MΦ-mediated *cis* and *trans* infection efficiency.

(A) SN MΦ- mediated *trans* infection efficiency and percentage of DC-SIGN positive MΦ were assessed for association by linear regression ($p = 0.0280$, $F = 7.177$, $R^2 = 0.4729$) (n = 10). (B) Anti-DC-SIGN and isotype control treated SN MΦ were pulsed with HIV-1 Bal, MOI 10^{-1}, and

cultured alone, or with MOI 10^{-3} and cultured with autologous $CD4^+$ T cells to assess *cis* (C) and *trans* infection, respectively (n = 5). (D) SN MΦ were treated with a monoclonal antibody against CD209 (DC-SIGN) or an isotype control then assessed by flow cytometry for DC-SIGN detectability (n = 2). *p ≤ 0.05. Histograms are expressed as mean ± SE.

5.1.6 Aim 2b. SIMV effect on MΦ *cis* infection

SIMV decreases MΦ *cis* infection

It has previously been shown that SIMV decreases total cholesterol and HIV-1 *trans* infection ability of DC and B cells. To test the role of cholesterol in MΦ *trans* infection, we assessed whether SIMV, an inhibitor of cholesterol synthesis, would interfere with MΦ *cis* or *trans* infection. For this, MΦ treated with SIMV prior to virus exposure were used in *cis* and *trans* infection assays, as described in the Materials and Methods. *Cis* infection of MΦ was significantly decreased by 90% (**Fig. 11**).

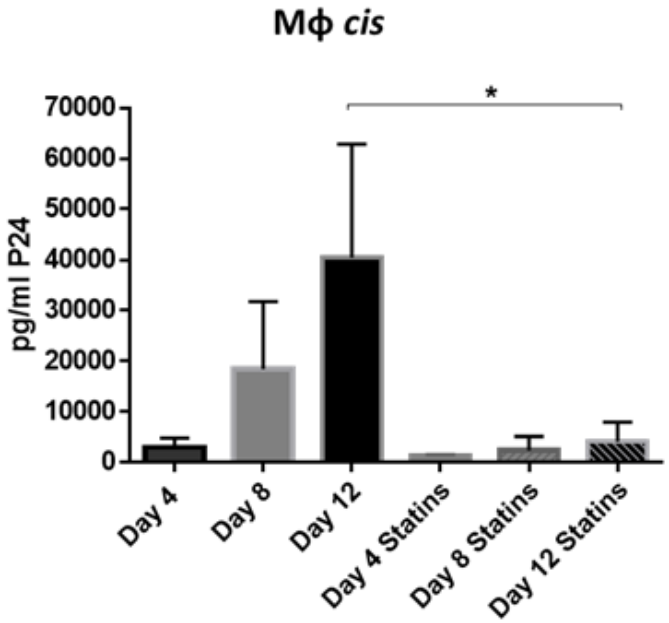

Figure 11. SIMV lowers MΦ *cis* infection.

SN MΦ left untreated or treated with 1ug/ml SIMV for 24 hours were assessed for *cis* infection. (n = 4). (C) *p ≤ 0.05. Histograms are expressed as mean ± SE.

5.1.7 Aim 2c. SIMV effect on MΦ - mediated *trans* infection

SIMV decreases MΦ *trans* infection

MΦ-mediated *trans* infection trended down sharply (**Fig, 12A**; p=0.06) in the presence of SIMV. We then assessed the impact of SIMV on the ability of MΦ to enhance infection of $CD4^+$ T cells in *trans* compared to cell free $CD4^+$ T cell *cis* infection. Consistent with our data in **Fig. 1D**, untreated MΦ loaded with HIV-1 significantly enhanced infection in *trans* compared to $CD4^+$ T cell *cis* infection by day 12 (**Fig. 12B**). However, SIMV-treated MΦ were unable to enhance infection in *trans* compared to *cis* infection in four of five SN samples tested (**Fig. 12C**). Although SIMV did not completely abrogate MΦ *cis* or *trans* infection, in both cases SIMV treatment lead to a decrease in P24 production. Most importantly, SIMV blocked the ability of MΦ to significantly enhance infection of $CD4^+$ T cells compared to *cis* infection.

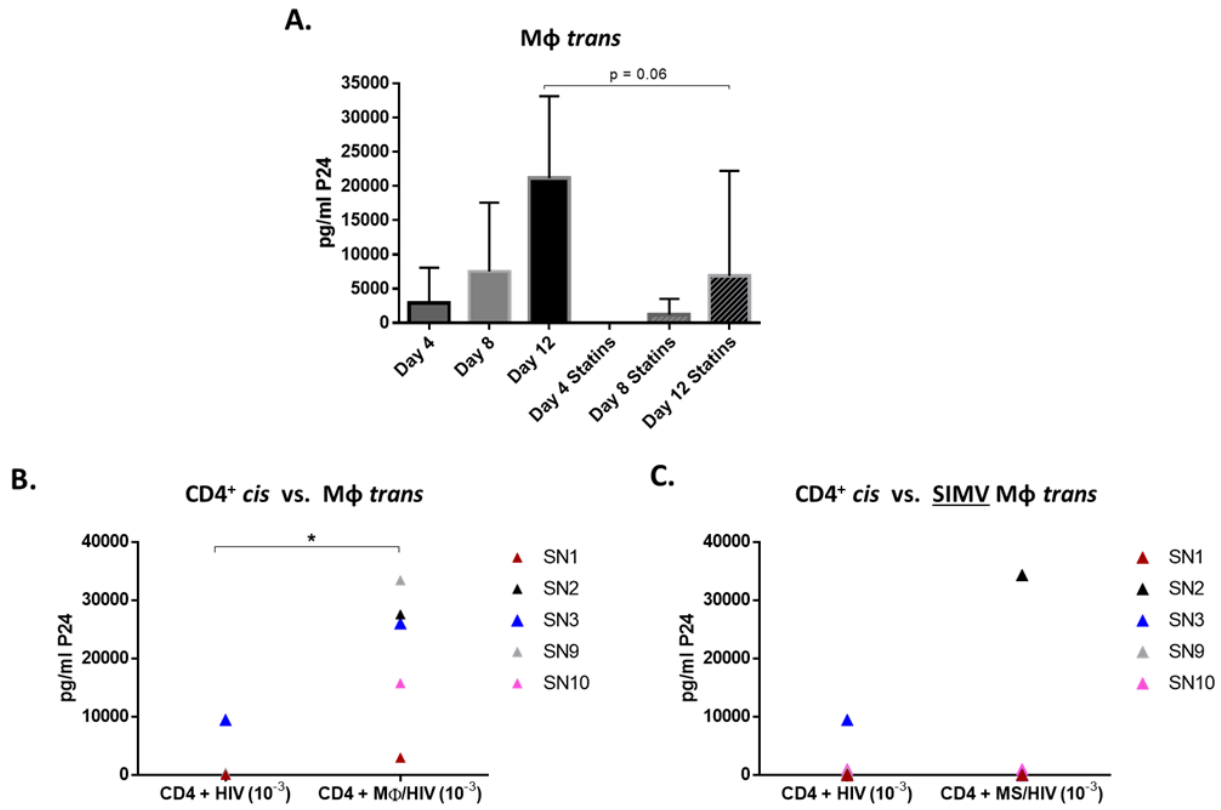

Figure 12. SIMV lowers MΦ *trans* infection.

(A) SN MΦ left untreated or treated with 1ug/ml SIMV for 24 hours were assessed for *trans* infection. (n = 4). (B) Untreated and (C) SIMV-treated MΦ (MS) *trans* infection was compared to autologous $CD4^+$ *cis* infection at day 12. *$p \leq 0.05$. Histograms are expressed as mean $\pm$ SE.

SIMV decreases DC-SIGN-expressing MΦ in a cholesterol-mediated manner

DC-SIGN RNA levels determined by RT-PCR were not different among SN, NP and PR (**Fig. 13A**). We sought to determine if SIMV treatment decreased *trans* infection by altering DC-SIGN protein expression on the surface of MΦ. SN MΦ treatment with SIMV followed by DC-SIGN analysis by flow cytometry revealed that SIMV decreased the number of DC-$SIGN^+$ MΦ compared to untreated cells (**Fig. 13B**). SIMV is a known competitor of HMG CoA reductase

and, in addition to serving as a source from which cholesterol is derived, HMG-CoA reductase also mediates protein prenylation in cells (228). Statins are known to interfere with this pathway in addition to cholesterol synthesis (229). Therefore, to elucidate which pathway SIMV was altering DC-SIGN expression, SIMV-treated MΦ were simultaneously treated with either squalene or geranylgeranyl pyrophosphate (GGpp), downstream products of the cholesterol synthesis and protein prenylation pathways, respectively. GGpp and squalene both abrogated the effect of SIMV on the number of DC-SIGN$^+$ MΦ, however the number of DC-SIGN$^+$ MΦ trended higher with the squalene treatment compared to GGpp (**Fig. 13C**).

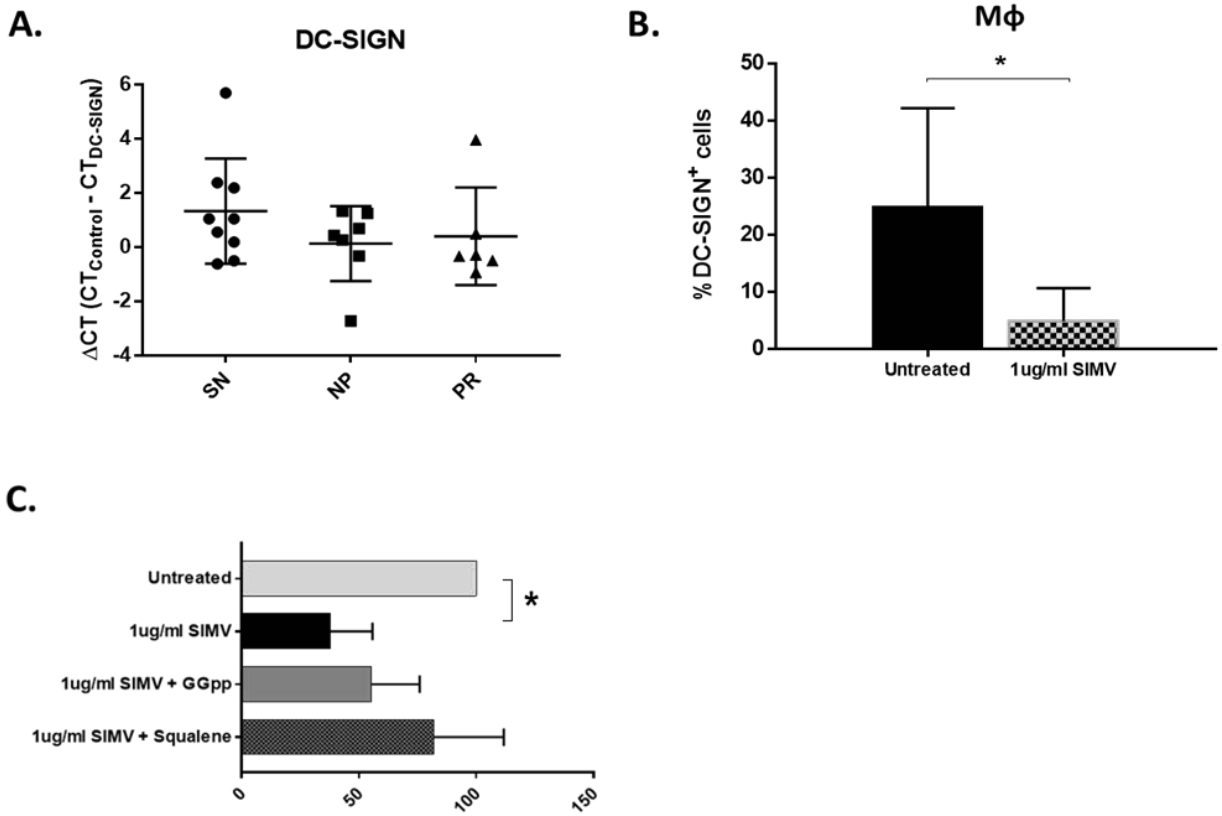

Figure 13. SIMV decreases the number of DC-SIGN$^+$ MΦ and *trans* infection in a cholesterol dependent manner.

(A) Total RNA extracted from SN, NP, and PR MΦ was used to measure DC-SIGN gene expression by RT-PCR. CT of DC-SIGN probe was subtracted from the CT of RNA polymerase II probe within each sample for a relative ΔCT value. (B) SN MΦ were untreated or treated with 1ug/ml SIMV (n = 5), or in the presence of (C) SIMV and GGpp or SIMV and squalene for 24

hours prior to analysis for DC-SIGN surface expression by flow cytometry (n = 4). Histograms are expressed as mean $\pm$ SE. *$p \leq 0.05$.

Cholesterol repletion recovers MΦ *trans* infection in NP and SN.

Due to the lack of *trans* infection observed by SN4 and SN5 (**Figs. 1D, 1E, 1F**), in order to further assess their similarity to NP, we compared their cholesterol levels and DC-SIGN expression. SN5 MΦ had lower levels of both DC-SIGN (**Fig. 14A**) and total cholesterol when compared to SN4 (**Fig. 14B**). To further assess whether altered cholesterol is responsible for the lack of MΦ *trans* infection in NP, we next tested whether squalene or GGpp could recover *trans* infection in SN4, SN5, and NP. Additionally, squalene, but not GGpp, recovered MΦ-mediated *trans* infection in SN5 but not SN4 (**Fig. 14C**). Squalene treatment of MΦ recovered detectable *trans* infection in NP (**Fig. 15**). Therefore, SN5 was both functionally and phenotypically similar to the NP group, while phenotyping suggests SN4's lack of *trans* infection is likely caused by a mechanism independent of cholesterol metabolism. This also strongly supports that low cholesterol in NP MΦ contributes to their inability to *trans* infect HIV-1.

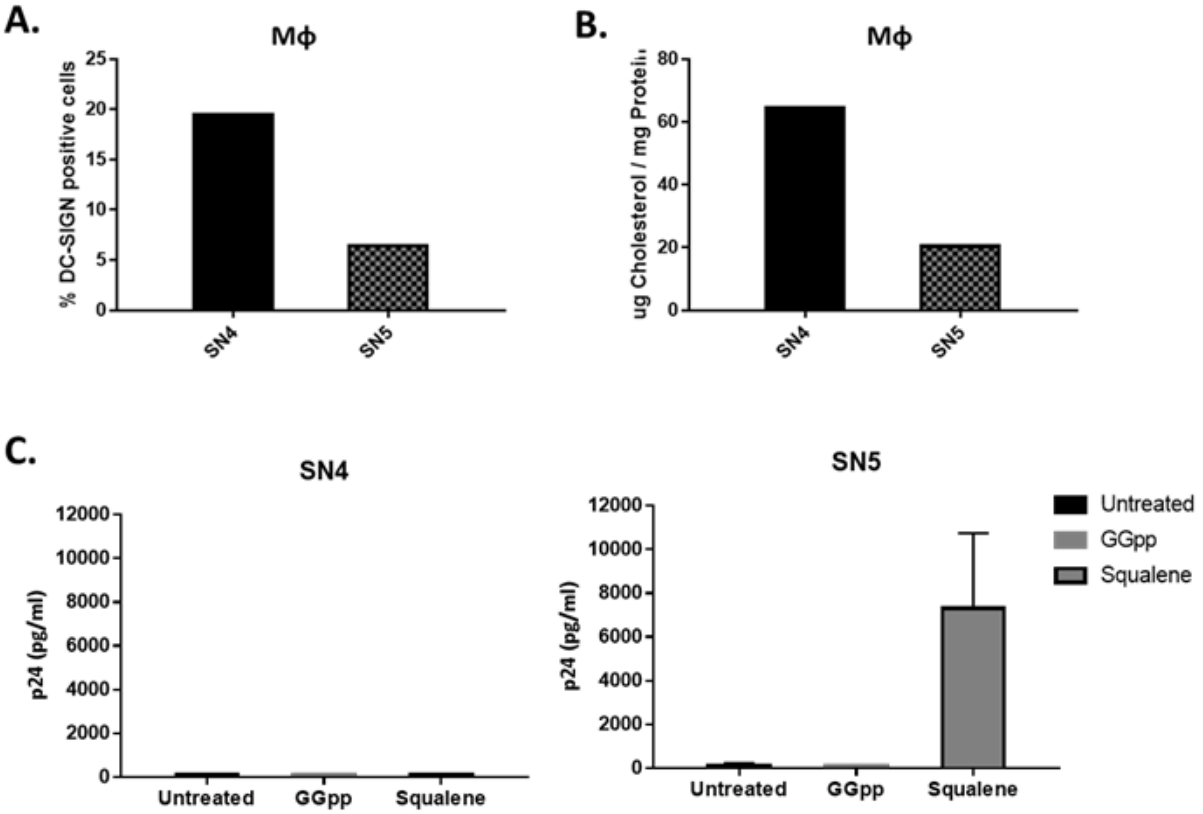

Figure 14. Squalene recovers MΦ-mediated *trans* infection in SN with low cholesterol.

(A) Percent of DC-SIGN+ MΦ and (B) total cholesterol of SN4 and SN5. (C) Day 12 *trans* infection with untreated or 24 hour GGpp- or squalene-treated SN4 and SN5 MΦ (Two independent experiments). Histograms are expressed as mean + SE. *$p < 0.05$.

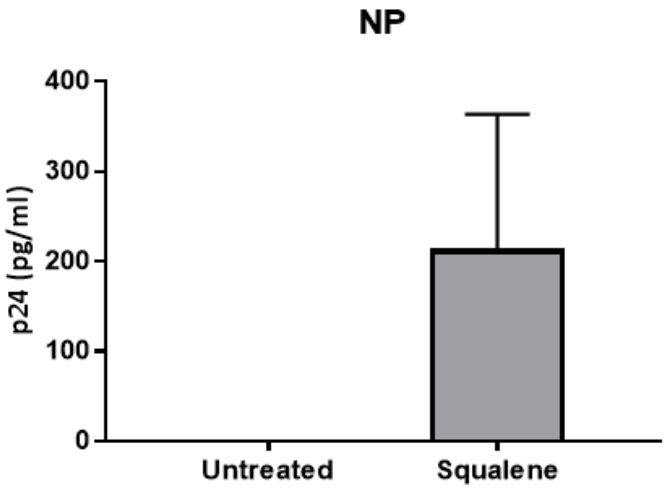

Figure 15. Squalene recovers MΦ-mediated *trans* infection in NP.

Day 12 *trans* infection with untreated or 24 hour squalene-treated NP MΦ (n = 2). Histograms are expressed as mean ± SE.

SIMV alters membrane-associated cholesterol and lipid rafting in MΦ

To further understand how SIMV alters MΦ cholesterol content and the role of cholesterol in MΦ-mediated *trans* infection, we analyzed the abundance of total cellular cholesterol compared to membrane-associated cholesterol in SIMV-treated MΦ. Total cholesterol content of SIMV-treated MΦ did not decrease across a wide range of SIMV concentration tested (0.5 – 10ug/ml) (**Fig. 16A**). However, cell membrane-associated cholesterol was lowered by SIMV (**Fig. 16B**) as demonstrated by analysis of cells treated with filipin III. Fluorescent CTx-B labeling of untreated and SIMV treated MΦ showed that SIMV also significantly reduced PM lipid rafting (**Fig. 16C**). Intriguingly, visualization of filipin III cholesterol staining revealed a remarkable dissociation of cholesterol clustering or rafting after SIMV treatment and confirmed that cholesterol rich lipid rafting was also lowered in SIMV-treated MΦ (**Fig. 16D**). These data suggest that SIMV effectively mimics the altered cholesterol state of MΦ observed in NP, the mechanism by which we propose SIMV significantly decreases HIV-1-mediated *trans* infection.

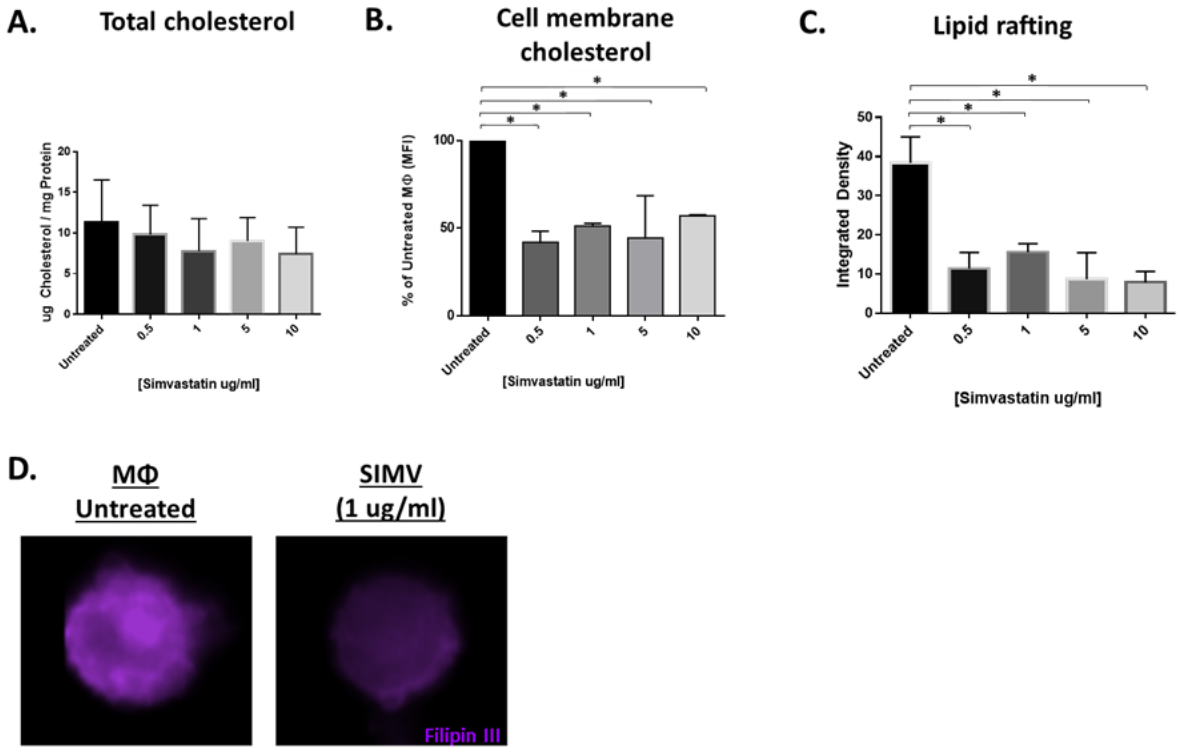

Figure 16. SIMV alters MΦ lipid rafting.

(A) SN MΦ were treated with 0.5, 1, 5, or 10ug/ml of Simvastatin and tested for total cholesterol concentration by Amplex Red (n = 2) and (B) cell membrane cholesterol-specific staining with Filipin III by flow cytometry (n = 2). (C) MΦ were treated with 0.5, 1, 5, or 10ug/ml of Simvastatin, exposed to CTx-B, and analyzed by confocal microscopy to quantify lipid rafting (n = 2). (D) Representative images from SN MΦ untreated or treated with 1ug/ml SIMV followed by Filipin III labeling (purple) and imaging with the Millipore ImageStream. *p $\leq$ 0.05. Histograms are expressed as mean $\pm$ SE.

5.1.8 Aim 2d. Effect of altered cholesterol homeostasis on MΦ virus binding and internalization in NP

NP and PR MΦ bind similar amounts of HIV-1

Our data thus far indicate that DC-SIGN is playing a role in HIV-1 interactions with macrophages. To further understand the role of virion – MΦ interactions in HIV-1 disease progression we assessed HIV-1 binding to MΦ. CCR5 – tropic HIV-1 binding trended lower in NP MΦ but was not significantly different compared to PR MΦ (**Fig. 17A**) as has been previously demonstrated (230). However, confocal microscopy visually supported less virus binding in NP (**Fig. 17B**).

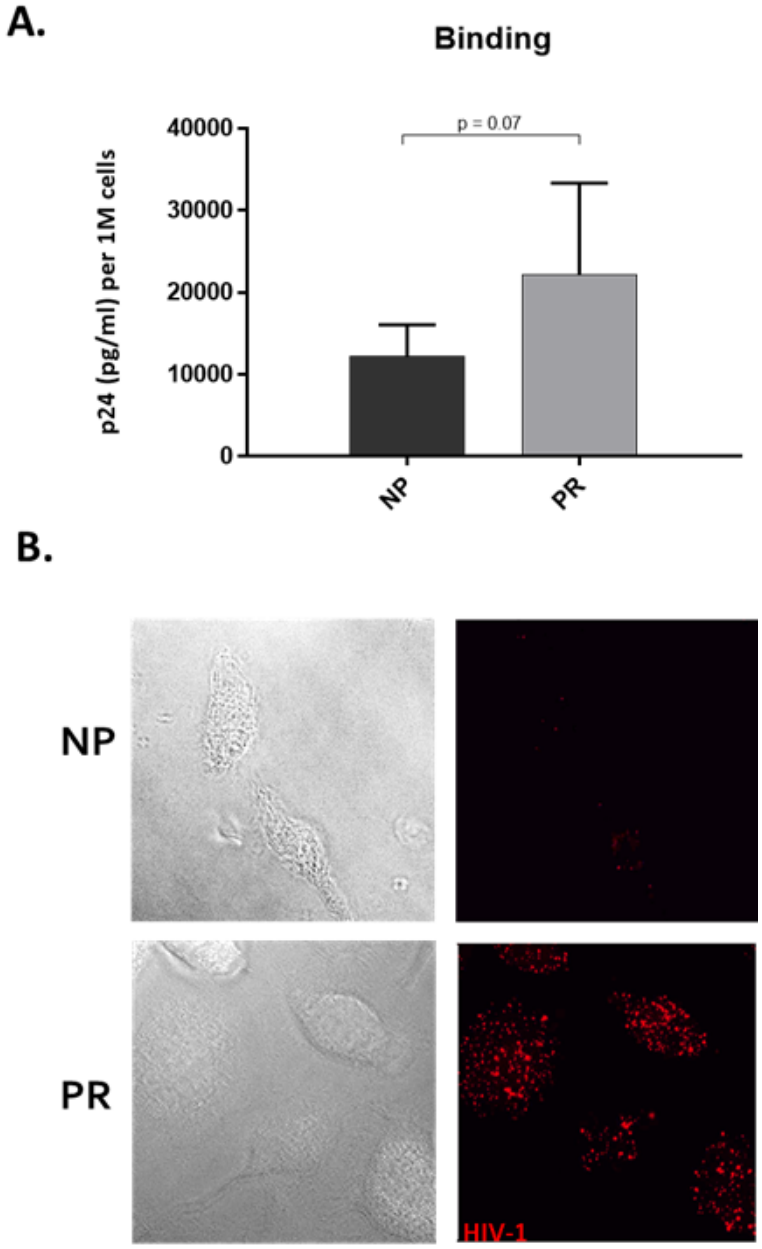

Figure 17. MΦ binding of HIV-1 trends lower in NP.

(A) NP and PR MΦ were exposed to 12ng p24 of AT-2 inactivated CCR5-tropic HIV-1 per 0.5 x 10^6 cells for 2 hours at 4°C, lysed, and analyzed for bound p24 by ELISA (n = 4). (B) Representative DIC and fluorescent images from SN MΦ treated with RFP-tagged AT-2 inactivated CCR5-tropic virus (red) for binding assays analyzed by confocal microscopy. *p $\leq$ 0.05. Histograms are expressed as mean $\pm$ SE.

NP MΦ internalize less HIV-1 into early endosomes

DC-SIGN is known to mediate endocytosis of intact HIV-1 virions into early endosomes (81). We next assessed HIV-1 internalization into NP and PR MΦ. We found that significantly less HIV-1 was internalized into NP MΦ (**Fig. 18A**). Additionally, very little internalized virus was visible in NP by confocal microscopy, however internalized virus was more abundant in PR and co-localized with early endosomes (**Fig. 18B**). Once we determined the associations of less virus binding and internalization into NP MΦ, we assessed whether binding and internalization was lower in the 2 SN that lack MΦ *trans* infection. Intriguingly, both binding and internalization of virus to and into MΦ was lower compared SN with efficient *trans* infection (**Fig. 19**). Together these data show that virus – MΦ interactions, specifically virus internalization into early endosomes, play a role in MΦ – mediated *trans* infection and thereby, disease progression.

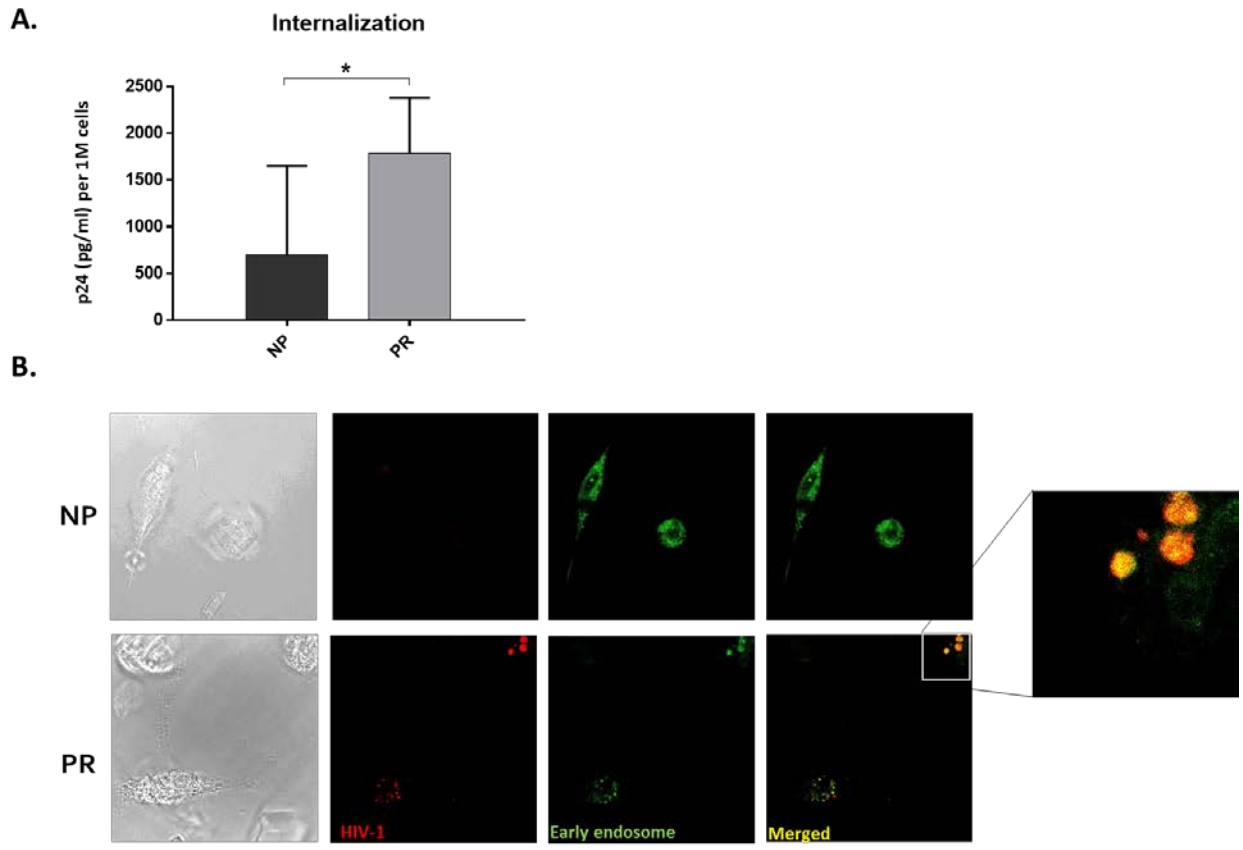

Figure 18. NP MΦ internalize less HIV-1 into early endosomes.

(A) NP and PR MΦ were exposed to 12ng X p24 of AT-2 inactivated CCR5 - tropic HIV-1 per 0.5 x 10^6 cells for 2 hours at 37°C, trypsinized, lysed, and analyzed for internalized p24 by ELISA (n = 5). (B) Representative DIC and fluorescent images from SN MΦ treated with RFP-tagged AT-2 inactivated CCR5-tropic virus (red) for virus internalization assays, co-stained for early endosomes (green), and analyzed by confocal microscopy. *p $\leq$ 0.05. Histograms are expressed as mean $\pm$ SE.

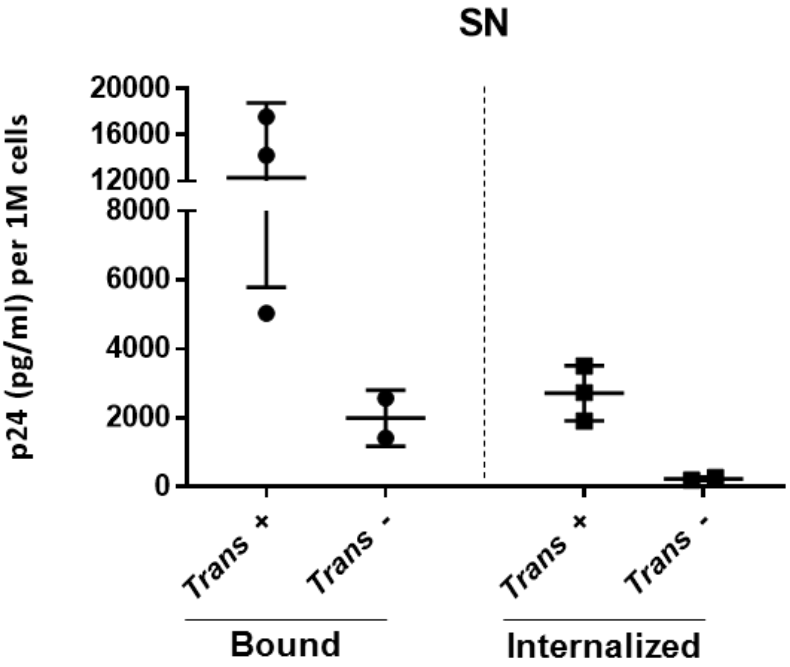

Figure 19. SN MΦ lacking *trans* infection bind and internalize less HIV-1.

MΦ from *trans* infection positive and *trans* infection negative SN were assessed for virus binding and internalization by p24 ELISA. *$p \leq 0.05$. Histograms are expressed as mean $\pm$ SE.

5.1.9 Aim 3a. Expression levels of proteins associated with lack of *trans* infection in NP by PCR

Palladin, Siglec-10, and CD1β are not differentially expressed in NP and PR MΦ

Archived transcriptome data generated from DC, B cells, and $CD4^+$ T cells from 8 NP and 8 PR was used to identify genes and protein involved in the function of these cells in HIV-1 progression. At the transcriptome-wide level, no genes were significantly differentially expressed in B cells and $CD4^+$ T cells of NP and PR. However, multiple genes were differentially expressed in NP and PR DC. The top three identified were siglec-10, palladin, and CD1β (**Fig. 20**), however only Palladin and CD1β were verified by PCR (completed by Marilee Clunk, *et.al.* unpublished). cDNA corresponding to Palladin and CD1β was generated from total RNA isolated from SN, NP, and PR MΦ was pre-amplified and further tested for expression levels by RT-PCR. Palladin mRNA levels were not different in NP and PR (**Fig. 21A**). CD1b was not found to be expressed in all of the MΦ samples. Intriguingly, CD1b expression was found in significantly more NP (4 of 7; 57%) than both PR (1 or 6; 17%) and SN (2 of 9; 22%) (**Fig. 21B**).

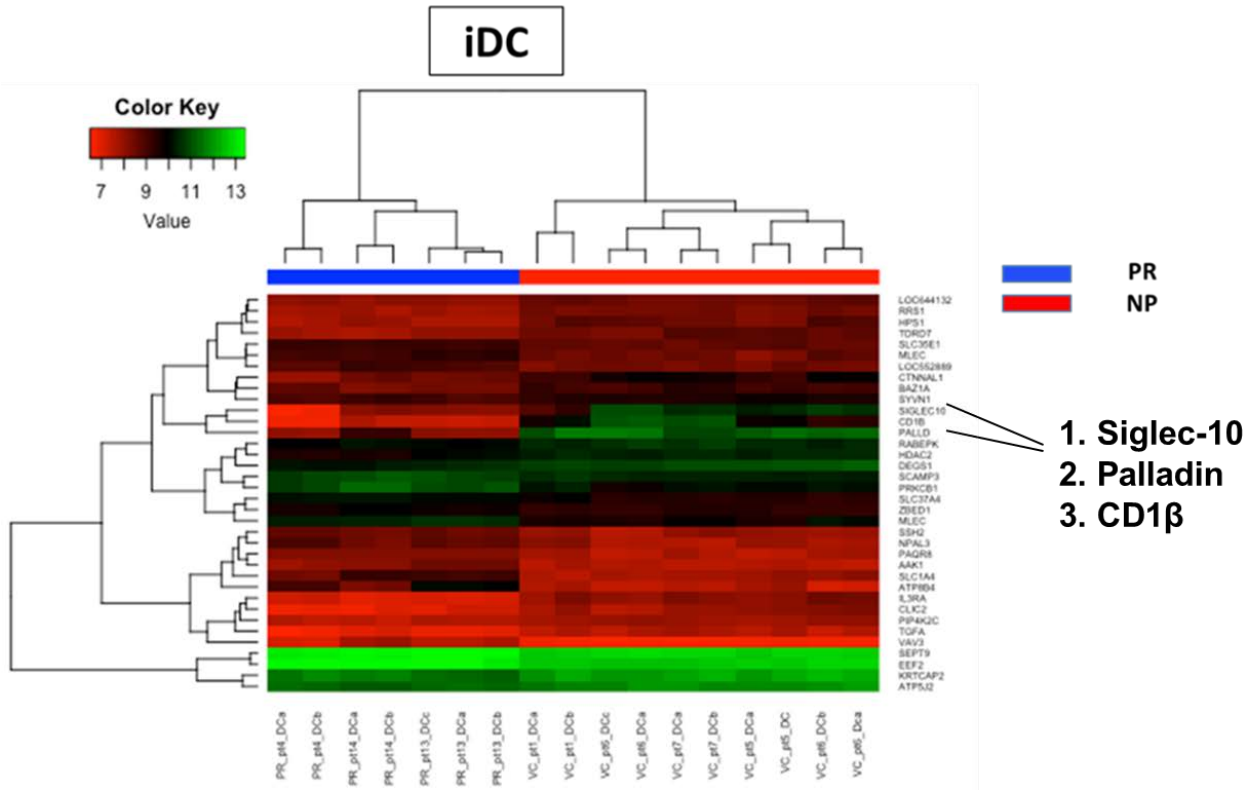

Figure 20. Transcriptome-wide analysis of NP and PR DC.

Viremic controller (VC) in this study is equivalent to NP.

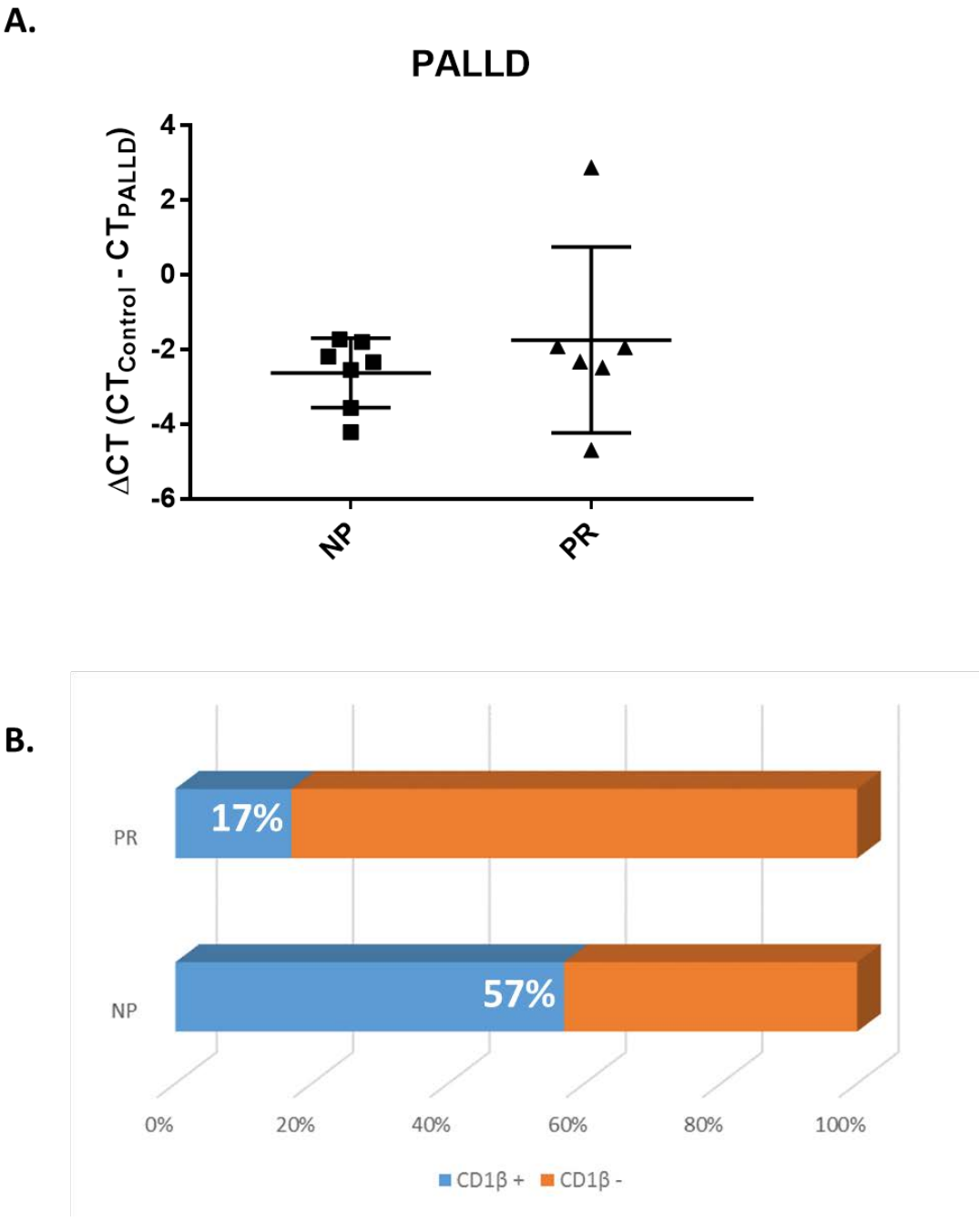

Figure 21. Fewer PR express CD1β than NP.

(A) Total RNA extracted from NP and PR MΦ was used to measure Palladin (PALLD) mRNA by RT-PCR. Histograms are mean of technical triplicates $\pm$ SE. (B) CD1β mRNA levels were assessed by RT-PCR and expressed as the number of samples with CD1β expressing MΦ. NP (n = 7) and PR (n = 6). *$p \leq 0.05$.

5.1.10 Aim 3b. Expression levels of proteins involved in MΦ cholesterol metabolism by PCR

PR MΦ express higher levels of PPARg mRNA

A focused analysis of cholesterol metabolism genes from archived transcriptome data from NP and PR DC also revealed that mediators of cholesterol efflux, including PPARγ and ABCA1 are differentially expressed in NP and PR (**Fig. 22**). We next sought to identify the mechanism by which levels of FC were being regulated differentially in NP and PR. cDNA corresponding to sterol regulatory element-binding protein (SREBP), ATP-binding cassette transporter 1 (ABCA1), ATP Binding Cassette Subfamily G Member 1 (ABCG1), low density lipoprotein receptor (LDLR), liver X receptor alpha (LXRa), peroxisome proliferator-activated receptor gamma (PPARg), 3-Hydroxy-3-Methylglutaryl-CoA reductase (HMGCR), apolipoprotein E receptor 2 (APOER2), CD36, liver X receptor beta (LXRb), and CD1b was generated from total RNA isolated from SN, NP, and PR MΦ was pre-amplified and further tested for expression levels by RT-PCR. We found that PPARg expression was significantly higher in PR than NP and SN, and LDLR expression trended higher in NP ($p = 0.08$) (**Fig. 23A**). Expression of SREBP, ABCA1, ABCG1, LXRα, HMCGR, APOER2, CD36, and LXRβ were not significantly different among the 3 clinical groups (**Fig. 23A**).

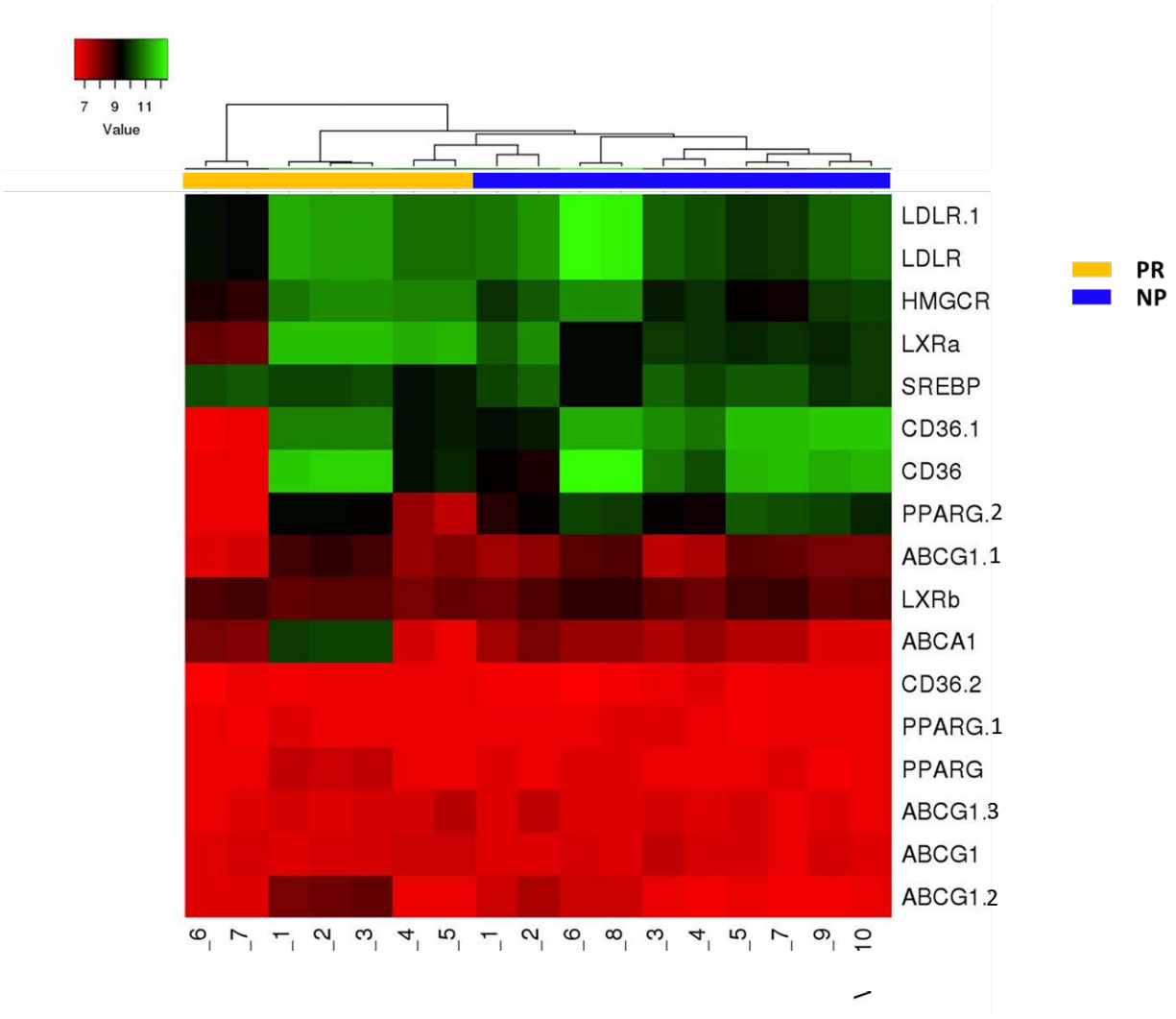

Figure 22. Focused analysis of cholesterol metabolism genes in NP and PR DC.

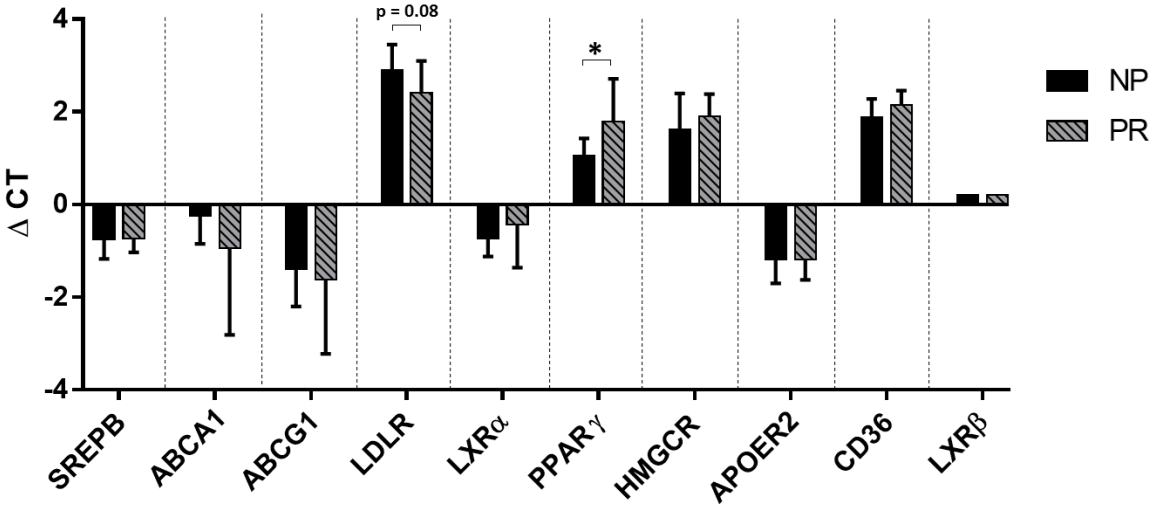

Figure 23. PR MΦ express higher levels of PPARγ mRNA.

(A) Total RNA extracted from NP and PR MΦ was used to measure SREBP, ABCA1, ABCG1, LDLR, LXRα, PPARγ, HMGCR, APOER2, CD36, and LXRβ mRNA by RT-PCR. Histograms are mean of technical triplicates $\pm$ SE. *$p \leq 0.05$.

5.1.11 Aim 3c. MiRNA screening of plasma and APC by NanoString

Based on the important role of miRNA in cholesterol metabolism and the link between cellular cholesterol homeostasis and HIV-1 *trans* infection, it is possible that the miRNA expression profile of NP and PR influence their *trans* infection efficiency and natural progression pattern. Since our targeted PCR data revealed very few inherent differences in MΦ gene expression of proteins directly involved in cholesterol uptake, synthesis, and efflux in our *in vitro* cultures we hypothesized that an extracellular plasma factor, such as miRNA, may influence MΦ cholesterol homeostasis in NP and PR. We sought to characterize the expression profile of miRNA in the plasma of 9 NP and 9 PR. Total RNA was extracted from NP and PR plasma and tested using human v3 miRNA Assay (NanoString Technologies). Four samples (2 NP and 2 PR) were excluded from analysis due to variances caused by undetectable hemolysis and subsequent red blood cell miRNA contamination. Following normalization of the remaining 7 NP and 7 PR samples, miRNA expression patterns revealed that the NP clustered in two distinct groups **(Fig. 24A)** indicating the NP studied may be further categorized based on miRNA profiles. Intriguingly, we compared average $CD4^+$ T cell count and viral load of all MACS visits prior to cART initiation of the two NP clusters and found that the clusters significantly differed in total $CD4^+$ T cell counts (**Fig. 24B**), but not viral load (**Fig. 24C**).

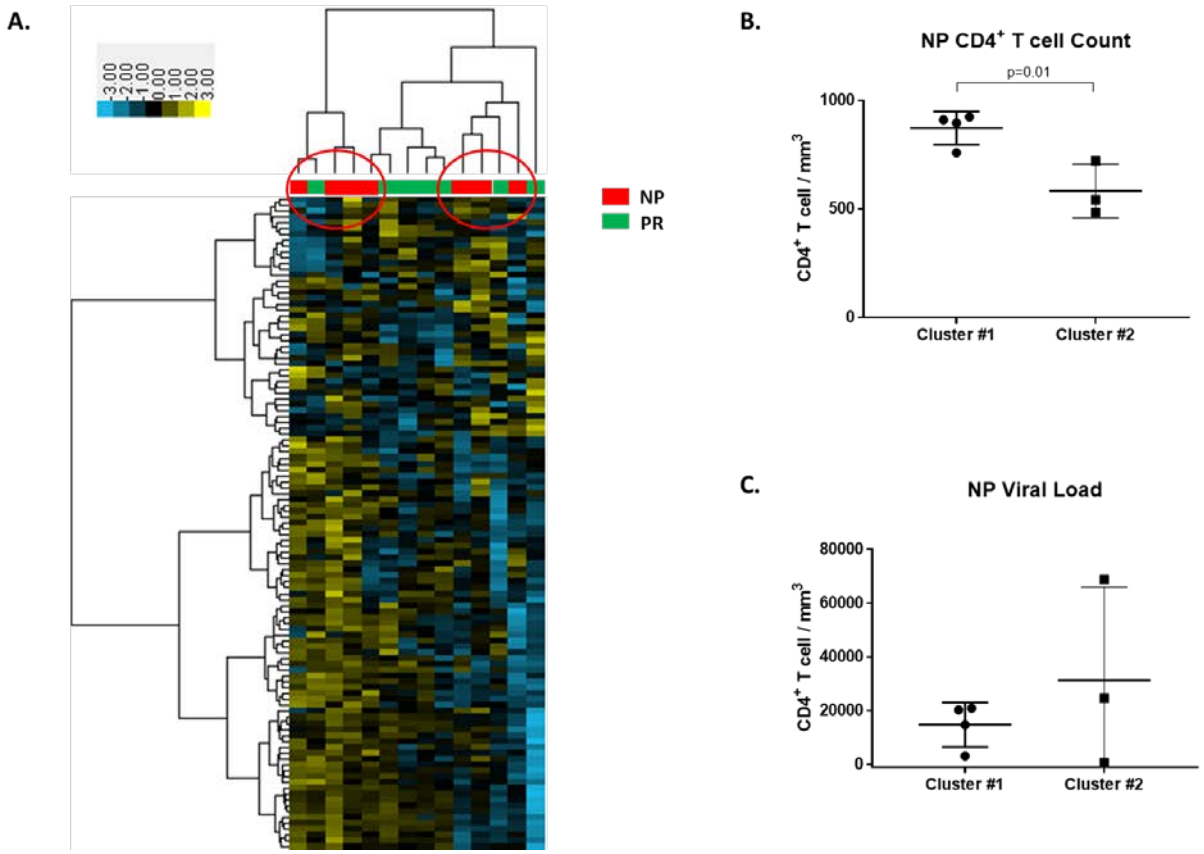

Figure 24. NP cluster into two groups based on miRNA profiles.

(A) Total RNA isolated from NP (n = 7) and PR (n = 7) plasma was tested using the NanoString Human V3 miRNA Assay. MiRNA with at least 135 counts/ml were included in the heat map. Gene clustering on the Y axis, as well as NP (red) and PR (green) sample clustering on the X axis was determined using Wards Minimum Distance. (B) Collective $CD4^+$ T cell count and (C) viral load prior to cART were compared among the NP in cluster #1 (n = 4; left side of heat map) and cluster #2 (n = 3; right side of heat map).

We next analyzed differences in miRNA expression of all NP and all PR tested. The top 10 expressed miRNA were very similar in NP (**Table 2**) and PR (**Table 3**). The majority of miRNA expressed in NP and PR appeared similar and highly correlated (**Fig.25)**, however several outliers suggested there were differentially expressed miRNA in NP and PR (**Fig. 25, red arrows**). We next performed a comparative analysis of miRNA profiles of NP and PR plasma. Two miRNA. MiR-29b-3p and miR-376a-3p, were significant differentially express in NP and

PR. Three additional miRNA with known effects on cholesterol metabolism, miR-150-5p, miR-144-3p, and miR-148-3p, were expressed in NP and PR, however total counts were not significantly different (**Table 4**). We then investigated known human gene interactions of these five miRNA. miR-29b-3p has been experimentally validated to interact with 86 human transcripts (**Appendix B, Fig. 29**), miR-376a-3p with 12 human transcripts (**Appendix B, Fig. 30**), miR-150-5p with 28 human transcripts (**Appendix B, Fig. 31**), miR-144-3p with 25 human transcripts (**Appendix B, Fig. 32**), and miR-148a-3p with 40 human transcripts (**Appendix B, Fig. 33**). These data represent preliminary results meant to screen for potential miRNA implicated in cholesterol metabolism in NP and PR. Future studies are planned to further mine the vast amount of data generated by the NanoString miRNA assay. Additionally, PCR validation of the differentially expressed miRNA identified and *in vitro* studies are also planned to further elucidate the role of these miRNA in APC cholesterol metabolism and HIV-1 *trans* infection.

Table 2. The 10 most highly expressed miRNA in NP plasma.

Gene name	Mean miRNA counts[a]	SD
hsa-miR-451a	4636.76	3005.06
hsa-miR-223-3p	3868.84	871.83
hsa-miR-1976	3490.01	1600.89
hsa-miR-16-5p	3072.94	828.57
hsa-miR-142-3p	2738.03	820.66
hsa-let-7a-5p	2321.73	983.13
hsa-miR-126-3p	2284.92	1220.63
hsa-miR-199a-3p+hsa-miR-199b-3p	1579.69	421.4
hsa-miR-191-5p	951.36	210.63
hsa-miR-23a-3p	898.04	40.94

[a]Input = 3ul of total RNA; equivalent to 150ul plasma.

Table 3. The 10 highest expessed miRNA in PR plasma.

Gene name	Mean miRNA counts[a]	SD
hsa-miR-451a	3910.4	1786.85
hsa-miR-1976	3612.16	3172.04
hsa-miR-223-3p	3224.14	509.38
hsa-miR-16-5p	3170.23	1034.61
hsa-miR-126-3p	2144.2	1275.69
hsa-miR-142-3p	1952.28	1039.99
hsa-let-7a-5p	1282.85	1400.71
hsa-miR-199a-3p+hsa-miR-199b-3p	1169.62	388.94
hsa-miR-23a-3p	882.32	148.5
hsa-miR-130a-3p	757.3	195.68

[a]Input = 3ul of total RNA; equivalent to 150ul plasma.

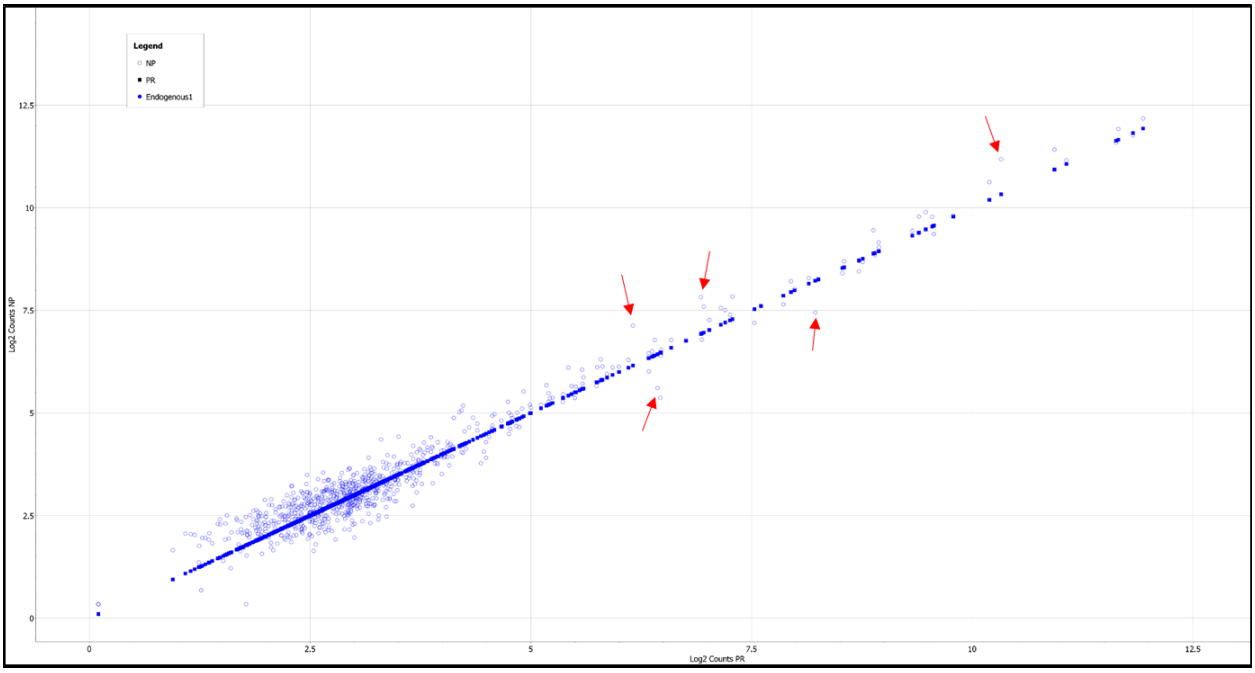

Figure 25. Correlation between NP and PR expressed miRNA.

Table 4. Differentially expressed miRNA comparing NP and PR.

Gene Name	Expression change	Log2 FC	*P* value
NP vs. PR (*overall*)			
hsa-miR-29b-3p	Increase	1.56	0.04
hsa-miR-376a-3p	Increase	1.49	0.04
hsa-miR-150-5p	Increase	1.79	0.07
NP vs. PR (*Cholesterol-related*)			
hsa-miR-144-3p	Decrease	-1.72	0.1
hsa-miR-148a-3p	Decrease	-1.24	0.2

Log2 FC = Log2 fold change using PR as the reference value. *P* value is unadjusted from an unpaired two-tailed T test with a Welch's correction.

6.0 MATERIALS AND METHODS

6.1 ETHICS STATEMENT

Biological samples were acquired and studied from consented individuals according to University of Pittsburgh International Review Board approved protocols. All recruited participants were over the age of 18 and provided written consent prior to sample collection or use.

6.2 PARTICIPANT SELECTION

We studied three groups of individuals (Table 1) within the Multicenter AIDS Cohort Study (MACS) and/or Pitt Men's Study (PMS) defined based on their HIV-1 serostatus and associated disease progression: **(1)** (PR) – HIV-1 seropositive individuals with a 100 cells/mm^3 annual decrease in $CD4^+$ T cell count prior to cART **(2)** NP – HIV-1 seropositive individuals displaying a lack of progression to AIDS and $CD4^+$ T cell count above 500 for at least 7 years post seroconversion without the aid of cART based on either $CD4^+$ T cell count. **(3)** (SN) – healthy HIV-1 negative individuals whose serostatus is checked twice a year (17). We recruited 10 SN, as well as 5 PR and 5 NP not on cART at the time of study or with frozen samples available prior to cART initiation. Participant reported data was used to rule out the use of cholesterol or lipid lowering medications during the time points of samples studied. HLA-type and CCR5 Δ32 genotype were considered for all participants as possible confounders of disease progression classification.

6.3 CELLS AND VIRUSES

6.3.1 Cell Isolation

$CD4^+$ T lymphocytes, B lymphocytes, and monocytes were positively enriched from freshly isolated or frozen peripheral blood mononuclear cells (PBMC) (fresh from consented Pittsburgh MACS participants, or frozen from the MACS repository) using anti-CD4, CD19, or CD14 monoclonal antibody (mAb)-coated magnetic bead separation (Miltenyi Biotech), respectively, according to manufacturer's instructions.

6.3.2 APC and T cell culture

MΦ and DC were derived from monocytes cultured with 20ng/ml M-CSF (Peprotech) for 7 days or 1000U/ml GM-CSF (Miltenyi Biotech) and 1000 U/ml rhIL-4 (R&D systems) for 5d, respectively, in AIM-V medium (Gibco). $CD4^+$ T cells and B cells were activated for 48 hours in RPMI with 10U/ml of delectinated IL-2 and 2ug/ml PHA (16) or 1000U/ml rhIL-4 and 0.1ug/ml CD40L, respectively.

6.3.3 Viruses

R5-tropic HIV-1 BaL grown in and purified from PM1 cells (American Type Culture Collection) (65), were used for cis and trans infection experiments. Virus stock titration and experimental p24 measurements were acquired by ELISA using the HIV-1 p24 Antigen Capture

Immunoassay kit (Leidos Biomedical Research Inc., Frederick National Laboratory for Cancer Research), per manufacturer's instructions.

6.4 *TRANS* INFECTIONS

Briefly, $1x10^6$ APC were incubated with a low concentration of HIV-1 (MOI 10^{-3}) for 2 h at 37C, then washed 3 times with medium. Virus-pulsed APC were co-cultured with autologous activated $CD4^+$ T cell targets at a 1:10 effector : target ratio (10^4 : 10^5) in AIM-V medium, and p24 was quantified in cell-free supernatants at days 4, 8, and 12 post co-culture.

6.5 *CIS* INFECTIONS

Briefly, $1x10^6$ APC or activated $CD4^+$ T cells were incubated with low (10^{-3}) or high (10^{-1}) MOIs of HIV-1 and cultured independently. P24 was quantified in cell-free supernatants at days 4, 8, and 12 post co-culture.

6.6 CELL PHENOTYPING

Cells were assessed for surface and intracellular protein expression. MΦ were incubated with an amine binding viability dye using the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular probes) for 20 minutes, then incubated with isotype controls or monoclonal antibodies against CD4 - V450, CD14 - APC-H7, CD16 - APC-H7, CCR5 – PE, CD169 – BB515 (BD Biosciences) and CD209 – APC or FITC (R&D Systems) diluted in wash buffer (PBS supplemented with 0.1% FBS and 0.1% NaN_3) for 30 minutes. Cells were then

permeabilized with Perm II (BD Biosciences) for 20 minutes and labelled intracellularly with anti-CD68 – BV421 (BD Biosciences) for 30 minutes. Cells were fixed in 1% PFA, acquired with the BD LSRFortessa, and analyzed with FlowJo v10. Fluorochrome compensation was performed using Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD Biosciences) and ArC Amine Reactive Compensation Bead Set (Thermo Fisher) prior to acquisition.

6.7 CELLULAR CHOLESTEROL QUANTIFICATION

6.7.1 Total cellular cholesterol

Cultured cells were washed two times with PBS to remove any residual media and lysed with 200ul of 0.1% triton X-100 for total cellular cholesterol quantification using the Amplex Red Cholesterol Assay Kit as per manufacture's instruction (Thermo Fisher). Total cholesterol content was normalized to total cellular protein quantified with the Micro BCA Protein Assay Kit as per manufacturer's instructions (Thermofisher). Briefly, total cholesterol (ug) was divided by total protein (mg) for each sample.

6.7.2 Membrane-associated cholesterol

Cultured cells were analyzed for membrane-associated cholesterol using filipin III from the Cholesterol Cell-Based Detection Assay Kit (Cayman Chemical), as per manufacturer's instructions, and analyzed by flow cytometry or confocal microscopy. For flow cytometric analysis, cells were incubated with viability dye, labelled filipin III, re-suspended in 1% PFA,

acquired with the BD LSRFortessa, and analyzed with FlowJo v10, or acquired with the EMD Milipore Amnis ImageStreamX and analyzed with IDEAS 6.2. For confocal microscopy analysis, MΦ were grown in glass bottom dishes coated with poly-D-lysine (MatTek). Cells were fixed and labeled with filipin III reagent according to the manufacturer's instructions. Cells were additionally stained for DC-SIGN with an anti-CD209 – FITC (BD Biosciences) were noted. Cells were acquired on a Nikon A1 confocal microscope at 40X with a 3.42 zoom at the University of Pittsburgh Center for Biological Imaging and analyzed using NIS Elements.

6.7.3 PM lipid rafts

In addition to cholesterol specific labels, the GM1 ganglioside was labelled using Vybrant ® Alexa Fluor ® 488 Lipid Raft Labeling Kit (Molecular Probes) and analyzed by flow cytometry or confocal imaging. Integrated density was calculated with ImageJ2 from an average of five cells per image using five images per sample.

6.8 CHOLESTEROL SYNTHESIS AND PROTEIN PRENYLATION STUDIES

6.8.1 Statin Treatment and Recovery

MΦ were washed free of AIM-V media after 6d of culture then treated for 24 h with a range of concentrations (0.5 – 10ug/ml) of SIMV (Sigma) in RPMI supplemented with charcoal-stripped FBS (Gibco) and M-CSF prior to downstream assays.

During 24 hour incubation with SIMV, cells were simultaneously treated with squalene (Sigma) to recover cholesterol, or GGpp (Sigma) to recover protein prenylation blocked by statin treatment. Cells were then washed extensively prior to use in downstream assays.

6.8.2 Cholesterol repletion

After day 7 of culture, MΦ were incubated with 300 ug/ml of soluble cholesterol in BCD (Sigma) for 2 hours, then washed prior to use in downstream assays.

6.9 CDNA PRE-AMPLIFICATION AND RT-PCR

Total RNA was extracted from MΦ with the RNeasy Mini Kit (Qiagen) and cDNA was generated as per the manufacturer's instructions. cDNA corresponding to human DC-SIGN, SREBP, ABCA1, ABCG1, PPARγ, LXRα, LXRβ, LDLR, CD36, HMG-CoA reductase, APOE receptor 2, and CD1β was pre-amplified using the TaqMan PreAmp Master Mix Kit (Applied Biosystems) as per manufacturer's instructions. CD1β was also pre-amplified using IDT Taq-Man® primers (primer 1: 5'ACTTTTGGGCTGATATCTT GGG-'3; primer 2: 5'-CTTCCTTGCTCCTTTTGCTATG-'3; probe: 5'-/56-FAM/CTCATG GGA/ZEN/TCTGATATGACCGGCG/31ABkFQ/-3'). Pre-amplified cDNA was then used for RT-PCR using Taqman® Gene Expression Assays (Applied Biosystems) (SREBP-Hs01088679_g1; ABCA1- Hs01059137_m1; ABCG1- HS00245154_m1; PPARγ-Hs01115513_m1; LXRα- Hs00172885_m1; LXRβ- Hs01027215_g1; LDLR - Hs00181192_m1; CD36- Hs00354519_m1; HMGCR- Hs00168352_m1; and APOER- Hs00182998_m1) and TaqMan Universal Master Mix II, no UNG. RNA polymerase 2 (RNA pol2) was amplified from

each sample as an internal control. ΔCT values were calculated by subtracting averaged CT values of the gene of interest from the RNA pol2 CT within each sample.

6.10 VIRUS BINDING AND INTERNALIZATION STUDIES

6.10.1 Virus Binding

MΦ were incubated with 12ng of p24 per 0.5 x 10^6 cells of AT-2 inactivated BaL HIV-1 for 2 hours at 4C, washed extensively with cold PBS, pelleted, and lysed with 0.1% triton X 100. Cell lysates were assessed for p24 content by ELISA. For confocal analysis, cells were incubated with AT-2 inactivated RFP-tagged BaL HIV-1 for 2 hours at 4C, washed, mounted, and analyze by confocal microscopy.

6.10.2 Virus Internalization

MΦ were incubated with 12ng of p24 per 0.5 x 10^6 cells of AT-2 inactivated CCR5 – tropic HIV-1 for 2 hours at 37C, washed extensively with PBS, trypsinized to remove surface bound virus, pelleted, and lysed with 0.1% triton X 100. Cell lysates were assessed for p24 content by ELISA. For confocal analysis, CellLight™ Early Endosomes-GFP, BacMam 2.0 (ThermoFisher) was added to on day 6 of culture and incubated overnight. Cells were then washed and incubated with AT-2 inactivated RFP-tagged CCR5-tropic HIV-1 for 2 hours at 37C, washed, mounted, and analyze by confocal microscopy.

6.11 HUMAN MICROARRAY

Total RNA was extracted from cultured NP and PR DC using the RNeasy Mini kit (Qiagen), per manufacturer's instructions and used to assess mRNA levels with the HumanHT-12 v4 Expression Bead Chip (illumina). Normalization of data was performed with Lumi software, and the heatmap was generated using the Limma software package in Bioconductor.

6.12 MIRNA STUDIES

6.12.1 MiRNA Extraction and Testing

Plasma samples used were archived samples initially collected from heparinized blood tubes and frozen at -80C. Frozen plasma samples were thawed and stored on ice until use. Sample volumes of 600ul were microcentrifuged for 10 minutes at 500g to remove cell debris. Supernatant was collected and store on ice until RNA extraction. Total RNA was extracted using the Plasma/Serum RNA Purification Midi Kit (Norgen Biotek) as per manufactures instructions. Two synthesized spike-in nonhuman control miRNA (Table 5) were used to control and normalize for extraction variation in downstream assays (Integrated DNA Technologies).

Table 5. Synthetic miRNA spike-in controls for NanoString Human v3 Assay.

miRNA	Sequence	Length	Accession Number
ath-miR159a	UUUGGAUUGAAGGGAGCUCUA	21aa	MIMAT0000177
cel-miR-248	AUACACGUGCACGGAUAACGCUCA	24aa	MIMAT0000304

Following the addition of the lysis buffer from the extraction kit, the sample was incubated at room temperature for 5 minutes. Then 20pg of each spike-in was added to each sample prior to continuation of the extraction protocol. Extracted RNA was diluted to 420ul with RNase free water, added to an Amicon Ultra-0.5 Centrifugal Filter Unit with a 3 kDa cut off, and microcentrifuged at 14,000 rcf for 90 minutes. The column was inverted and transferred to a fresh collection tube and microcentrifuged at 8,000 rcf for 2 minutes. Eluted volume was brought to 20ul with RNase free water if needed. Extracted RNA (3ul) was analyzed using the Human v3 miRNA Assay (NanoString Technologies) and quantified using the nCounter® MAX Analysis System to assess counts of a panel of approximately 770 human miRNAs derived from miRBase. The assay was performed by the University of Pittsburgh Genomics Research Core.

6.12.2 NanoString Data Analysis

Raw NanoString RCC data files were analyzed using nSolver v3.0 Software. Technical negative and positive controls for counting and ligation were used to normalize for slight variances in counting and ligation across the samples. Spike-in counts were also used to normalize for extraction variances across samples. Log2 fold change values were generated using the PR group as the reference. Significant differences in miRNA counts between NP and PR calculated by nSolver were generated with a student's two-tailed T test with a Welch's correction.

6.12.3 MiRNA gene interaction networks and visualization

Gene ontology and microRNA gene set enrichment networks were generated and assessed with miRNet (231). Network visualization were generated with a forced atlas layout.

6.13 STATISTICAL ANALYSES

An analysis of variance (ANOVA) was used to determine overall significant differences between SN, NP, and PR samples for *trans* infection, *cis* infection, phenotyping, and cholesterol content. ANOVA testing was followed by a post hoc test when required. A two-tailed Student t-test will be used for comparisons of less than three groups. MiRNA assay statistical testing is described above under "**NanoString Data Analysis**".

7.0 DISCUSSION

Highly efficient *trans* infection of HIV-1 from APC to $CD4^+$ T cells may contribute to disease progression. Previously, we found that altered cholesterol homeostasis is linked to poor DC and B cell HIV-1 *trans* infection of $CD4^+$ T cells in NP (17). However, the extent to which *trans* infection by other APC, such as MΦ, is implicated in HIV-1 disease progression, and the mechanisms by which cholesterol is differentially regulated in individuals with different disease progression patterns is unknown.

Given the intimate interactions between MΦ and T cells in lymphoid tissue (232), and previous reports that MΦ from SN mediate HIV-1 *trans* infection (21, 183), we sought here to determine if MΦ *trans* infection would also be deficient in NP. Due to the central role of MΦ in cholesterol metabolism we also sought to determine the role of cellular cholesterol in MΦ HIV-1 *trans* infection ability, and what potential mechanisms regulate cholesterol homeostasis in NP compared to PR. Through this work, we showed for the first time that MΦ HIV-1 *trans* infection was deficient in NP and thus associated with lack of disease progression. Additionally, the mechanism of poor MΦ *trans* infection of autologous $CD4^+$ T cells observed was a result of altered cell cholesterol homeostasis. We further demonstrated that NP MΦ had significantly less FC and PM lipid rafting, which lead to fewer DC-SIGN expressing MΦ and lower virus internalization into MΦ, resulting in a lack of *trans* infection. Lastly, we identified several miRNA differentially expressed in NP and PR plasma that may serve as regulators of cholesterol homeostasis in NP and PR.

Collectively our data support they hypothesis that cholesterol-facilitated MΦ *trans* infection of HIV-1 to $CD4^+$ T cells could be implicated in HIV-1 disease progression. Here I

discuss how our data, in concert with previous studies, illuminate the ways in which MΦ – mediated *trans* infection can contribute to HIV-1 infection, pathogenesis, and disease progression. Additionally, I discuss some potential mechanisms by which cholesterol facilitates *trans* infection and how targeting these pathways may have therapeutic value.

Analysis of *in vitro* models of HIV-1 *trans* infection

Studying *trans* infection *in vivo* poses several technological challenges. Identifying *trans* infection as a mechanism by which T cells become infected requires evidence of cell-to-cell passage of virus. Visualizing this transfer of virus requires live cell imaging and is limited to animal models. However, *in vitro* methods have been used to study *trans* infection for decades, and although they have been beneficial, many of them have also been flawed. We eliminated these flaws with our *in vitro* model of HIV-1 *trans* infection used in these studies.

MΦ – mediated *trans* infection of HIV-1 has previously been demonstrated by multiple groups, using several *in vitro* models. However, early methods did not use purified or activated $CD4^+$ T cells as *trans* infection targets (183) or differentiate well between monocyte-derived MΦ and DC (183, 233). By the use of activated, purified $CD4^+$ T cells and M-CSF-differentiated MΦ derived from purified $CD14^+$ monocytes, our model eliminated this ambiguity.

Past *trans* infection *in vitro* models were unable to differentiate between different routes of *trans* infection. *Trans* infection is thought to occur through two pathways. The first involves endocytosis of intact virions which can then be passed between cells through intercellular connections, such as nanotubes (78), or regurgitated into a synapse between two immune cells (16). The second involves budding of new virions as a result of productive *cis* infection into a similar synapse (16). In prior studies, virus concentrations used for *trans* infection assays were

capable of *cis* infecting MΦ (130, 183, 233). While MΦ-mediated *trans* infection likely can occur in the presence of high concentrations of virus, using concentrations that result in MΦ *cis* infection enables the discrimination between *trans* infection resulting following *de* novo infection of MΦ or following endocytosis without productive infection. The model we used (17, 68), exposed APC to low amounts of virus prior to co-culture with activated $CD4^+$ T cells, and allowed us to assess whether HIV-1 uptake by APC, in the absence of APC *cis* infection, could be passed in *trans* to $CD4^+$ T cells. We showed in SN individuals that MΦ *trans* infection enhanced virus production by autologous $CD4^+$ T cells compared to *cis* infection of T cells with cell free virus. Using this model, our data not only confirmed that MΦ can mediate HIV-1 *trans* infection of $CD4^+$ T cells, but also revealed that the transfer of virus can occur efficiently in the absence of MΦ *cis* infection.

In vitro trans infection mediated by APC may serve as a potential tool for predicting HIV-1 disease progression. Notably, two of the 10 SN (SN4 and SN5) studied here lacked *trans* infection by all three types of APC. We have previously identified lack of APC-mediated *trans* infection as an inherent characteristic of NP, in that *trans* infection ability is similar pre- and post-seroconversion (17), suggesting *trans* infection could potentially be a predictor of disease progression. Indeed, MΦ from one individual (SN5) had cholesterol levels and DC-SIGN expression similar to NP, and *trans* infection efficiency was restored upon recovery of cholesterol synthesis but not protein prenylation. Additionally, both SN4 and SN5 exhibited lower HIV-1 internalization compared to *trans* infection efficient SN, However SN4 displayed no further NP characteristics, and likely has a cholesterol-independent blockade in the *trans* infection pathway. The high prevalence of impaired *trans* infection in our SN cohort (20%) appears to be a chance anomaly but supports the conclusion that the SN population is

heterogeneous and comprised of individuals with genetically determined NP characteristics. Therefore, SN lacking *trans* infection represent a unique group of individuals, from which novel factors of HIV-1 disease progression control can be identified.

MΦ-mediated *trans* infection and systemic HIV-1 spread

HIV-1 *trans* infection is challenging to study and demonstrate *in vivo*. However, given the intimate interactions between APC, such as MΦ, and $CD4^+$ T cells in lymphoid tissue (232), it would be advantageous for the virus to utilize its association with APC to more efficiently contact. Our data support the hypothesis that MΦ-mediated *trans* infection of $CD4^+$ T cells could facilitate many stages of HIV-1 infection including host virus entry, inward trafficking, amplification, and dissemination.

Trans infection and acute HIV-1 infection

DC-SIGN facilitates *trans* infection of HIV-1 from DC (69, 74), as well as B cells (68), to $CD4^+$ T cells. DC-SIGN also interacts with and is a receptor for infection of several other viruses (100, 101). The C-type lectin is expressed on MΦ in adult lung and uterine tissue (103), which suggests that DC-SIGN expressing MΦ could aid in the uptake and trafficking of HIV-1 from the female reproductive tract. Our data showed the number of DC-SIGN positive MΦ was positively associated with disease progression and correlated with the magnitude of SN MΦ-mediated *trans* infection in SN, which supports they hypothesis that DC-SIGN-mediated HIV-1 *trans* infection could be a mechanism of virus dissemination.

With immunohistochemistry and live-cell imaging, MΦ have been visualized passing retroviruses to B cells in lymph nodes (72). Additionally, follicular $CD4^+$ T helper cells (Thf)

have recently been identified as a reservoir of HIV-1, as well as an essential $CD4^+$ T cell subset that supports HIV-1 replication and increased viral load (73). Thf are found primarily in lymph node T cell regions where they can interact with B cells at the edge of B cell follicles. It is possible that HIV-1 passed from MΦ to B cells could be further passed to Thf in primary and secondary lymphoid organs, such as the spleen and lymph nodes, which are known major sites of HIV-1 replication *in vivo*.

DC-SIGN is constitutively expressed on MΦ in lymph nodes (102). We found that the number of DC-SIGN surface expressing MΦ was associated with HIV-1 disease progression. The percentage of DC-$SIGN^+$ MΦ cultured from PR monocytes was significantly higher compared to that of NP, and positively associated with *trans* infection efficiency in SN, which indicates that this characteristic is not an effect of HIV-1 infection. Our data support that DC-SIGN facilitated *trans* infection of HIV-1 by MΦ could contribute significantly to infection of Thf and the robust amplification of virus typically seen in acute infection upon HIV-1 dissemination to lymphoid tissue.

In addition to trafficking to lymphoid tissue, MΦ are one of the few immune cells that can gain access to the central nervous system, including the brain. MΦ have been shown to carry HIV-1 from the periphery into the brain and microglia, as well as perivascular MΦ support HIV-1 infection (234). MΦ – mediated *trans* infection of brain-resident MΦ or neuronal cells could assist in virus replication in the brain and the pathology of HAND. Elimination or control of HIV-1 in the brain and limiting cognitive impairment associated with HIV-1 is challenging, even in the presence of cART (235). Limiting the efficiency of MΦ to carry HIV-1 to the brain pass virus in *trans* could help lower the reservoir of HIV-1 in the brain and potentially improve HAND outcomes in infected individuals.

Trans infection and chronic HIV-1 infection

In addition to robust virus replication, *trans* infection at low levels may help aid in establishment of the reservoir as well as reservoir maintenance. In SN MΦ, we showed that *trans* infection occurred with high efficiency when MΦ were loaded with a concentration of HIV-1 insufficient for *cis* infection of MΦ and $CD4^+$ T cells. *Trans* infection of HIV-1 can be highly efficient and can be a way to reduce the susceptibility of HIV-1 to cART (14). Additionally, HIV-1 *trans* infection can occur in the presence of certain antiretroviral drugs (99). Additionally, $CD4^+$ T cells can mediate infection in *trans* of other $CD4^+$ T cells (236), which could contribute further to virus dissemination throughout the body.

MΦ-mediated *trans* infection and disease progression

Due to the intimate interactions between MΦ and T cells in lymphoid tissue (232), we hypothesized MΦ *trans* infection would be deficient in NP. Our data showed that MΦ-mediated HIV-1 *trans* infection was undetectable in NP and therefore associated with HIV-1 disease progression. However, the mechanism(s) by which a lack of *trans* infection influences HIV-1 disease progression is unknown. Here we speculate the ways in which MΦ-mediated *trans* infection could influence immune activation and $CD4^+$ T cell loss, well-known hallmarks of HIV-1 disease progression.

According to the MACS, NP can be classified by their ability to control viral load or maintain healthy $CD4^+$ T cell counts (see introduction section "***Disease progression***" for more detail). However, there is some overlap among subtypes of NP. For example, a healthy $CD4^+$ T cell count can be strongly associated with undetectable viral load, therefore some LTNP can concurrently be categorized as EC. However, some LTNP maintain healthy $CD4^+$ T cell counts

despite detectable and even high viral load. We studied NP with healthy $CD4^+$ T cell counts regardless of viral load because $CD4^+$ T cell count is indicative of HIV-1 disease progression. They also lack previously determined genetic factors associated with disease progression, and therefore represent a valuable population from which novel mechanisms of disease control can be identified. However, the heterogeneity of NP clinical characteristics, as well as the collective published work studying NP, suggest the manifestation of nonprogression is multifactorial and not the same for all individuals. Studying different groups of HIV-1 NP would further our understanding of the role of *trans* infection in HIV-1 disease and progression.

$CD4^+$ T cell count decline is the primary feature of HIV-1 disease progression to AIDS, and the cause of $CD4^+$ T cell loss is believed to be multifactorial. HIV-1 replication and virus-mediated apoptosis are believed to contribute, however immune activation is more highly associated with $CD4^+$ T cell count decline than viral load (237). Additionally, chronic T cell activation, characterized by increased expression of HLA-DR, CD38, CCR5, and PD-1, is believed to be the primary force driving immune activation (238). In some individuals, a low level of virus replication can persist and contribute to chronic immune activation despite therapy (239). If HIV-1 *trans* infection can facilitate low levels of virus replication under cART, it can likely contribute to greater immune activation, which is more commonly observed in PR compared to NP (240). Consequently, lower activation of $CD4^+$ T cells could result in fewer susceptible T cell targets for HIV-1 infection and lower viral load.

MΦ-mediated *trans* infection of HIV-1 may also contribute to $CD4^+$ T cell activation and HIV-1 – associated pathogenesis in the gastrointestinal tract (GIT). Gut-associated lymphoid tissue (GALT) is the primary site of HIV-1 replication in human tissue, an abundant reservoir of $CD4^+$ T cells and other lymphocytes, and a major site of $CD4^+$ T cell loss (241). MΦ densely

populate the intestinal mucosa, and while gut MΦ are considered less susceptible to HIV-1 infection compared to their blood-derived counterparts (242), they can carry latent virus, even in the presence of cART (243). Interactions between such cells and GALT $CD4^+$ T cells following virus uptake by of infection of gut-associated MΦ in the GIT and GALT could promote HIV-1 *trans* infection from those MΦ to other cells. Although we analyzed blood-derived MΦ, it is likely that gut-resident MΦ trafficking virus to GALT may also be capable of HIV-1 *trans* infection. Therefore, HIV-1 *trans* infection could be a driving mechanism of virus production and subsequent pathogenesis in the GIT and GALT.

Pyroptosis is a proinflammatory form of cell death induced mediated specifically by caspase-1. Pyroptosis of $CD4^+$ T cells undergoing abortive HIV-1 infection can result in chronic inflammation and chemo attraction of target $CD4^+$ T cells (244, 245) which could enhance the infection of other target cells and further cell death. This process is deemed feasible by mathematic models as a mechanism of slow gradual $CD4^+$ T cell decline observed in many HIV-1 infected individuals (246). Abortive infection as a result of HIV-1 transfer in *trans* infection can occur (247), therefore a lack of APC-mediated *trans* infection in NP could help decrease abortive $CD4^+$ T cell infection and subsequent pyroptosis and thereby maintain healthy cell counts.

Unlike B cells and DC, *ex vivo* tissue-resident MΦ and *in vitro* monocyte-derived MΦ are susceptible to HIV-1 infection (248), and *trans* infection can occur when virus is released into the infectious synapse following *cis* infection of a susceptible APC, such as MΦ (*see introduction section* "**MΦ *trans* infection *in vitro* and *in vivo***"). As previously demonstrated (230), we observed that SN and PR MΦ are significantly more susceptible to *cis* infection than NP MΦ. However, in SN MΦ *cis* infection did not correlate with *trans* infection efficiency, as 5

of 8 SN with efficient MΦ *trans* infection had undetectable MΦ *cis* infection. Therefore MΦ susceptibility to *cis* infection was not required for *trans* infection. Although it may contribute, MΦ susceptibility to *cis* infection is likely not the sole factor responsible for the complete lack of *trans* infection observed in NP.

MΦ replication and production of HIV-1 in tissues can be a source of virus during AIDS development, and the presence of opportunistic infections and interactions of MΦ with such antigens can increase MΦ HIV-1 production (249). The greater MΦ *cis* infection observed in PR could provide more virus for *trans* infection, immune activation, and subsequent $CD4^+$ T cell loss throughout disease progression. As concordance with our previous study (17), we demonstrated that T cell susceptibility to *cis* infection was not different in SN, NP, and PR, which further supports the hypothesis that altered virus - APC interactions in NP is more likely to be responsible for poor *trans* infection, than a lack of $CD4^+$ T cell susceptibility. Although NP $CD4^+$ T cells may produce infectious virus, T cell – to – T cell infection is far less efficient than APC – to – T cell infection.

Collectively, the association of MΦ *cis* and *trans* infection with HIV-1 disease progression suggests that MΦ interactions with HIV-1 are more complex than that of B cells and DC, due to their lack of and low susceptibility to *cis* infection, respectively. However, since MΦ, B cells, and DC display the same *trans* infection phenotype in NP, *trans* infection occurring following virus endocytosis, the *trans* infection pathway known to be shared by all three cell types (16), is likely to be more heavily implicated in HIV-1 disease progression.

Cholesterol: A key factor in HIV-1 pathogenesis

Previously, we showed that altered cholesterol homeostasis is linked to poor DC and B cell HIV-1 *trans* infection of $CD4^+$ T cells in NP (17). In addition to aiding in immune surveillance and immune responses during infection, MΦ are heavily involved in lipid and cholesterol metabolism (156). MΦ can internalize excessive lipoproteins and become cholesterol-rich foam cells (18). Additionally, HIV-1 can alter MΦ ability to maintain cellular cholesterol homeostasis through normal necessary cholesterol transport (179). Our cellular cholesterol characterization and manipulation data support that MΦ cholesterol shapes *trans* infection efficiency, is associated with nonprogression, and could serve as an target for HIV-1 therapies.

MΦ cholesterol content influence on HIV-1 trans and cis infection

Cholesterol is recognized as an important factor in HIV-1 infection and disease. Cholesterol in the PM of target cells is essential for interactions with HIV-1, and in the envelope of the virion it aids in virus fusion and determines infectivity (128, 129). However, the impact of MΦ cholesterol content on MΦ-mediated *trans* infection was unknown until now. We showed for the first time that treatment of SN MΦ with SIMV decreased MΦ-mediated HIV-1 *trans* infection, and squalene-enhanced cholesterol synthesis recovered MΦ *trans* infection ability in NP. Our data suggest that decreased cholesterol synthesis can hinder *trans* infection by MΦ, and similar to DC and B cells, MΦ can also use a cholesterol-mediated mechanism of *trans* infection. Additionally, our data demonstrated that total cholesterol was lower in NP MΦ than PR MΦ, which further supports the relevance of cholesterol in HIV-1 disease progression.

Although measuring total cholesterol can provide insight with regard to the overall difference in cellular cholesterol metabolism, it can not indicate the presence or location of specific cholesterol types, such as esterified and unesterified FC. If not esterified and effluxed, FC in the cytosol is rapidly transported to the PM where it contributes to lipid raft formation which influences membrane integrity and fluidity, as well as the function of many surface proteins (113). We showed NP MΦ had significantly lower FC and membrane lipid rafting than PR MΦ. Intriguingly, although SIMV treatment did not decrease MΦ total cholesterol, SIMV treatment did significantly decrease MΦ FC, as well as lipid rafting. Together, this suggests that PM-localized cholesterol, such as FC-rich lipid rafts, could be a regulator of HIV-1 *trans* infection and serve as a targetable component of MΦ-mediated HIV-1 *trans* infection.

Changes in PM fluidity due to altered cholesterol and sphingolipid compositions can alter function of many cell surface proteins (110). HIV-1 interacts with several molecules and proteins of the MΦ surface, and such virus – cell interactions are essential for both MΦ *cis* and *trans* infection. This data supports that less lipid rafting in the PM of NP MΦ can result in lower or less functional cell surface expression of proteins or molecules important for virus – MΦ interactions, such as DC-SIGN mediated virus endocytosis.

DC-SIGN localizes in cholesterol-rich lipid rafts on the cell surface (50) and relies on rafting for efficient internalization of ligands (50, 145). Our data showed that SIMV treatment lowered the number of DC-SIGN expressing MΦ from SN and *trans* infection efficiency. Additionally, DC-SIGN blocking significantly decreased MΦ *trans* infection. Furthermore, fewer DC-SIGN expressing MΦ were detected in NP compared to PR. Taken together, this data support that low FC content of NP MΦ membranes can hinder lipid raft formation and thus functional surface expression of DC-SIGN which, in turn, can decrease the efficiency of trans

infection in NP **(Figs. 27 and 25)**. Exposure of MΦ to oxidized low density lipoprotein can also decrease DC-SIGN expression (250). Due to the reliance of DC-SIGN surface expression and function of PM lipid rafting, decreased lipid rafting in NP MΦ could directly hinder proper optimal surface expression of DC-SIGN and negatively affect DC-SIGN-facilitated *trans* infection of HIV-1 by MΦ, thereby decreasing disease.

Decreased host cell PM cholesterol content can decrease surface expression and function of CD4 and CCR5 (144, 251, 252). Although we did not see a difference in CD4 or CCR5 surface expression by NP and PR MΦ, decreased lipid rafting could increase membrane fluidity in NP MΦ and decrease function of both proteins contributing to the decreased *cis* infection observed in NP. We postulate that DC-SIGN can enhance *cis* infection of MΦ, if not by directly facilitating viral envelope binding and fusion, then by increasing or stabilizing virus interactions on the surface of the cell through interactions with gp120 (253).

MΦ in peripheral tissue, secondary lymphoid tissue, and the central nervous system can harbor HIV-1 (254), and MΦ *cis* infection can occur in the presence of cART (255). DC-SIGN – facilitated *cis* and *trans* infection of HIV-1 mediated by the MΦ reservoir in PR could further explain persistence and maintenance of the T cell reservoir in such individuals despite cART. More generally, this mechanism of *trans* and *cis infection* may be hijacked by other viruses to enhance viral dissemination as DC-SIGN (100, 101) and cholesterol are important for infection of many viruses (131, 132, 256).

MΦ from 1 of the 4 NP we studied exhibited detectable *cis* infection, however that individual was not capable of *trans* infection of $CD4^+$ T cells. Others have proposed that despite NP susceptibility to HIV-1 infection, the virus subsequently produced by NP may be poor or less infectious virus. HIV-1 virions require cholesterol in their envelopes which is derived from the

host cell membrane following budding of new virus. Due to the decreased level of FC in the cell membranes of NP MΦ, the virus produced from such cells may be less infectious and therefore less likely to efficiently infect other cells, including $CD4^+$ T cells, in *trans.* Although we did not test the cholesterol content or infectivity of the virus isolated from NP2 MΦ, it would be of interest for future studies.

A unique challenge of studying the impact of MΦ cholesterol content of HIV-1 produced by NP is that because NP $CD4^+$ T cells do not have lower cholesterol content compared to that of PR (17), virions produced by NP $CD4^+$ T cells likely have high envelope cholesterol levels and good infectivity. Therefore, analysis of the cholesterol content of virus isolated from the blood or tissue of NP could lead to spurious results due to the unknown cellular origin of the virus. However, testing cholesterol content and infectivity of virus produced by *in vitro* derived MΦ or tissue isolated MΦ from NP would be of interest for future studies, because decreasing virus infectivity through manipulation of cholesterol metabolism represents another potential benefit of targeting cholesterol with novel HIV-1 therapeutics.

Importantly, SIMV treatment and DC-SIGN blocking did not completely abrogate MΦ *trans* infection. HIV-1 can bind DC in a cholesterol dependent manner, independently of CD4, DC-SIGN, mannose binding C-type lectin receptors, and heparan sulfate proteoglycans expression (142). While DC-SIGN facilitated *trans* infection, it is not the only mechanism by which MΦ mediate *trans* infection. CD169 (siglec-1) can mediate the capture of retroviruses by macrophages lining the sinus region of LN and virus passage to B cells within LN follicles (72),\. However, MΦ CD169 expression was not associated with *trans* infection in our studies. Analyses of MΦ isolated from lymphoid tissue from SN, PR, and NP will help to better

understand the role of CD169 expression in HIV-1 *trans* infection and its relationship to disease progression.

MΦ – virus interactions and HIV-1 trans infection

Although we have identified a cholesterol - dependent DC-SIGN pathway of MΦ *trans* infection, how disruption of this pathway alters MΦ – virus interactions is unknown. B cells do not and DC only minimally support HIV-1 replication. Therefore, we hypothesized that the endocytosis pathway of *trans* infection is likely to be more heavily implicated in HIV-1 disease progression as it is the pathway shared by all three APC.

We previously have shown that DC and B cells binding of HIV-1 was not different in NP and PR (17). Here we showed that NP and PR MΦ bound HIV-1 at similar levels, which further supports that total virus binding to NP APC is not responsible for altered *trans* infection. Alternatively, we showed that NP MΦ internalized significantly less HIV-1 into early endosomes compared to that of PR. Importantly, DC-SIGN has been shown to mediate HIV-1 endocytosis into early endosomes (81). Therefore, decreased FC and lipid rafting in MΦ could result in less binding of HIV-1 to DC-SIGN and thereby decrease DC-SIGN-facilitated endocytosis and *trans* infection.

Treating HIV-1 with SIMV

We showed that SIMV significantly decreased FC, lipid rafting, the number of DC-SIGN expressing MΦ, as well as MΦ ability to enhance T cell infection in *trans* compared to *cis* infection. SIMV is a frequently prescribed FDA-approved drug used to treat hypercholesterolemia (257), that could feasibly be repurposed for the treatment of HIV-1

infection. Statins were beneficial in several HIV-1-focused clinical trials, and could influence virus production and immune activation, as well as all the downstream consequences of these two factors on nonprogression that we previously discussed.

Statins have been assessed for their ability to lower chronic inflammation and immune activation associated with HIV-1 infection (147-149), combat cART-associated dyslipidemia and cardiovascular disease risk (150, 151), as well as decrease overall mortality in HIV-1 seropositive individuals (152). These studies collectively suggest that the use of statins does not significantly augment current HIV-1 treatment regimes. Yet, a recent study found that individuals currently taking statins had fewer documented cases of viral rebound after therapeutic viral suppression (153). Importantly, all of these studies were conducted on groups of HIV-1 infected individuals on cART, but certain antiretroviral drugs can significantly alter statin pharmacokinetics (154). Furthermore, a study on the effect of statins in a small group of HIV-1 infected, therapy naïve individuals, showed a reduction of viral load under statin treatment, which rebounded after therapy interruption (155).

Interestingly, we observed that SIMV treatment decreased the population of DC-SIGN^{+} MΦ without decreasing total MΦ cellular cholesterol. Statins competitively inhibit HMG-CoA reductase (258). Although HMG-CoA reductase is an essential enzyme for cholesterol synthesis in MΦ, it is also essential for protein prenylation (229). Protein prenylation is a form of lipid modification that can increase the ability of proteins to interact with cell membranes (229), and statins can decrease HIV-1 release from the monocyte-like U1 cell line by inhibiting the protein prenylation pathway (259). We showed that despite an insignificant decrease in total MΦ cholesterol following SIMV treatment, recovering cholesterol synthesis, unlike protein prenylation, also recovered DC-SIGN surface expression. This data supports that clinical SIMV

treatment of HIV-1 infection individuals may also decrease virus replication in this manner and positively influence disease outcome. Statins in combination with cART, under alternative administration routes less affected by cART drugs, could reduce MΦ dissemination of HIV-1 and should be reconsidered for prophylaxis and therapy regimens. Manipulation of ABCA-1–mediated cholesterol efflux with nuclear receptor agonists, or LDLR–mediated cholesterol uptake with the herb-derived compound, berberine (260), to decrease cellular cholesterol and lipid rafting may serve a purpose similar to SIMV.

Regulation of cholesterol metabolism in NP APC

Thus far, I have discussed how MΦ cholesterol content and localization can influence HIV-1 *trans* infection in relation to disease progression. However, in addition to being a contributor to HIV-1 infection, MΦ cholesterol content can also be a reflection of HIV-1 infection. HIV-1 proteins, such as Nef, can interrupt cellular cholesterol homeostasis leading to intracellular cholesterol sequestering and an increase in cell-association cholesterol content (179). Yet, despite HIV-1 infection, NP MΦ maintain lower levels total cholesterol, FC, and lipid rafting than PR, consistent with other APC, such as DC and B cells (17). Therefore, NP APC likely have a unique ability to naturally reduce cellular cholesterol or counteract the HIV-1 mechanisms of cholesterol sequestering. MΦ cholesterol homeostasis requires balancing of several mechanisms of cholesterol regulation including cholesterol uptake, efflux, and *de novo* synthesis. Our cholesterol metabolism-associated gene and miRNA expression data provide further insight into the pathway(s) through which NP MΦ may maintain a low level of cholesterol content during HIV-1 infection.

Cholesterol sensing in NP MΦ

Through cholesterol sensing, a cell can respond to the intra- and extracellular cholesterol environment, as well as induce cholesterol uptake, synthesis, or efflux to achieve cholesterol levels needed by the cell. Our data support that MΦ from both NP and PR can efficiently sense their intracellular cholesterol levels. NP MΦ had greater LDLR gene expression which, in response to low cholesterol, could increase cholesterol uptake. Complementarily, PR MΦ had greater PPARγ gene expression which, in response to high cholesterol could increase cholesterol efflux. Nuclear receptor induced cholesterol efflux by ABCA1 can decrease HIV-1 *trans* infection by DC (130). However, our data suggests that the differential expression of PPARγ by NP and PR MΦ is a result of or a response to the different NP and PR MΦ cholesterol content rather than the cause of lower cholesterol content and *trans* infection in NP compared to PR. Increased expression of the efflux-mediating cholesterol transport protein, ABCA1 is a characteristic of NP DC (17).

We also observed that more NP had CD1β expressing MΦ, which better equips these cells for lipid antigen presentation because MΦ can generate and present lipid-based antigens using CD1β. HIV-1 nef and vpr can down-regulate CD1 expression on DC (261), which may represent a mechanism used by the virus to evade immune detection of infected cells by CD1-restricted NKT cells (262). Better lipid antigen presentation by NP MΦ due to a greater abundance of CD1β-expressing cells could contribute to better anti-HIV-1 immune responses and slower disease progression, two characteristics of NP (263). However, our data assessing gene expression levels of essential genes involved in cholesterol uptake, efflux, and synthesis did not explain the mechanisms contributing to the differences in cholesterol levels we see in NP and PR MΦ either. Exosomes disseminated from HIV-1 infected cells can facilitate HIV-1 *trans*

infection (264), as well as transport proteins and RNAs, including miRNA (265). We hypothesized that the low cholesterol state of NP APC is a result of extracellular factors, such as miRNA, signaling molecules, or Apolipoproteins, all of which have been shown to alter cellular cholesterol metabolism (201, 266, 267).

MiRNA and HIV-1 non-progression

Several miRNA can regulate gene expression of proteins involved in human cellular cholesterol homeostasis (201, 268, 269), including miR-148a-3p (270, 271), miR-144-3p (272, 273), and miR-150-5p (274). miR-148a-3p directly negatively regulates LDLR and ABCA1 expression in Huh7 cells, and *in vivo*, single nucleotide polymorphisms in the miRNA promoter have been associated with altered levels of circulating LDL (275). miR-144-3p can also directly target and decrease ABCA-1 levels in hepatic cells and has been associated with a decrease in circulating HDL levels (272), indicative of decreased ABCA-1 mediated efflux of cholesterol from hepatocytes to extracellular HDL molecules. Our data showed that both miR-148a-3p and miR-144-3p expression trended higher in PR plasma compared to NP, which suggests that these miRNA could be implicated in the cholesterol metabolism differences in NP and PR APC, particularly PR DC which express less ABCA-1 than NP DC (17). MiRNA, such as miR-148a, expressed by and released from cells in exosomes could act on cells encountered in circulation, and effect MΦ, DC, and B cells. Overall, lower concentrations of circulating miR-148a-3p and miR-144-3p in NP, could limit down-regulation of proteins involved in cholesterol metabolism leading to higher levels of cholesterol efflux, lower APC cellular cholesterol, and inefficient HIV-1 *trans* infection.

In addition to regulators of cholesterol metabolism, miR-148a-3p and miR-144-3p have been categorized as an immune-related miRNA (273, 276). miR-148a can bind and decrease

expression of HLA-C, and variance in the HLA-C UTR can decrease miR-148a binding ability, and is associated with better HIV-1 control (277, 278). These studies, along with our data, suggest that the down-regulation of miR-148a expression therapeutically, may serve to combat HIV-1 disease by lowering APC cholesterol, limiting HIV-1 *trans* infection, and increase HLA-C-mediated anti-HIV-1 immune responses.

MiRNA have also been implicated in or associated with HIV-1 pathogenesis and disease directly. MiR-29b-3p can reduce HIV-1 production from 293T cells (279) and is essential for IL-21-mediated suppression of HIV-1 in $CD4^+$ T cells (280). Although we did not observe a difference in $CD4^+$ T cell production of virus in NP and PR, we described higher expression of miR-29b-3p in NP compared to PR plasma, which supports that circulating miR-29b-3p could impact HIV-1 disease progression by influencing HIV-1 production by other immune cells such as MΦ.

miR-150-5p can directly target and decrease expression of CXCR4, altering the ability of cells to interact with stromal cell-derived factor-1 resulting in a decrease in cell mobility and migration (281). MiR150-5p is primarily produced by MΦ and adipose tissue and can decrease cholesterol efflux in THP-1 cells through the down-regulation of Adiponectin receptor 2 (274). Importantly, down-regulation of adiponectin receptor 2 can induce expression of LXRα and thereby APO E production (274). Our data demonstrating an upward trend in miR-150-5p expression in NP plasma compared to PR support that an increase in the extracellular cholesterol acceptor, APO E, in NP could facilitate increased cholesterol efflux from MΦ, resulting in lower MΦ cholesterol in the absence enhanced ABCA-1 expression.

We also reported significantly higher levels of miR-376a-3p in the plasma of NP compared to PR. miR-376a-3p represses insulin-like growth factor receptor (282), and therefore

has been proposed as an anti-cancer agent. However, other studies have also found that miR-376a-3p down-regulates KLF15 (283), a known suppressor of cell proliferation, caspase 3, and caspase 8 (283, 284), thereby acting as a potential enhancer of cancer cell proliferation. Although there is currently no evidence in the literature that miR-376a-3p is directly involved in HIV-1 pathogenesis or cholesterol metabolism, miR-376a is significantly lower in the serum of obese individuals compared to healthy controls and inversely associated with serum cholesterol and LDL-C levels (285), which suggests miR-376a-3p could be involved in cholesterol metabolism in ways that have not yet been determined.

Overall, our miRNA expression data suggest that the miRNA expressed in NP plasma that we discussed could cause increased MΦ cholesterol efflux and ultimately lower MΦ FC and lipid rafting, if not due to increased ABCA-1 expression, then due to increased production and presence of extracellular apolipoproteins (**Fig. 26)**. Additionally, our data support that specific miRNA expression profiles of NP plasma could be a factor contributing to lower APC cholesterol levels we have observed in NP. Further interrogation of the pathway(s) through which NP MΦ maintain a low level of cholesterol content during HIV-1 infection could provide greater insight into how we can target *trans* infection with novel therapies.

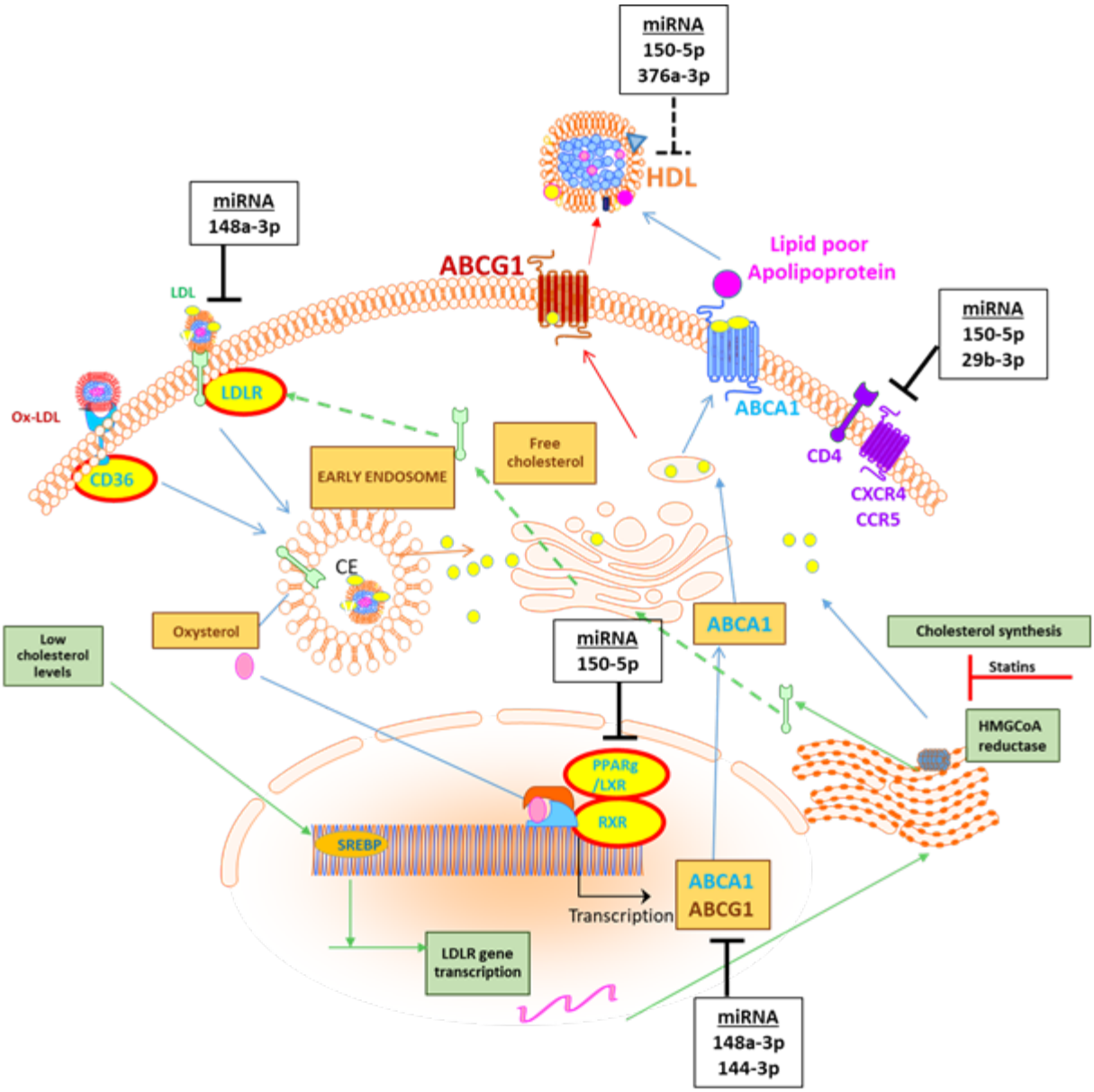

Figure 26. Summary schematic of the potential cholesterol homeostasis pathway components targeted by miRNA differentially expressed in NP and PR.

Conclusions and future work

We evaluated the involvement of MΦ-mediated HIV-1 *trans* infection in disease progression, assessed the role of cellular cholesterol in MΦ *trans* infection efficiency, and identified a potential mechanism by which cholesterol homeostasis is differentially regulated in NP and PR APC. We showed that MΦ-mediated *trans* infection is associated with HIV-1 disease progression. We also demonstrated the importance of FC and lipid rafting in MΦ for efficient DC-SIGN – mediated endocytosis of HIV-1 and subsequent *trans* infection of $CD4^+$ T cells in PR, which is lacking in NP (**Figs. 27 and 28**). Additionally, we showed SIMV treatment not only lowered the DC-$SIGN^+$ MΦ population, but also limited MΦ *cis* and *trans* infection efficiency, two features characteristic of NP. Lastly, we identified differential expression of miRNA involved in HIV-1 infection and cholesterol metabolism, miR-29b-3p and miR-376a-3p, in NP and PR, which likely contributes to the altered cholesterol homeostasis observed in NP. By altering APC cholesterol metabolism we can decrease HIV-1 *trans* infection and potentially further reduce dissemination of virus and the size of the latent reservoir.

Although significant advancements have been made in our understanding of cholesterol regulation of HIV-1 *trans* infection by MΦ, our data has generated new questions and promising leads that need to be further pursed. Future directions for this work include furthering our understanding of APC-mediated HIV-1 *trans* infection *in vivo* and the methods by which cholesterol lower agents can be used to decrease HIV-1 *trans* infection. Understanding HIV-1 *trans* infection in different MΦ types, such as tissue resident and lymph node surveying MΦ, may be important for assessing the required reach therapies targeting the elimination of the HIV-1 reservoir.

Further defining the role of cholesterol in lack of HIV-1 endocytosis in NP and targeting HIV-1 endocytosis with cholesterol manipulation in PR would also be of interest for future studies. Additionally, determining the influence of the miRNA identified by our work on APC cholesterol metabolism and *trans* infection ability through functional studies *in vitro* would help determine if there is a novel role for these miRNA in HIV-1 disease progression and whether targeting their expression has anti-HIV-1 therapeutic potential.

PR

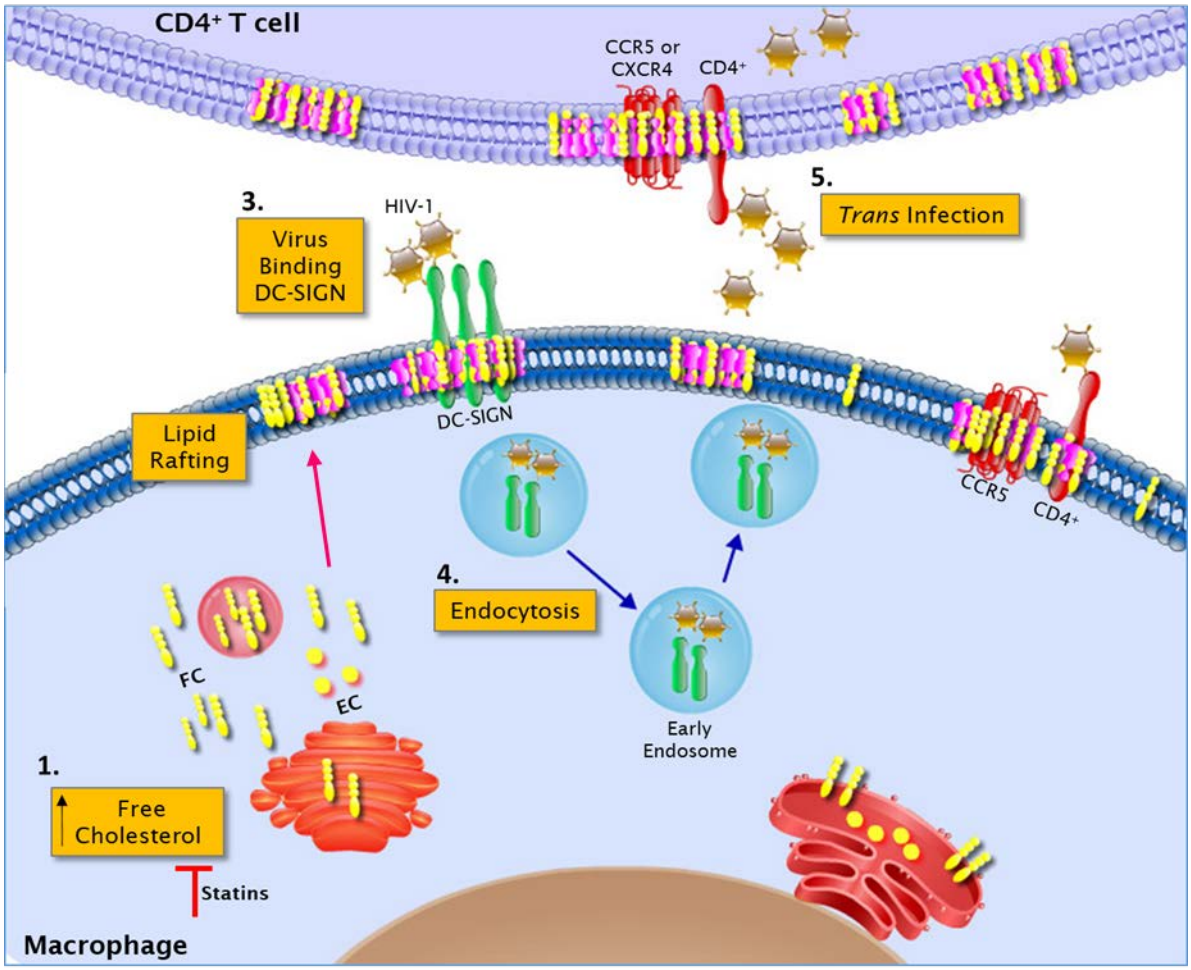

Figure 27. Schematic of the role of cholesterol in HIV-1 *trans* infection by MΦ in PR.

In HIV-1$^+$ PR, **1)** unesterified free cholesterol (FC) and esterified cholesterol (EC) are abundant in the MΦ and T cell, specifically **2)** in the PM where it aggregates to form lipid rafts. **3)** Abundant lipid rafting allows for abundant DC-SIGN expression on the MΦ surface and HIV-1 binding. **4)** This leads to DC-SIGN - mediated virion endocytosis, intracellular transport, and release of virus into the virological synapse where **5)** optimal infection of CD4$^+$ T cells occurs in *trans*. At low MOIs, *trans* infection occurs in the absence of MΦ and T cell *cis* infection.

NP

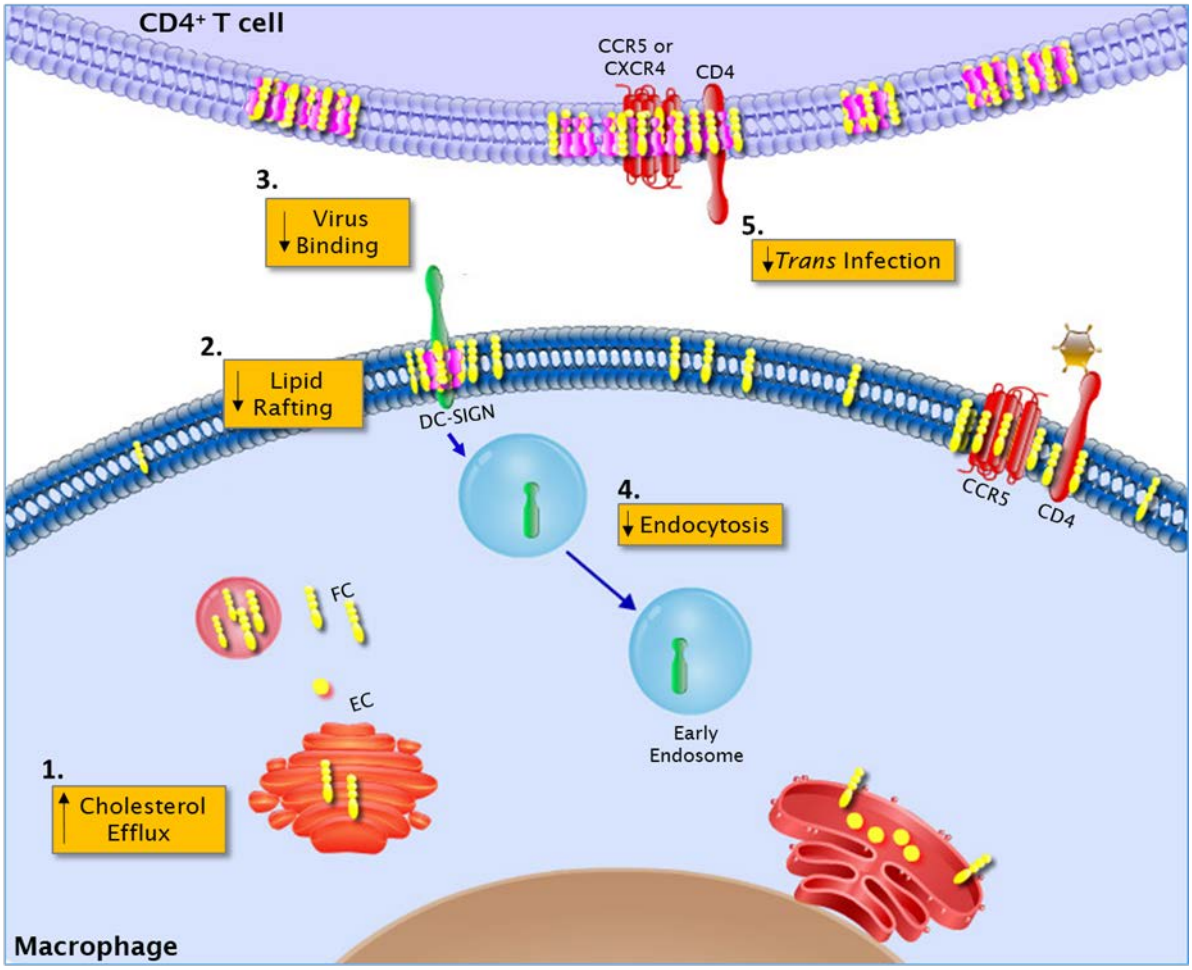

Figure 28. Schematic of the role of cholesterol in HIV-1 *trans* infection by MΦ in NP.

In HIV-1$^+$ NP, **1)** MΦ display significantly lower levels of total cholesterol which we hypothesize is mediated by extracellular elements such as miRNA, signaling molecules, or Apolipoproteins facilitating greater cholesterol efflux. **2)** Subsequently, lower levels of FC in the PM results in significantly less lipid rafting and DC-SIGN expression. **3)** Consequently, DC-SIGN - mediated virion **4)** endocytosis does not occur at a rate sufficient enough to **5)** mediate CD4$^+$ T cell infection in *trans.*

8.0 PUBLIC HEALTH IMPORTANCE

Despite the success of combination antiretroviral therapy, neither a vaccine nor a cure for HIV-1 infection has been developed, demonstrating a need for novel prophylactic and therapeutic strategies to further lower the public health burden of HIV-1 infection. The functional breadth of the reservoir of latently infected MΦ under cART is becoming more relevant for cure and therapeutic research (286-288). Actively infected MΦ, including DC-SIGN expressing MΦ in mucosal membranes (289), transmit and disseminate HIV-1 (248). MΦ-mediated *trans* infection is a feasible source of HIV-1 replication and dissemination in the presence of very low quantities of virus. Understanding how this cell-to-cell spreading of virus by MΦ impacts disease progression is of importance for development of novel treatment and curative HIV-1 therapeutics.

Here we showed that efficiency of MΦ-mediated HIV-1 *trans* infection of $CD4^+$ T cells is a unique characteristic associated with control of disease progression and impaired in HIV-infected NP. *In vitro* treatment of MΦ from healthy donors with SIMV lowers their cholesterol content which results in a strongly reduced *trans* infection ability, similar to the levels of MΦ from NP. Taken together, my data support the hypothesis that MΦ-mediated HIV-1 *trans* infection plays a role in HIV-1 infection and disease progression. Furthermore, the use of SIMV, or other cholesterol lowering agents, to decrease this mechanism of virus transfer should be considered for future HIV-1 therapeutic development, as well as for incorporation into methods for the elimination of the HIV-1 reservoir and ultimately an HIV-1 cure.

9.0 ACKNOWLEDGMENTS

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APPENDIX A: ABBREVIATIONS

ABCA1:	ATP-binding cassette transporter 1
ABCG1:	ATP Binding Cassette Subfamily G Member 1
AIDS:	Acquired Immunodeficiency Syndrome
APC:	Antigen presenting cell
APOBEC3G:	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
APOER2:	Apolipoprotein E receptor 2
ART:	Antiretroviral therapy
B cell:	B lymphocytes
BCD:	β-cyclodextrin
cDNA:	complementary deoxyribonucleic acid
CTx-B:	Cholera Toxin subunit B
DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific Intercellular adhesion molecule-3-grabbing non-integrin
ELISA:	Enzyme-linked immunosorbent assay
FBS:	Fetal bovine serum
FC:	Unesterified free cholesterol
GALT:	Gut-associated lymphoid tissue
GGpp:	Geranylgeranyl pyrophosphate
GIT:	Gastrointestinal tract

GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HAND:	HIV-associated neurocognitive disorder
HESN:	Highly-exposed seronegative
HIV-1:	Human immunodeficiency virus-1
HMGCR:	3-Hydroxy-3-Methylglutaryl-CoA reductase
IFN:	Interferon
IL:	Interleukin
kDa:	Kilodalton
LDLR:	Low density lipoprotein receptor
LTNP:	Long term nonprogressor
LXRα:	Liver X receptor alpha
LXRβ:	Liver X receptor beta
mAb:	Monoclonal antibody
MACS:	Multicentered AIDS Cohort Study
M-CSF:	Macrophage colony-stimulating factor
miRNA:	microRNA
MOI:	Multiplicity of infection
MS:	SIMV-treated MΦ
MΦ:	Macrophage
NP:	HIV-1 seropositive nonprogressor
PALLD:	Palladin
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline

PFA:	Paraformaldehyde
PM:	Plasma membrane
PPARγ:	Peroxisome proliferator-activated receptor gamma
PR:	HIV-1 seropositive progressor
RCT:	Reverse cholesterol transport
RFP:	Red fluorescent protein
RNA:	Ribonucleic acid
RNAi:	RNA interference
RT-PCR:	Reverse transcriptase polymerase chain reaction
SIMV:	Simvastatin
SIV:	Simian immunodeficiency virus
SN:	HIV-1 seronegative
SREBP:	Sterol regulatory element-binding protein
VC:	Viremic controller

APPENDIX B: MIRNA GENE INTERACTION NETWORKS

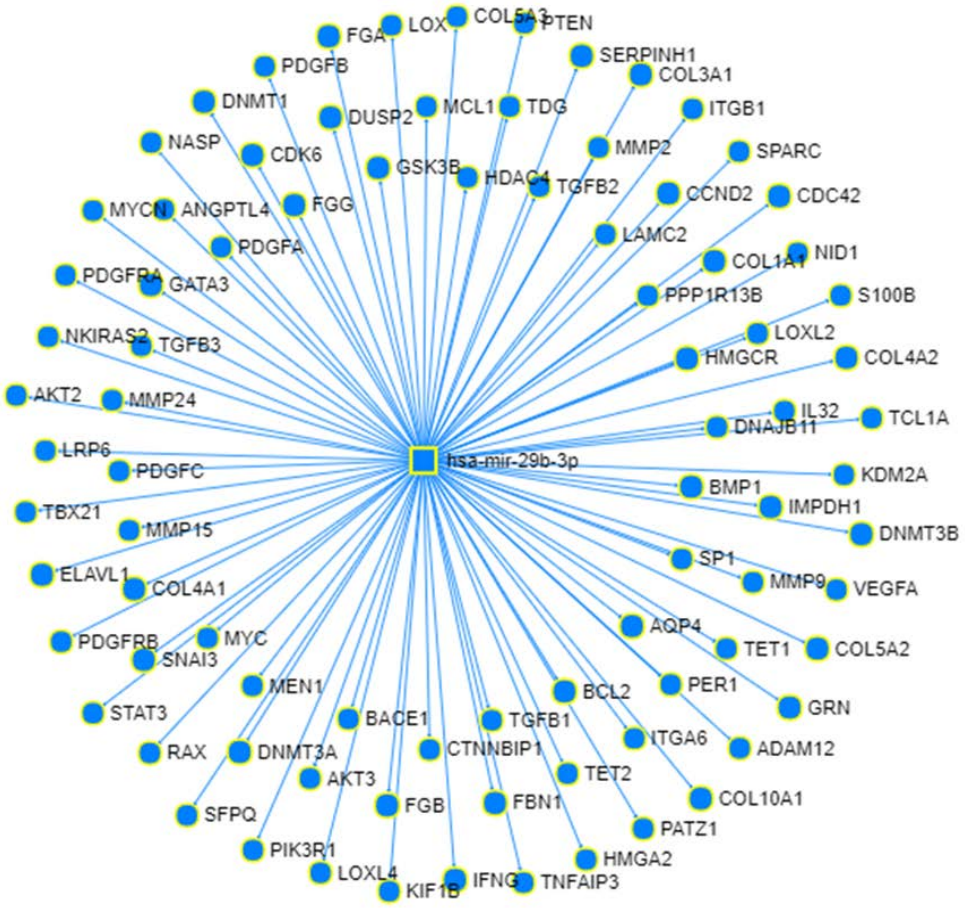


Figure 29. Has-miR-29b-3p experimentally verified human gene interactions.

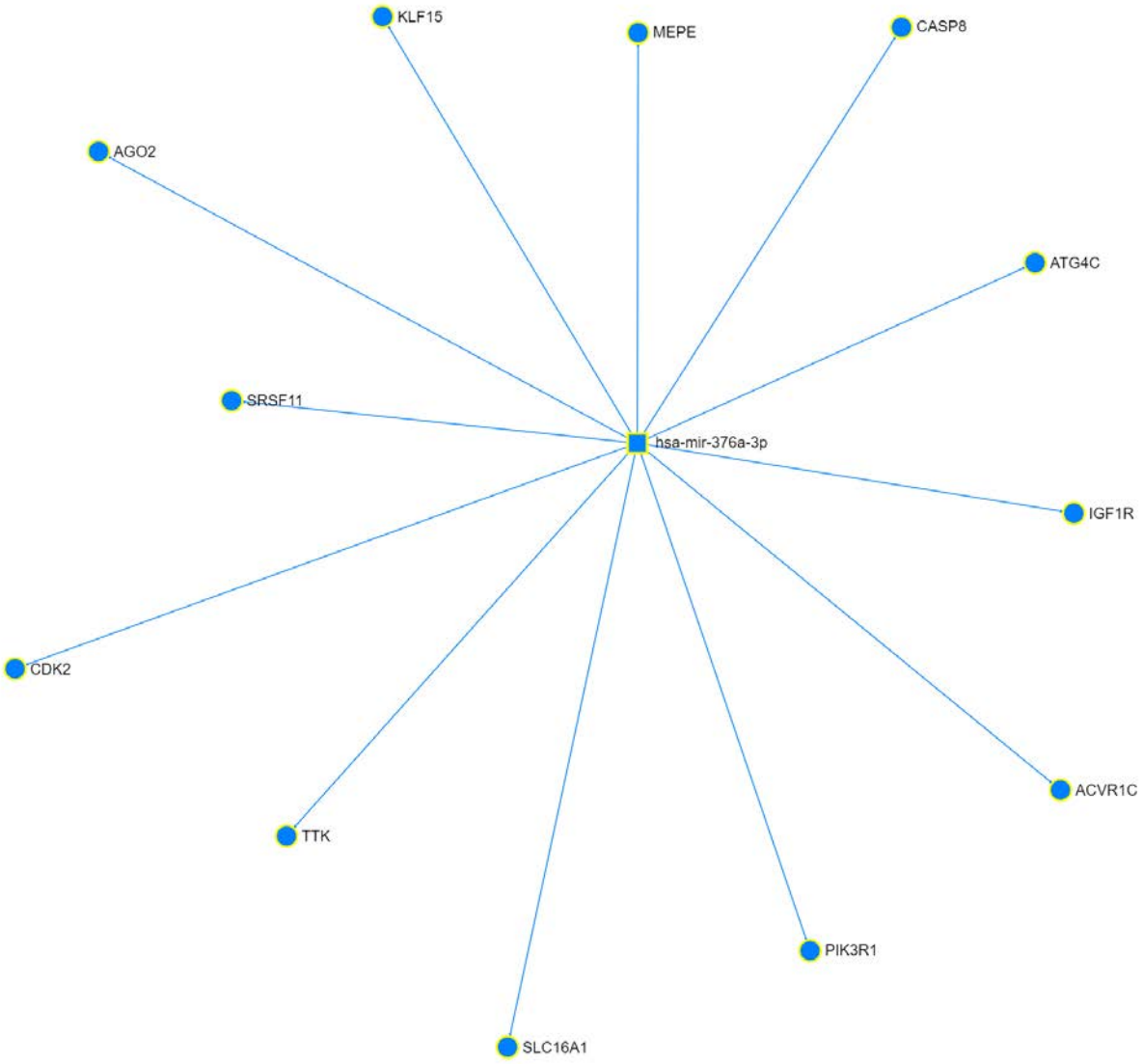


Figure 30. Has-miR-376a-3p experimentally verified human gene interactions.

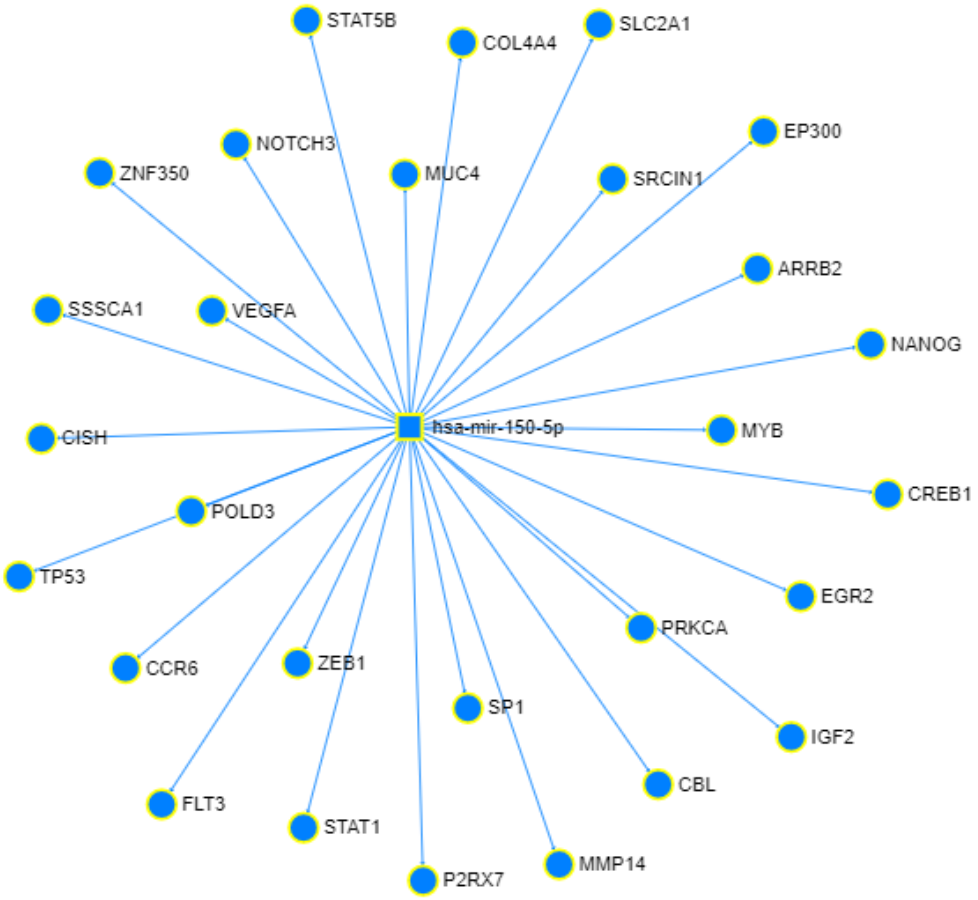


Figure 31. Has-miR-150-5p experimentally verified human gene interactions.

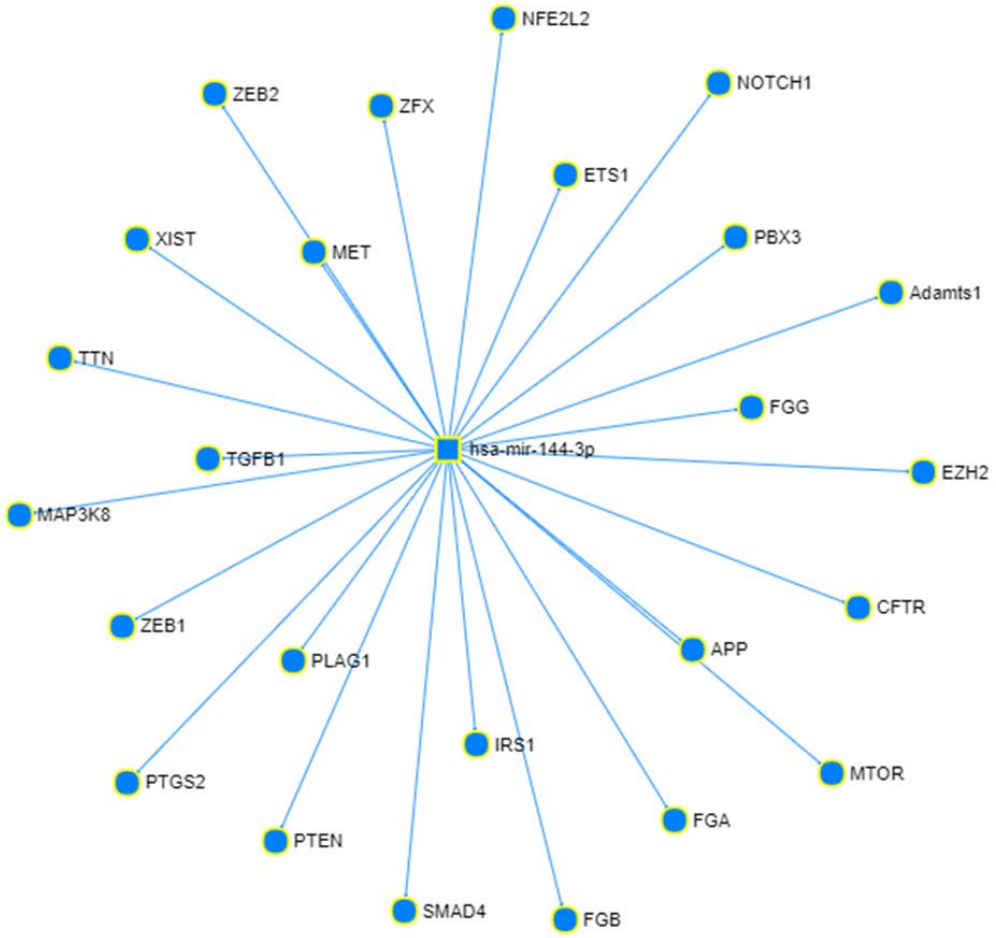


Figure 32. Has-miR-144-3p experimentally verified human gene interactions.

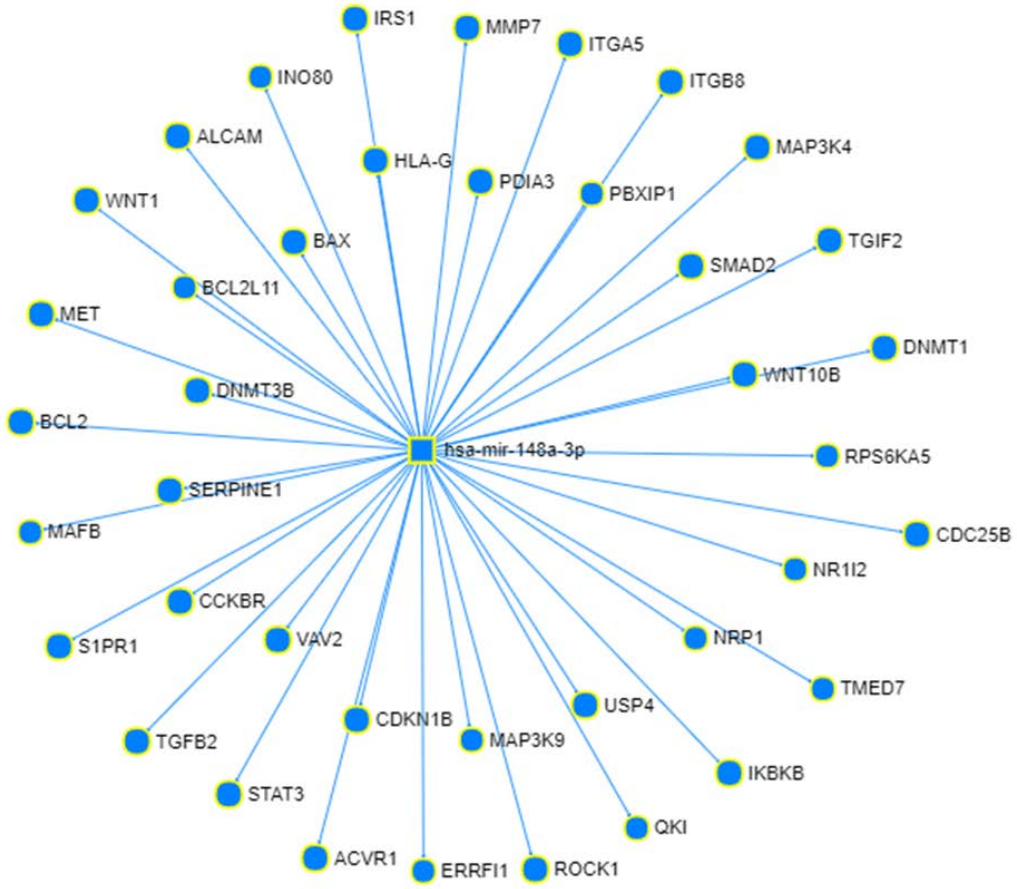
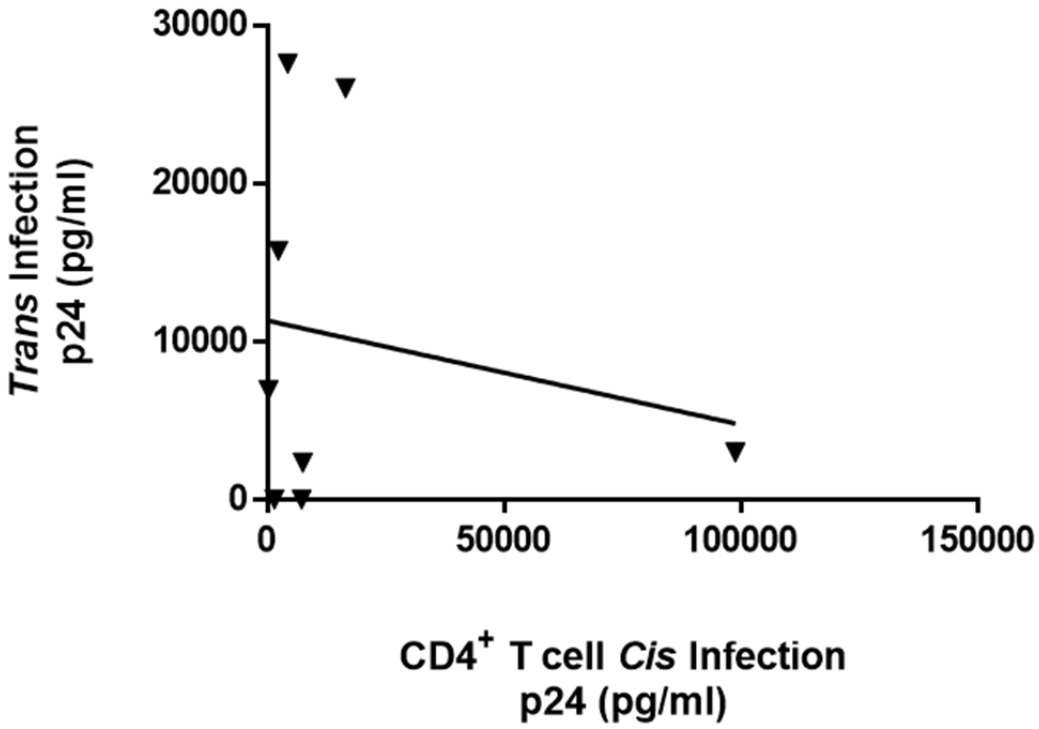


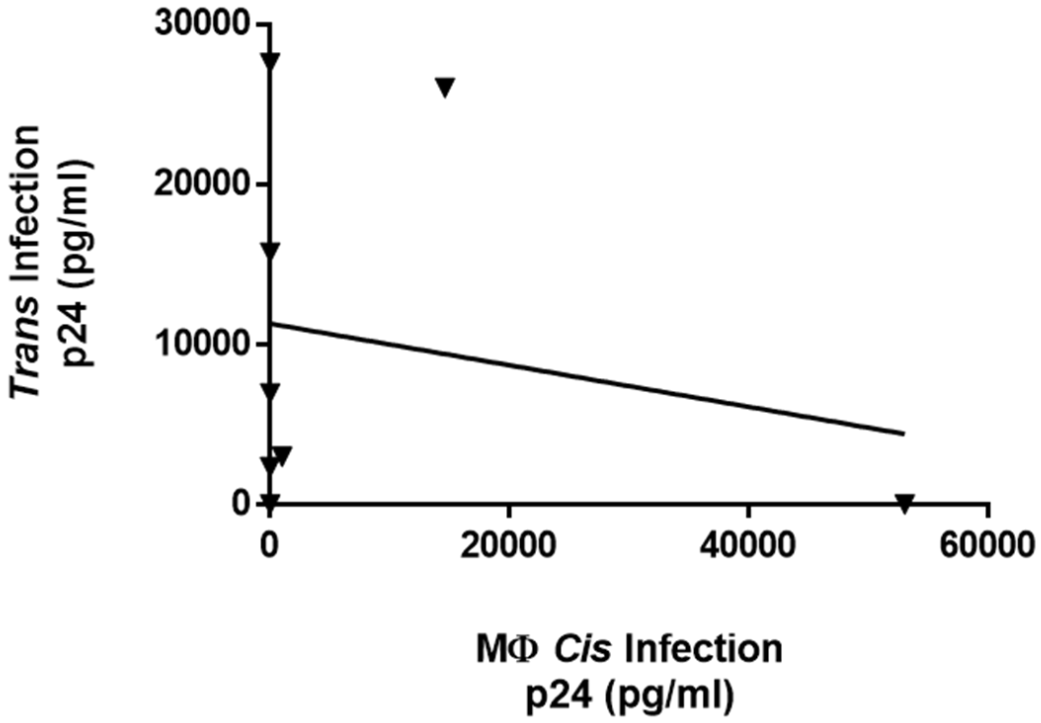
Figure 33. Has-miR-148a-3p experimentally verified human gene interactions.

APPENDIX C: SUPPLEMENTAL DATA



Supplemental Figure 1. MΦ *trans* infection efficiency is not associated with CD4$^+$ T cell *cis* infection efficiency.

MΦ- mediated *trans* infection efficiency association with CD4+ T cell cis infection (MOI 10^{-1}) (p =0.6486, F =0.2299, R2 =0.0369)



Supplemental Figure 2. MΦ *trans* infection efficiency is not associated with MΦ *cis* infection efficiency.

Mφ- mediated trans infection efficiency association with Mφ cis infection (MOI 10-1) (p =0.6140, F =0.2827, R2 =0.045)

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