# SIGLEC-10, CD1B, AND PALLADIN ARE DIFFERENTIALLY EXPRESSED BETWEEN HUMAN IMMUNODEFICIENCY VIRUS POSITIVE (HIV+) PROGRESSORS AND NONPROGRESSORS

by

**Marilee Ja Clunk**

BS in Molecular Biology, University of Pittsburgh, 2015

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This thesis was presented

by

**Marilee Ja Clunk**

It was defended on

**June 19, 2017**

and approved by:

**Committee Member:**
Bernard Macatangay, MD
Assistant Professor of Medicine
Division of Infectious Diseases, Department of Medicine
School of Medicine
University of Pittsburgh

**Committee Member:**
Giovanna Rappocciolo, PhD
Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

**Thesis Director:**
Jeremy Martinson, DPhil
Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Jeremy Martinson, DPhil

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## ABSTRACT

HIV plasma viremia and CD4+ lymphocyte counts dictate the classification of HIV+ disease progression to Acquired Immunodeficiency Syndrome (AIDS), i.e. progressor (PR) vs. non-progressor (NP). In the absence of antiretroviral therapy, NP CD4+ counts remain stable and plasma viremia is substantially reduced. Recent data affirms that NPs exhibit decreased trans-infection efficiency due to reduced cellular cholesterol levels in antigen presenting cells (APCs) through cholesterol efflux protein, ABCA-1. Because defective *trans* infection in NPs was also observed prior to seroconversion, it is believed that nonprogression is influenced by a genetic trait. Further transcriptome analysis of Multicenter AIDS Cohort Study (MACS) immature dendritic cells (iDCs) reveals elevated expression profiles for Siglec-10, CD1B, and Palladin (PALLD) in NPs compared to PRs. While the explicit mechanism remains elusive, our aim is to elucidate the genetic origin of NP viremic control. I have hypothesized that upregulated expression of Siglec-10, CD1b, and Palladin in HIV+ MACS NPs is responsible for control of HIV progression by attenuating *trans* infection between iDCs and CD4 lymphocytes through unrelated molecular and immunological mechanisms.

MACS CD14+ monocytes were cultured with IL-4 and GM-CSF to yield iDCs. Protein expression of Siglec-10, CD1B, and PALLD in seronegative (SN), PR, and NP iDCs has been assessed using flow cytometry. Quantitative PCR (qPCR) analysis of MACS iDCs RNA is used

for corroboration of gene expression data from the transcriptome findings. TaqMan® SNP genotyping assays examined if there is a link between NP disease state and single base-pair variations within DNA.

Flow cytometry shows no significant difference in Siglec-10, CD1b, or PALLD protein expression levels between PR and NP donors. SyBr green qPCR primers for resulted in artefactual amplicons for control and experimental melt curves. IDT Taq-Man® primers were subsequently designed. Analysis of qPCR data shows significant differences in CD1b and PALLD expression levels between NPs and PRs ($p < 0.05$). Haplotype analysis of CD1b SNPs between NP, PR, and SN individuals via TaqMan Genotyping was not statistically significant.

**Public Health Significance:** Identifying the underlying genetic factors that drive HIV-1 disease progression could reveal potential therapeutic targets with the long term aims of improving HIV health outcomes.

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# PREFACE

I would like to graciously thank past and present members of the Martinson, Rappocciolo, and Rinaldo Labs for their constant support and guidance in my research endeavors, namely Blandine Victor, M.S.; Diana DeLucia (Campbell), M.S., PhD Candidate; Kathleen Hartle, B.S.; Patrick Mehta, B.S., and James Hayes. I want to specifically recognize Diana, Kathleen, Patrick, and James in assisting me with the herculean task of retrieving MACS samples from the liquid nitrogen freezers. I would also like to extend my gratitude to N. Jayanth Venkatachari, PhD for his technical assistance. Most importantly, I would like to recognize the vital contribution of all past and present MACS donors—without whom, this research would not be possible.

# 1.0 INTRODUCTION

In 1981, Acquired Immunodeficiency Syndrome (AIDS) captured the attention of the medical and scientific community[2]. Initially, it was exclusively associated with homosexual men with unique opportunistic infections and a rare type of endothelial cancer known as Kaposi's sarcoma[3]. Concrete epidemiological data purported that the etiological agent was a sexually transmitted pathogen in which homosexuality and bisexual activity served as the greatest risk factor[3]. HIV, the causative agent of AIDS, was isolated from patients two years later[4]. Current research has established that HIV is transmitted through several definitive routes: sexual (i.e. between heterosexual and homosexual individuals), perinatal (i.e. mother to fetus transmission), and percutaneous (i.e. drug users)[5]. Despite modern advances in the treatment and maintenance of HIV through highly active antiretroviral therapy (HAART), over 2.5 million individuals worldwide are infected annually[6]. As researchers continually search for a sustainable cure, investigating the underlying molecular and genetic factors that drive HIV infection remains a top priority.

## 1.1 HIV PATHOGENESIS

HIV's viral life cycle has been studied extensively, elucidating entry and attachment factors, as well as transmission[7,8]. In the vast majority of infections, HIV invades host immune

cells through mucosal exposure (e.g. vaginal or anal mucosa in adults), and it replicates within helper T-cells, macrophages, and monocytes[9,10]. More specifically, binding of the CD4 T-cell receptor and CCR5/CXCR4 co-receptors via HIV gp120/41 spikes are crucial factors in cell entry as well as virulence, disease progression, and cell tropism[7,8].

Receptor mediated fusion of host and viral envelopes facilitates capsid entry into the host cytoplasm. As a single stranded RNA retrovirus, the HIV genome is converted into double stranded DNA via viral RNA dependent DNA polymerase, also known as reverse transcriptase. Viral integrase incorporates the new dsDNA into the host genome, forming an undetectable provirus. Host cell machinery is used to transcribe and translate the provirus in order to create transmissible, infectious progeny, which undergo further processing and maturation by HIV protease prior to budding. Hallmark indicators of HIV infection include depleted CD4+ lymphocyte counts and persistent, elevated levels of plasma viremia[11].

## 1.2 DCS FUNCTION AS IMMUNOLOGICAL SENTINELS

DCs bridge the gap between the innate and adaptive immune response by surveying lymphoid tissues, blood, and various organs; broadly recognizing pathogenic features; and activating CD4 helper T cells through antigen presentation[12,13]. In order to display antigens on the cellular surface, microbial and viral pathogens are degraded into smaller peptide fragments and transported to the dendritic cell surface through highly specific mechanisms, namely through the Major Histocompatibility Complex (MHC) I and II pathways.

DCs exist in different states based on expression of surface markers and cytokine profile production[14]. Immature dendritic cells are primarily responsible for maintaining immune tolerance

by preventing strong reactivity with host antigens. When immature dendritic cells phagocytize foreign pathogen encountered in peripheral tissues, they migrate to regional lymph nodes and subsequently undergo activation via upregulated expression of chemokine receptors, co-stimulatory molecules, adhesion molecules, and MHC molecules[15]. Mature dendritic cells are responsible for priming and activating T-cells through antigen presentation, which allows for highly specific and coordinated host response against foreign pathogens.

DC subtypes, while all originating from bone-marrow (hematopoietic), can be further classified into two subtypes on the basis of functionality, specialization, and phenotype: classical DCs (cDCs)—also known as myeloid DCs (mDCs)—and plasmacytoid DCs (pDCs)[16–18]. Each have distinct roles within the immune response.

cDCs account for roughly one to five percent of tissue cells within the body, and they patrol both lymphoid and non-lymphoid tissues[17]. They are capable of antigen processing and presentation to T cells, priming naïve T cells, and exhibit upregulated HLA-DR (MHC II), CD11c, CD13, CD33, and CD11b expression[16,19].

In contrast, pDCs are found primarily within the bloodstream and can travel to lymphoid tissues[17]. Unlike like their classical counterparts, steady state pDCs express much lower levels of CD11c and HLA-DR, and they can be distinguished by their enhanced expression of CD123, CD303, and CD304[16,17]. They also have been associated with upregulating Type 1 IFN response upon viral infection[20]. Although antigen presentation is possible, priming of naïve T cells is far less robust in pDCs than mDCs[15].

### 1.2.1 Models of DC Mediated HIV Infection: *cis* versus *trans*

Given the diverse role of dendritic cells in immunological surveillance, it has been shown that HIV exploits the interaction between dendritic cells and helper T-cells, which increases T-cell susceptibility to infection[14]. Classical *cis* infection involves the direct entry and binding of the CD4 receptor in target cells, fusion of host and viral envelope, productive viral replication, and transmission of progeny[21].

In contrast, dendritic cells form highly productive infectious synapses that target CD4 lymphocytes through *trans* infection via C-type lectin receptors and the host endocytic pahway[14,21,22]. Viral gp120 binds C-type lectin receptor DC-SIGN with high affinity, which allows the internalization of HIV into early endosomes and DC transportation into lymphoid tissue[14]. It has been observed that the internalized virus has persisted for several days in an infectious state within subcellular compartments, while hiding from host surveillance—also known as the Trojan Horse Hypothesis[14,23]. Following entry into lymphoid tissue, retrograde transport of HIV shuttles the virus to the DC surface, which buds from the host cell and is transferred across the infectious synapse to infect the CD4 T-cell. Canonical lysosomal degradation of the endocytosed virus by host defenses is inhibited during *trans* infection by redirecting the virus during trafficking[24].

Although DCs are responsible for transmission of HIV to CD4 lymphocytes, they are drastically less susceptible to infection than T-cells. This is partially due to upregulated interferon responses and decreased CCR5 expression in mature DCs[25]. Differences in susceptibility to HIV infection can also be observed between DC subtypes. Myeloid dendritic cells must be infected with to ten to one-hundred times more HIV than iDCs.[26] However, research has shown that levels of mDCs directly correlate with HIV disease progression rates[27]. That is, mDCs levels in the blood remained persistently low despite ART in rapid and untreated PRs, while LTNPs exhibited higher

numbers. Plasmacytoid DC numbers were similar to CD4+ lymphocyte counts for all donor types. This implies that HIV continually affects mDCs during infection despite ART and slowing of disease progression.

## 1.3 NATURAL HISTORY OF UNTREATED HIV INFECTION

HIV infection progresses through three stages that vary in length: acute, asymptomatic chronic, and symptomatic. Each stage presents distinct clinical and molecular manifestations. Acute or primary infection lasts roughly two to six weeks in HIV+ patients, and plasma viremia peaks at this stage[28]. Roughly fifty to eighty percent of infected individuals experience influenza-like symptoms during the initial weeks of acute infection, including but not limited to: fever, myalgia, headache, rashes, and pharyngitis[29]. Circulating CD4 lymphocytes quickly decline to less than 500 cells per $mm^3$ due to HIV's cytopathic effects, but are eventually restored to roughly 800 cell per $mm^3$ as the acute infection is controlled through CTL response and antibody production during seroconversion[28,30,31].

After three to four months of infection, individuals enter the asymptomatic chronic stage in which the virus persists at low levels within the body[28]. The viral set point, or the amount of virus within the blood at the start of the asymptomatic chronic stage, is a strong indicator of disease progression and prognosis for HIV+ patients[28,32]. Clinical latency can persist anywhere from six months to twenty years after infection[15].

When CD4 levels fall below 500 cells per μL plasma, individuals have entered the symptomatic stage of HIV infection. HIV-infected helper T cells present as a foreign invader that

must be cleared either by CTL response or programmed cell death. Production of new CD4 lymphocytes is severely limited at this stage of infection[33].

### 1.3.1 Acquired Immunodeficiency Syndrome (AIDS)

Once an individual's CD4 counts have fallen below 200 cells per μL, he or she is diagnosed with AIDS[34]. At this stage, opportunistic infections are highly lethal for patients due to ablation of immune competence. They are also at higher risk of developing secondary conditions, such as neurological complications through HIV-Associated Neurocognitive Disorders (HAND), *Pneumocystis carinii* pneumonia, tuberculosis, and cancer[29]. Unless viral replication is suppressed through HAART, the vast majority of individuals will develop AIDS.

### 1.3.2 Antiretroviral Therapy

According to the U.S. Department of Health and Human Services, all HIV infected individuals are recommended to undergo HAART regardless of CD4 counts with initial medication combinations of an integrase strand inhibitor (InSTI) and two non-nucleoside reverse transcriptase inhibitors (NNRTIs)[35]. Evidence suggests that initiation of HAART prior to seroconversion depletes the latent viral reservoir and protects circulating memory T-cells[36]. Strict adherence to HAART regimens (i.e. use of 95% or more of the prescribed drugs) is required for maximizing survival rates in HIV+ individuals. Non-adherence is the leading cause of HAART failure in patients, and it has been a primary focus of targeted inteventions[37]. Determinants of non-adherence have been linked to sociodemographic and psychosocial factors ranging from age,

gender, education level, and employment status to degree of social support, fear of discrimination or stigmatization to use of traditional health remedies and access to healthcare[37].

Initially introduced in 1986 as an anticancer medication, zidovudine (AZT, retrovir) was the first FDA-approved medication for HIV. Nearly every stage of the HIV viral life cycle can be targeted through HAART via six types of drugs: (1) nucleoside reverse-transcriptase inhibitors (NRTIs), (2) non-nucleoside reverse-transcriptase inhibitors, (3) protease inhibitors (PIs), (4) integrase strand inhibitors (InSTIs), (5) co-receptor antagonists, and (6) fusion inhibitors[38]. Viral entry via gp120/41-mediated binding of host receptors can be targeted by fusion inhibitors, small molecule and antibody-based inhibitors, and chemokine receptor antagonists. After viral entry and fusion, viral RT converts the ssRNA genome into dsDNA, which can be integrated into the host genome via integrase. As the names suggest, NNRTIs and NRTIs target RT, while InSTIs inhibit integrase. PIs inhibit cleavage of viral structural and non-structural proteins in order to inhibit viral maturation.

Pharmacogenetics, which focuses on the role of genetics on an individual's drug response, has been invaluable in determining HAART sensitivity[39]. Most notably, an increased sensitivity to NRTI abacavir is associated with multiple allelic variants for major histocompatibility molecules (MHC) and immunological complement protein C4A6[38–40]. Understanding drug susceptibility, delivery, and efficacy through pharmacogenetics is vital for optimizing personalized therapeutics and management of HIV.

## 1.4 CLASSIFICATION OF DISEASE STAGES DURING HIV INFECTION

Clinical progression from HIV to AIDS is best predicted by assaying HIV viral levels—also known as viral titer or load—in plasma and CD4 lymphocyte numbers[11]. PRs, as the name aptly indicates, are individuals that develop AIDS roughly two to ten years after initial infection and typically contain more than 10,000 copies of HIV per mL of plasma[11,41]. The median duration of developing AIDS as a PR is 8.9 years. PRs can be subdivided into three categories: rapid, intermediate, and late progressors. Ten to fifteen percent of PRs are an extreme phenotype of HIV that develop AIDS within two to three years, also referred to as rapid PRs[31]. Intermediate PRs account for the majority of HIV+ patients (seventy to eight percent), and they develop AIDS within six to ten years post-infection with gradual declines in CD4 counts and increases in plasma viremia[31,41]. Lastly, five percent of progressors are classified as late progressors, and they maintain consistent CD4 counts and viremia for nearly ten years before entering symptomatic stage of HIV infection[31].

In the absence of HAART, NPs can maintain low levels of viremia that generally vary from less than fifty to 2000 copies per mL plasma and stable CD4 counts, which range from 350 to more than 500 cells per mL plasma[11,41]. Like PRs, NPs can be further classified into three types: intermediate or viremic controller (VC), elite controller (EC), and long-term NP (LTNP). VCs contain roughly 50 to 2000 copies of virus per mL plasma[41]. While ECs account for less than one percent of HIV+ individuals, they maintain virtually undetectable viremia at less than 50 copies of virus per mL and more than 500 CD4 cells per mL[31,41]. LNTPs are classified as individuals that maintain low viremia levels and stable CD4 counts in the absence of HIV/AIDS-related symptoms for more than ten years. Due to the vague definition of LNTPs, classification of individuals can overlap.

Lastly, seronegative (SN) individuals lack specific antibodies against HIV despite exposure to the virus, which can range from single-event occurrences (e.g. laboratory technician) to continual exposure (e.g. intravenous drug user or promiscuous sexual practices)[42,43]. It is important to note that SN status can be dictated by a variety of factors, such as lifestyle choice (e.g. usage of Pre-Exposure Prophylaxis), minimizing (or zero) exposure to HIV, any of the biological and genetic factors listed for HIV resistance, or delayed seroconversion[42–44]. A small subset of SN individuals, known as highly exposed SNs, are repeatedly exposed to HIV, but do not contract the virus[43].

## 1.5 HOST GENETIC FACTORS THAT INFLUENCE HIV INFECTIVITY

Resistance to HIV infection has been influenced by a wide variety of conditions, including but not limited to: host genetics, biological receptor mutations, and immunologic factors[31,45,46]. While there are a wide variety of genes that affect HIV progression (e.g. entry, cytokine receptors, innate and adaptive immunity), only a small fraction will be discussed in detail. When the immune system detects foreign antigens, a specific set of cellular surface proteins known as Human Leukocyte Antigen (HLA) are responsible for recognizing and presenting non-self antigen to CD4 helper T-cells. This coordinates a highly specific immunological response against the foreign invader. HLA class I molecules (A-C) are expressed co-dominantly within cells, and receptor expression can greatly affect prognosis[15]. That is, HIV+ individuals that express protective HLA*B57 or HLA*B27 allelic variants have a better prognosis by delaying the onset of AIDS. In contrast, homozygosity for HLA Type I molecules, as well as expression of HLA*B37 allelic variant results in faster progression.

Another genetic host factor, CCR5-Δ32 mutation, has been associated with variations in HIV/AIDS protection or resistance based on expression profiles. CCR5-Δ32 causes a truncated version of CCR5, which diminishes HIV binding and fusion with host cells[47]. The frequency of the Δ32 allele is around 0.09 in Caucasian populations, while it is non-existent in Japanese and West African populations[48,49]. Homozygosity of the Δ32 mutation (approximately 1% of individuals) prevents HIV infection altogether by ablating CCR5 expression entirely, while heterozygosity (10% of individuals) delays AIDS by reducing the amount of wild type receptor[15,47,50].

## 1.6 MULTICENTER AIDS COHORT STUDY (MACS)

The MACS is a collaborative, prospective investigation of HIV infection in homosexual and bisexual men established at the University of Pittsburgh Graduate School of Public Health, The Ohio State University Medical Center, Northwestern University Feinberg School of Medicine, UCLA Fielding School of Public Health, and Johns Hopkins University Bloomberg School of Public Health. The study aims to evaluate HIV infection in the presence and absence of HAART, i.e. treated and natural histories of the viral infection. Participants' semi-annual visits to study sites consist of a physical examination, neurological and psychological assessment, demographic and health practice questionnaires, and blood draws. Since its inception in 1984, nearly 7,000 individuals have enrolled in the MACS cohort[51].

## 1.7 CELLULAR CHOLESTEROL METABOLISM LINKED TO NON-PROGRESSOR DISEASE STATE DURING *TRANS*-INFECTION

While studying MACS donors, NPs explicitly exhibited decreased metabolic cholesterol levels in their antigen presenting cells compared to PRs and SNs *in vitro*[1]. Moreover, NPs were found to have drastically less efficient dendritic cell-mediated *trans* infection, as well as elevated expression levels of ABCA1 protein, which modulates cholesterol and lipid concentrations within the cell. More specifically, ABCA1 is responsible for efflux of lipids and free cholesterol from the cell into the serum. It was hypothesized that decreased cellular cholesterol levels are disrupting the integrity of lipid rafts and shuttling of the virus —therefore, attenuating HIV *trans* infection and contribution to the NP state.

## 1.8 IDENTIFICATION OF TARGET GENES

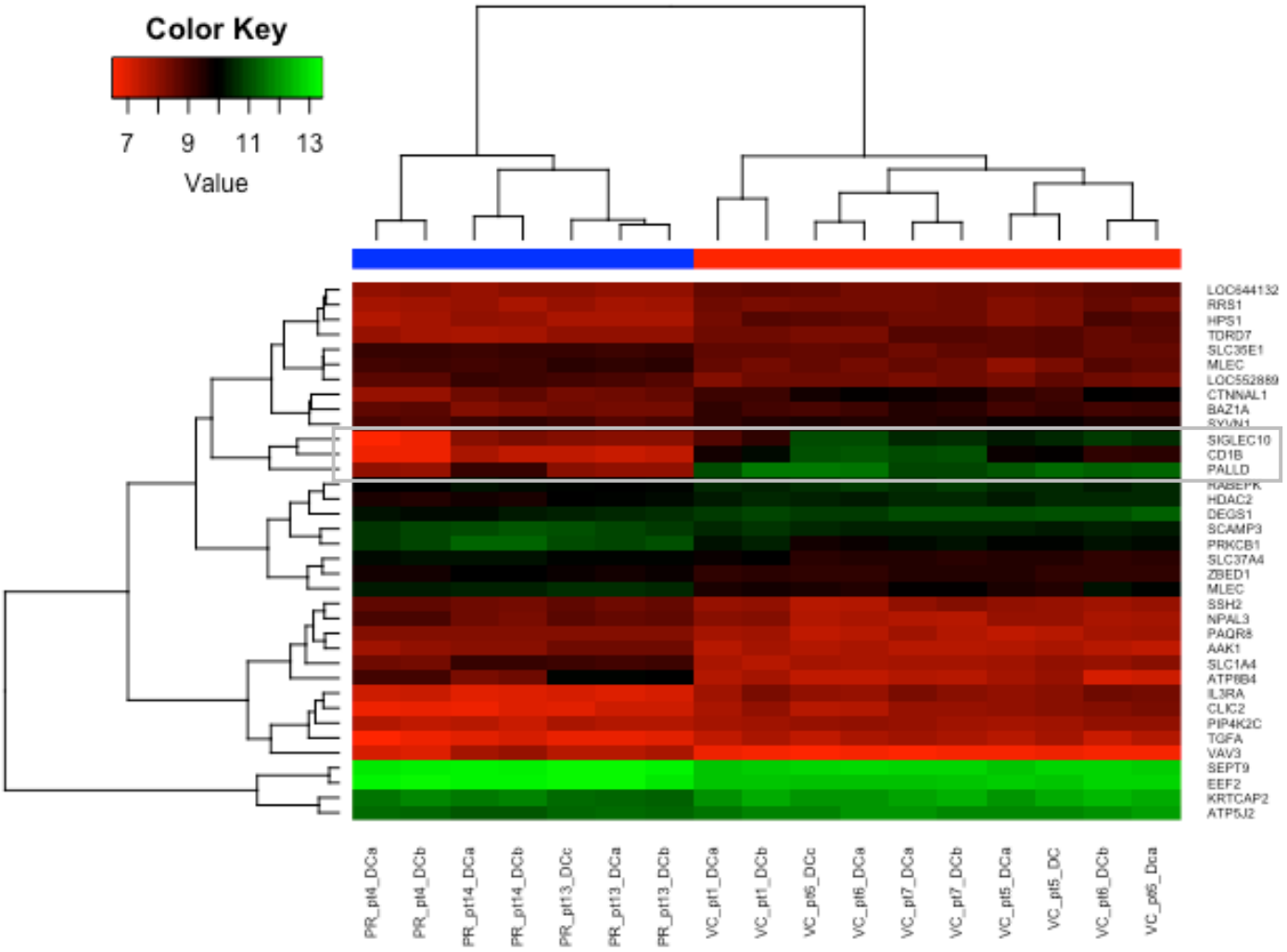


**Figure 1. Heat Map of Genes Expressed Differentially between MACS PRs and NPs**

Total RNA was extracted from the same subset of MACS donors analyzed by Rappocciolo et al. in 2014. Transcript levels were assayed using Illumina's Human HT-12 BeadChip, which facilitated genome-wide transcriptional coverage of candidate genes. The HT-12 assay utilizes a 3' *in vitro* translation (IVT) system, which probes the 3' UTR trailer sequence (i.e., polyadenylation signal). Data was read, normalized, and transformed via Lumi software package. Limma software was used to generate the heat map in Figure 1. Cholesterol metabolism data

asserted that a genetic trait or traits were responsible for HIV nonprogression. In turn, the heat map generated from the same set of donors had highlighted potential genes that were contributing to HIV nonprogression.

In Figure 1, gene expression levels in iDC subsets of MACS nonprogressor (denoted VC for viremic controller and below red horizontal header) and progressor (PR and below the blue horizontal header) are depicted. All genes along the vertical axis on the right exhibit highly significant differences in expression between donors with $p < 0.0025$. Note that red indicates decreased levels in expression, while green indicates increased levels in expression.

Siglec-10, CD1b, and PALLD (outlined in gray) display the most visually drastic disparity in gene expression levels between PRs and NPs. Note that NPs (denoted as VCs) exhibit elevated expression levels for each target gene when compared to PRs.

### 1.8.1 Siglec-10

Siglec-10 is a human cellular surface protein that mediates protein carbohydrate interactions, namely by binding sialic acid residues in oligosaccharide chains. It is comprised of five Ig-like domains, transmembrane domain, and cytoplasmic ITIM motif[52]. It is broadly expressed in all peripheral blood leukocytes (i.e., granulocytes, monocytes, lymphocytes, and APCs). It is known to have at least nine unique splice variants[53].

Recent data revealed that Siglec-10 expressed in human DCs bind pseudaminic acid, a sugar structurally related to sialic acid, and promotes regulatory cytokine IL-10 production by DCs[54]. HIV-1 infected monocyte derived DCs have been found to produce IL-10, while Siglec-7 (another member of the Siglec family) has been shown to bind HIV gp-120 in a sialic-acid mediated manner[55,56]. In relation to HIV pathogenesis, IL-10 has been shown to modulate viral

persistence by altering T-cell activity. Moreover, IL-10 promoter SNPs associated with upregulated IL-10 production have been linked to higher T-cells counts and protection against HIV progression[57,58]. I have hypothesized that upregulated Siglec-10 expression in NPs is indirectly decreasing *trans* infection efficiency by increasing IL-10 production in iDCs and thus stabilizing T-cell counts.

### 1.8.2 CD1b

CD1b is a cellular surface protein whose transmembrane glycoprotein dimerizes with β2-microglobulin, a relatively small protein that is non-covalently bonded to the alpha subunit of the MHC Class 1 molecule[59,60]. As a member of the CD1 family of proteins, it facilitates presentation of self and non-self lipid antigens by scanning the endocytic pathway in order to activate specific T cells through the adaptive immune response[60]. Its expression is limited to antigen presenting cells, i.e., DCs, B-cells, and macrophages.

When virions bud from the host membrane during *trans* infection, transport directly relies on cholesterol synthesis. Endocytic vesicles expressing CD1b derived from HIV+ iDCs were found to be nearly ten times more infectious than virions during *trans* infection[61]. HIV-1 accessory protein Nef has been linked to viral infectivity by enhancing cholesterol synthesis and lipid raft transport[62]. Because Nef downregulates MHC-1 molecules, there is reduced NK and CD8-mediated targeting of HIV+ cells[63]. In turn, it has been well established that loss of function Nef mutants result in attenuated viral infection[64].

Research on homologs in the CD1 family of proteins has also shown promising results: CD1a, which shares the same group classification CD1b, was found to colocalize with Nef, suggesting that endocytosis of HIV in Nef-dependent[65]. Nef has also been observed to decrease

expression of various surface cellular markers, including CD1b[65]. This also contributes to modulation of intracellular trafficking through the endo-lysosomal system to escape degradation[66]. More importantly, LTNPs have been associated with defective Nef mutations that attenuate viral infectivity[67].

I have hypothesized that upregulated CD1b expression in NP is a result of an upstream mutant viral Nef [which ultimately decreases trans infection efficiency by decreasing lipid raft transport and cholesterol synthesis]. Furthermore, upregulated CD1b is indicative of increased activity within the endolysosomal system, which reduces viremia via increased lysosomal processing.

### 1.8.3 Palladin (PALLD)

PALLD is an intracellular protein that modulates the binding of monomeric and filamentous actin[68]. The actin cytoskeleton is responsible for various cellular processes such as motility, adhesion, and contractility. Unlike Siglec-10 and CD1b, Palladin gene has three transcription initiation sites, and it can undergo extensive alternative splicing[69]. Currently, there are three major isoforms of PALLD: 90-92 kDa, 140 kDa, and 200 kDa. Additional minor isoforms have been isolated as well, and all isoforms exhibit tissue specific preferences[69].

During HIV infection, the virus has been found to utilize actin structures at the infectious synapse during *trans* infection through membrane extensions and nanotubules[70–72]. Evidence has shown that elevated palladin protein levels correlate with increased actin-based motility[73]. Furthermore, the actin cytoskeleton serves as a physical barrier that HIV must navigate in order to facilitate infection[74]. I have hypothesized that elevated Palladin expression levels in NPs are

indicators of upregulated actin in iDCs. Because the virus cannot overcome the upregulated cortical actin cytoskeleton as efficiently, HIV transmission by NPs is reduced.

# 2.0 STATEMENT OF PROJECT

Given current transcriptome data, we hypothesize that upregulated expression of Siglec-10, CD1b, and Palladin in HIV+ NPs is controlling HIV progression by attenuating *trans* infection between iDCs and CD4 lymphocytes through distinct molecular and immunological mechanisms. While the explicit mechanism of HIV disease progression remains unclear, our goal is to elucidate the genetic origin of NP viremic control using MACS-derived iDCs *in vitro*.

## 2.1 AIM #1

Validate HT-12 RNA expression results for Siglec-10, CD1b, and PALLD using RT-qPCR.

## 2.2 AIM #2

Phenotype Siglec-10, CD1b, and PALLD protein expression between MACS PRs and NPs via flow cytometry.

## 2.3 AIM #3

Analyze and identify potential association between genetic polymorphisms and HIV nonprogression using TaqMan® SNP genotyping assays.

# 3.0 RESEARCH DESIGN

## 3.1 MAGNETIC CELL SEPARATION

MACS donor blood was processed by a standard centrifugation protocol that utilized sterile filtered polysucrose and diatriozote solution (Lymphocyte Separation Medium), then was aliquoted and frozen in FBS/10% DMSO. 1-mL aliquots of peripheral blood mononuclear cells (PBMCs) were thawed at 37°C in a water bath, re-suspended in 15-mL RP-10 media (10% FBS, 25mM Hepes in RPMI media), and centrifuged at 1200 RPM for 10'. Pelleted PBMCs were re-suspended again in 15-mL RP-10 and incubated for 10' at RT before undergoing separation. Magnetic labeling and isolation of CD14+ cells followed MACS Miltenyi Biotec protocol using MACS Miltenyi Biotec CD14 Human Microbeads, MACS LS column, and Buffer A (PBS, 2.0 mM EDTA, 0.5% BSA). Viability of CD14+ and CD14- monocytes were calculated by the Beckman Coulter Vi-CELL™ Cell Counter and Analyzer. 1-mL aliquots of CD14- monocytes (maximum $5 \bullet 10^{-6}$) were frozen in Nalgene cryovials in FBS/10% DMSO in -80°C for 24 hours, then transferred to -140°C liquid nitrogen freezers for long-term storage. PR (N=5) and NP (N=5) PBMC samples that were selected to undergo magnetic cell separation and other experiments were the same donors studied by Rappocciolo et. al in 2014.

## 3.2 IMMATURE DENDRITIC CELL CULTURE

CD14+ monocytes were cultured in 1-mL AIM V media per 1 million cells at 37°C. Monocytes were fed with $10^3$ units/mL IL-4 and GM-CSF n Day 1 and Day 3. iDCs were harvested on Day 5. In order to facilitate detachment from the plate, iDCs were incubated in PBS for 20' at 37°C, then harvested by strongly pipetting with P1000 and P200 until all cells detached from the plate. iDCs were counted with a hemocytometer. iDCs reserved for qPCRs were frozen in FBS/10% DMSO at stored at -80°C for 24 hours, then transferred to -140°C freezers for long-term storage.

## 3.3 RNA EXTRACTION

RNA extraction from iDCs was conducted using Ambion by Life Technologies Pure Link® RNA Mini Kit and followed manufacturer's protocol. Note that frozen iDCs were re-suspended and incubated in RPMI media prior to manual homogenization at RT for 12,000*g* for 5'. Purified RNA was eluted with 25 µL RT Nuclease-Free Water (Ambion) and stored at -80°C. Thermo-Scientific NanoDrop Microvolume spectrophotometer and NanoDrop 2000 was used to evaluate RNA purity (Table 1).

**Table 1. RNA Sample Analysis via NanoDrop**

| Disease Status | Nucleic Acid | A260 (Abs) | A280 (Abs) | 260/280 | 260/230 |
|---|---|---|---|---|---|
| NP-1 | 23.9 ng/µl | 0.597 | 0.304 | 1.96 | 0.39 |
| NP-2 | 11.7 ng/µl | 0.293 | 0.141 | 2.08 | 0.16 |
| NP-3 | 54.2 ng/µl | 1.355 | 0.64 | 2.12 | 1.15 |
| NP-4 | 128.7 ng/µl | 3.218 | 1.544 | 2.08 | 0.87 |
| NP-5 | 21.9 ng/µl | 0.548 | 0.256 | 2.14 | 0.37 |
| PR-1 | 3.2 ng/µl | 0.079 | 0.034 | 2.3 | 0.09 |
| PR-2 | 31.7 ng/µl | 0.792 | 0.369 | 2.15 | 0.23 |
| PR-3 | 93.5 ng/µl | 2.338 | 1.105 | 2.12 | 1.06 |
| PR-4 | 13.6 ng/µl | 0.34 | 0.151 | 2.26 | 0.13 |
| PR-5 | 15.1 ng/µl | 0.377 | 0.192 | 1.96 | 0.18 |

## 3.4 COMPLEMENTARY DNA (CDNA) PREPARATION

Prior to hood usage, all materials were subjected to UV light for 15', then treated with RNase ZapWipes, and rinsed with Milli-Q water. Applied Biosystems TaqMan® Reverse Transcriptase Kit reagents (N8080234) were thawed on ice. 50 µM random hexamers were chosen in lieu of oligo-dTs or gene-specific reverse primers. Optional addition of 100 mM DTT was omitted from the master mix and was compensated with Ambion Nuclease-Free RT water. Reagent amounts in Table 2 were customized for (20) 20µL reactions, i.e. 15 µL of the master mix solution and 5 µL template RNA were added per 0.2-mL tube. Aliquots were amplified using the Applied Biosystems GeneAmp PCR system 9700. cDNA preps were stored at 4°C.

**Table 2. Master Mix of Reverse Transcriptase Reagents for cDNA synthesis**

| RT Master Mix | |
|---|---|
| Nuclease Free H2O | 210 µL |
| 10X RT Buffer | 20 µL |
| 25mM MgCl2 | 14 µL |
| 10mM dNTP | 40 µL |
| Rnase Inhibitor | 10 µL |
| Multiscribe RT | 10 µL |
| 50mM Random Hex | 10 µL |

## 3.5 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

Integrated DNA Technologies PrimeTime® qPCR TaqMan probe-based assay was executed per manufacturer protocol. Fluorescent TaqMan probes permit concurrent quantification of the internal control (RNA Polymerase 2) and target gene (Siglec-10, CD1b, PALLD) within a desired well due to distinct fluorophores (i.e. HEX and FAM respectively). IDT PrimeTime Master Mix (2X) solution with reference dye, target and internal control probes (20X), and nuclease-free water were combined and added to Applied Biosystems MicroAmp® Fast Optical 96-Well Reaction Plate using Eppendorf Repeater®/Combitips Advanced® system (Table 3). Target cDNA was added to each well, and the plate was sealed with Applied Biosystems Optical Adhesive Covers (Optical Adhesive Cover Starter Kit), smoothed with a roller to ensure proper seal, and centrifuged briefly at 1800 RPM. The qPCR was conducted using the Applied Biosystems StepOne Plus Real-Time PCR system and StepOne™ Software v2.1 was used to record amplification and fluorescence data. Specific probe info can be found in Table 4.

PR, NP, and SN ΔCT values were compared by means of One Way ANOVA and plotted in GraphPad Prism 7. ΔCT values are calculated as the difference of CT values between housekeeping gene (i.e., RNA Polymerase II) and gene of interest (i.e., Siglec-10, CD1b, PALLD).

**Table 3. IDT TaqMan® qPCR Probes and Primers for qPCR**

| | | *Sequence* | *Molecular Weight* | *GC Content* |
|---|---|---|---|---|
| RNA Pol 2 | Probe | 5'-/5HEX/ACTGAAGCG/ZEN/AATGTCTGTGACGGAG/31ABkFQ/-3' | 9.40 kDa | 52.00% |
| | Primer 1 | 5'-CAGTTCGGAGTCCTGAGTC-3' | 5.82 kDa | 57.90% |
| | Primer 2 | 5'-TCGTCTCTGGGTATTTGATGC-3' | 6.43 kDa | 47.60% |
| Siglec-10 | Probe | 5'-/56-FAM/AGTGGCAGT/ZEN/AGCATCTCCGCATAG/31ABkFQ/-3' | 8.80 kDa | 54.20% |
| | Primer 1 | 5'-GCACTCGTATCCAGAATCTCC-'3 | 6.32 kDa | 52.40% |
| | Primer 2 | 5'-CAGCACGACCAGAGAACAG-3' | 5.82 kDa | 57.90% |
| CD1b | Probe | 5'-/56-FAM/CTCATGGGA/ZEN/TCTGATATGACCGGCG/31ABkFQ/-3' | 9.12 kDa | 56.00% |
| | Primer 1 | 5'-ACTTTTGGGCTGATATCTTGGG-'3 | 6.79 kDa | 45.50% |
| | Primer 2 | 5'-CTTCCTTGCTCCTTTTGCTATG-'3 | 6.61 kDa | 45.50% |
| PALLD | Probe | 5'-/56-FAM/TTTTGGCTT/ZEN/GGTGCTCTGTGACTG/31ABkFQ/-3' | 8.81 kDa | 50.00% |
| | Primer 1 | 5'-CTGCTTTGATGTCTAGTCCCTG-3' | 6.68 kDa | 50.00% |
| | Primer 2 | 5'-ACACCAGGAGAACAAATACCC-3' | 6.37 kDa | 47.60% |

**Table 4. qPCR Prime Time Master Mix of Reagents**

| PrimeTime qPCR Master Mix | | | |
|---|---|---|---|
| | 10 µL rxn | 1/2 plate | 96-well plate |
| PrimeTime MM (2X) | 5.0 µL | 250.0 µL | 500.0 µL |
| Probe of Interest (20X) | 0.5 µL | 25.0 µL | 50.0 µL |
| Nuclease Free H2O | 4.5 µL | 225.0 µL | 450.0 µL |
| | | | |
| cDNA/DNA template | 2.0 µL | 2.0 µL / well | 2.0 µL / well |

Flow cytometry conditions were optimized in five SN donors before using MACS PR and NP samples. For samples from HIV+ participants, the 96-well plate was taped during centrifugation, supernatant was always aspirated, and any materials in contact with virus was treated with bleach and incubated under UV light overnight. Cellular compensation was used in lieu of beads, and staining conditions were identical to the experimental conditions.

Aliquoting cells: After counting, re-suspended iDCs in AIM-V were transferred to 96 well V-bottom plates at 50,000 – 100,000 cells per well. Each target protein was stained in separate wells. If cells weren't being actively stained, identical amounts of monoclonal antibody (mAb) wash (2% FBS, 0.01% $NaN_3$ in HBSS) wash were added.

Viability Stain: iDCs were stained with 5 µL of 1 µL LIVE/DEAD Aqua Viability in 500 µL PBS for 20' in the dark at RT, and washed with PBS and centrifuged at 2500 RPM for 5'.

Surface Stain: 5 µL FITC Mouse Anti-Human CD1b (BD555969) or BioLegend PE Anti-Human Siglec-10 antibody (347604) were combined separately with 5 µL mAb wash in the dark. 5 µL of the surface stain mix were added to the target well(s). Stained wells incubated for 25' RT in the dark, then subsequently washed and centrifuged at 2500 RPM for 5'.

Permeabilization and Intracellular Stain: Cells were re-suspended and permeabilized with Becton-Dickson Perm Buffer II in PBS, incubated for RT at 20', add mAb wash, and centrifuged at 2500 RPM for 5'. Cells were stained with 5 µL of (1:100) Abcam Anti-Palladin antibody (ab154827) in mAb wash and incubated for 25' RT in the dark. After washing and centrifugation at 2500 RPM for 5', cells were re-suspended with 5 µL of (1:2) BD BV-421 Goat Anti-Rabbit IgG (Palladin secondary antibody) in mAb wash. After 25' incubation in the dark, cells were washed and centrifuged at 2500 RPM for 5'.

Fixation of cells: 240 µL of 1% PFA were used to re-suspend each pellet. Cells were stored at 4°C until analysis.

Isotype control: The negative control was used to assess inherent antibody stickiness for unstained cells. Cells were stained with isotype mice antibodies for FITC (CD1b) and PE (Siglec-10). Due to the unavailability of a commercial isotype for Palladin secondary antibody, 5 µL of undiluted secondary Palladin antibody (BV-421) served as the isotype.

### 3.5.1 Fluorescent Activated Cell Sorting (FACS)

Flow cytometry analysis was conducted on the BD LSR FORTESSA in conjunction with BD FACS DIVA software. Results were further analyzed using FlowJo® v10.3. PR, NP, and SN

Mean Fluorescence Intensity (MFI) values were analyzed by means of One Way ANOVA in GraphPad Prism 7. A parametric distribution of MFI values was anticipated. All isotype control MFI values were subtracted from target protein MFI values to normalize data and control for background fluorescence.

### 3.5.2 Flow Cytometry Gating Hierarchy

All donors were analyzed under the following gating template on DIVA and FlowJo software with variations expected between donors (Figure 2). Figure 2 depicts fluorescence in the isotype control in a SN donor. Thus, fluorescence and MFI values for Siglec-10, CD1b, and Palladin are generally shifted to the right in NP and PR donors [for histograms displaying Siglec-10, CD1b, and Palladin-stained cells]. Viability is assessed through Am-Cyan fluorescence. PALLD is assessed via Pacific Blue fluorescence, CD1b via Alexa Fluorophore 488 fluorescence, and Siglec-10 via PE-Yellow Green fluorescence. The singlet gate was used to eliminate any clumped cells from analysis that could provide artificially higher fluorescence levels, while the monocyte gate isolated CD14+ monocyte-derived iDCs. iDC viability was assessed as a function of Am/Cyan fluorescence; dead cells uptake the Aqua Live/Dead stains, fluoresce, and cause a shift upwards on the gate. Histograms for each target protein were gated from the live cells and were used to enumerate the percentage and Mean Fluorescence Intensity (MFI) of positively-expressing cells.

It may appear as though viable singlet cells have been omitted from the gating strategy in Figure 2, and thus imply that I have omitted them from MACS donors. However, analysis of these cells on downstream gates has revealed them to inviable granulocytes, which would not prove valuable for iDC analysis.

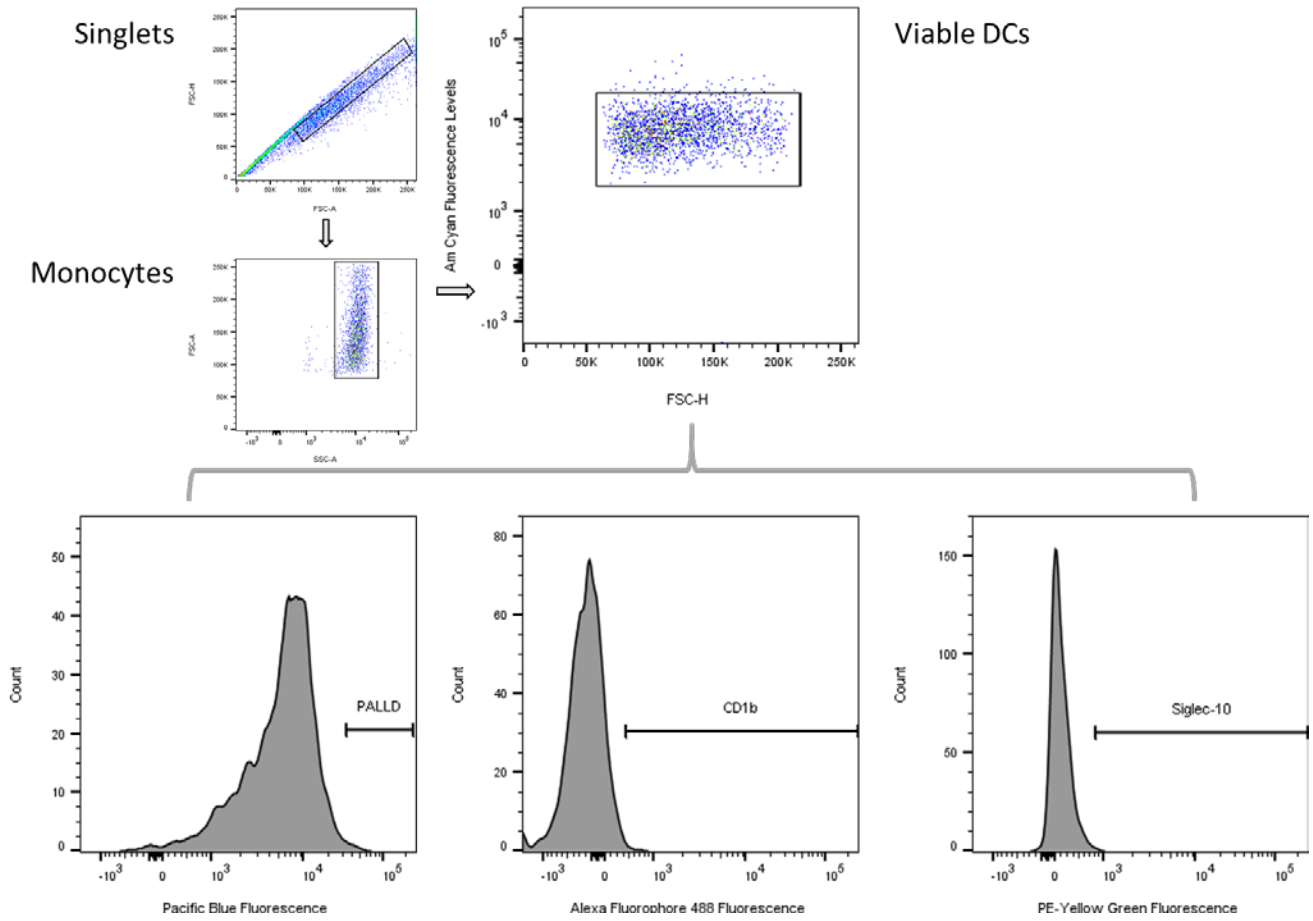


**Figure 2. Representative Gating Hierarchy for PR, NP, and SN Donors**

## 3.6 TAQMAN® SNP GENOTYPING® ASSAY

We developed a SNP genotyping assay to detect tagging SNPs encompassing the entire CD1B gene and promoter. The goal of this assay is to construct haplotypes that summarize all genetic variation in CD1B, and to relate this to transcript expression levels and NP/PR status. The assay was conducted in accordance with Applied Biosystems Custom TaqMan® SNP Genotyping Assays protocol and amplified in the Applied Biosystems StepOne Plus Real-Time PCR

thermocycler. StepOne™ Software v2.1 was used to generate Allelic Discrimination Plots for each CD1b SNP in relation to MACS PR, NP, and SN (1:50) genomiphi DNA. HaploView software was used to conduct haplotype analysis of the relationship between allelic frequencies and NP disease state. Seven SNPs were analyzed: rs10797007, rs16840096, rs10908647, rs11583390, rs2569512, rs11085748, and rs8113029. Similar assays are under development for the Siglec-10 and Palladin genes.

# 4.0 RESULTS

## 4.1 REDUCED CD1B AND PALLD EXPRESSION IS OBSERVED IN PROGRESSORS WHEN COMPARED TO NONPROGRESSORS VIA QPCR

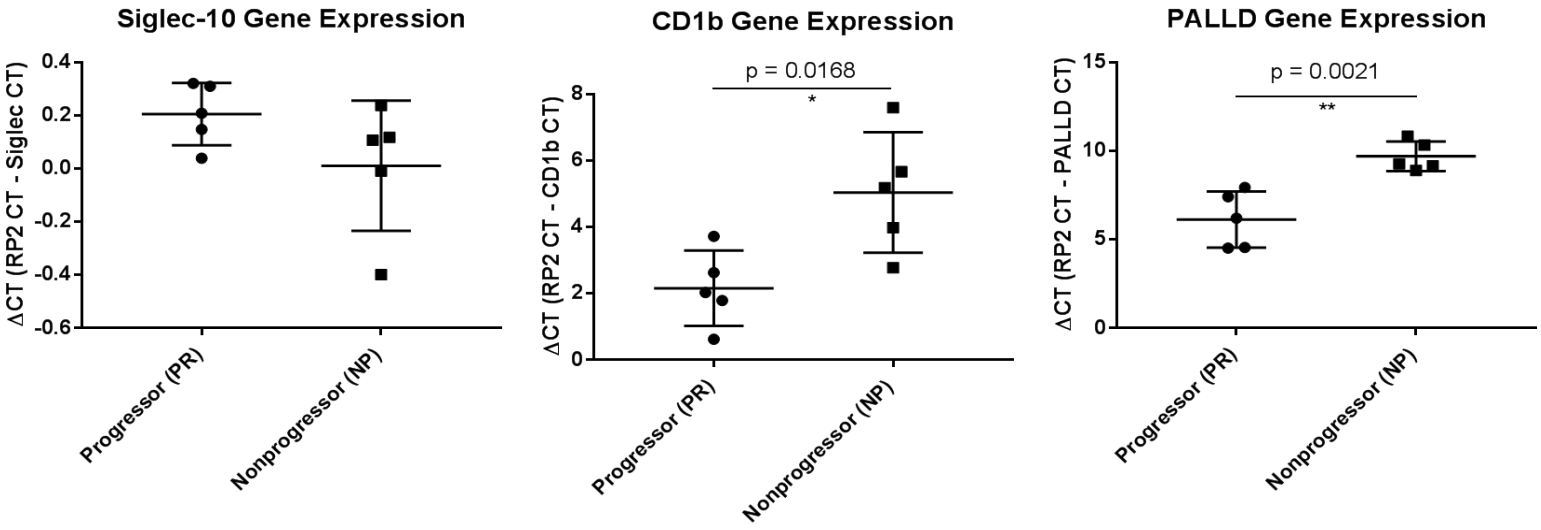


**Figure 3. Comparison of ΔCT Values between MACS Donors**

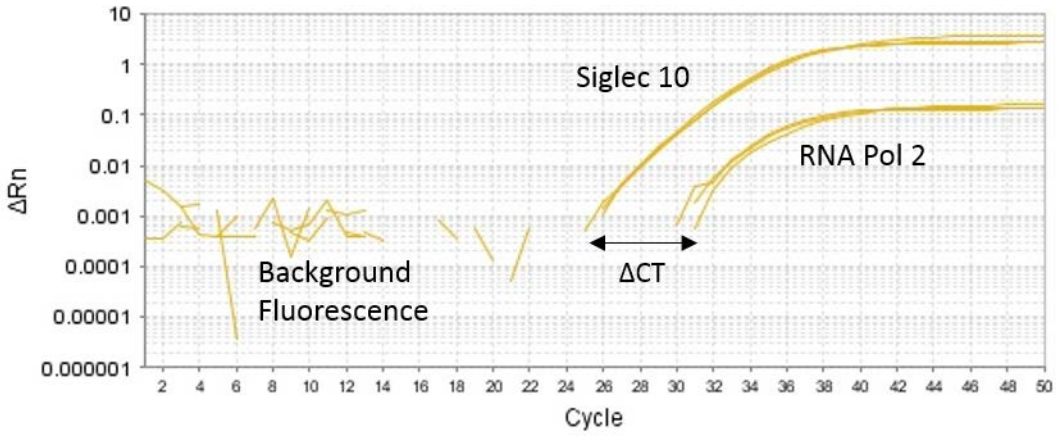


**Figure 4. Representative qPCR Amplification Plot**

Cycle threshold (CT) refers to the cycle number in which quantifiable fluorescence exceeds background levels, and it is inversely proportional to the level of nucleic acid in the sample. That is, CT values < 29 indicate abundant levels of target nucleic acid, while CT values from 30-37 and 38-40 indicate moderate and minimal amounts of nucleic acid, respectively. CT values for the target gene and housekeeping gene are transformed accordingly using the ΔCT method (Figure 3). The ΔCT method enables relative comparison between the target gene expressed in the PR or NP iDC sample and housekeeping gene (RNA Polymerase II), while normalization of ΔCT values is ensured by consistent loading of cDNA and probe samples. A representative amplification plot for one PR donor for Siglec-10 illustrates consistency during triplicate cDNA sample loading, as indicated by the uniform amplification curves for RNA Polymerase II and Siglec-10 (Figure 4).

Furthermore, the use of differently labeled TaqMan probes enable simultaneous quantitation of multiple genes in one sample, which eliminates discrepancies caused by differences in cell numbers during RNA extraction. Unlike SyBr green primers, TaqMan probes allow sequence-specific annealing and prevent non-specific amplicons. During primer extension, Taq polymerase cleaves the fluorophore from the molecular probe and releases it from the proximity of the quencher, which results in a detectable fluorescent signal. Because probes for RNA Polymerase II and target genes have distinct fluorophores (i.e. HEX and FAM respectively), one can quantify gene expression levels of RPII and candidate genes in the same sample.

Noting that positive ΔCT values indicate increased gene expression, while negative ΔCT values refer to decreased gene expression. Quantitative PCR analysis revealed highly significant differences in ΔCT values between donors for CD1b and Palladin. More specifically, CD1b and PALLD gene expression levels were elevated in NPs when compared to PRs ($p = 0.0168, 0.0021$ respectively). In contrast, Siglec-10 gene expression was relatively consistent between PRs and

NPs with the exception of one NP donor (Figure 3). When comparing ΔCT values between target genes, PALLD expression levels are the highest with CD1b as the intermediate and Siglec-10 expression as the lowest.

SN donors (N = 9) that were used to optimize qPCR conditions were omitted from ΔCT analysis for several reasons, namely variability of SN status, differences in cell types, and non-significant transcriptome data. Due to the large variability in the causes of SN status, it could not be confidently discerned that SN was a result of lifestyle choices or immunological or biological factors. Because optimization of TaqMan qPCR probes used SN activated CD4 T-cells in lieu of iDCs, it could not be accurately compared to MACS PRs and NPs iDC expression profiles. Activated T-cells were used due to the length of DC culture and limited availability of PBMCs and thus CD14+ monocytes. Lastly, no significant differences in gene expression were observed when comparing transcriptome data between a separate set of SN donors and MACS PRs and NPs (data not shown).

Transcriptome analysis indicated that NPs universally exhibited elevated PALLD expression levels when compared to PRs, while some NPs showed transcript levels similar to PRs for Siglec-10 and CD1b (Figure 1). The transcript profiles for each target gene showed upregulated expression levels for the majority of NPs compared to PRs. Quantitative PCR expression results for CD1b and PALLD are relatively consistent with the data generated through the heat map.

## 4.2 PROTEIN LEVELS ASSESSED BY FLOW CYTOMETRY DO NOT EXHIBIT SIGNIFICANT DIFFERENCES BETWEEN PROGRESSORS AND NONPROGRESSORS

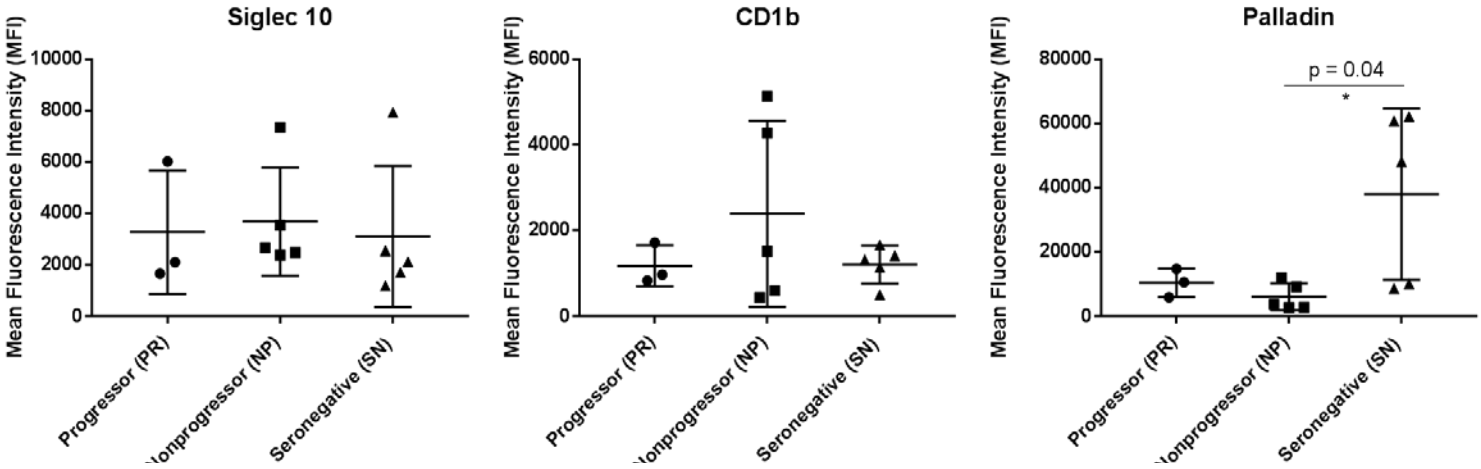


**Figure 5. Comparison of Target Protein Expression via MFI between MACS Donors**

Expression of target proteins in iDCs were assayed using flow cytometry between PRs (N=5) and NPs (N=5) via Mean Fluorescence Intensity (MFI) values. Every donor was analyzed under identical SSC and FSC voltages and sample flow rate to ensure consistency. During sample analysis on the BD LSR Fortessa, injection of PR-1 and PR-5 iDC samples into the port was immediately impaired despite recent re-suspension in 1% PFA, and it subsequently resulted in a loss of more than 70% of the samples. MFI of target proteins could not be analyzed due to unreliable singlet gating. That is, rather than a uniform linear distribution of singlets, cells were scattered randomly throughout the gate, which is indicative of blockage. Once a proper singlet distribution was observed after repeated attempts, there were too few recorded events in order to yield reliable MFI data for Siglec-10, CD1b, and PALLD (Figure 6). As a result, PR-1 and PR-5

were removed from the one way ANOVA comparison of MFI values between the three donor groups (Table 5**,** Figure 6).

It is plausible that clumping during flow cytometry analysis of PR-1 and PR-5 was caused by an excess of undifferentiated and/or unviable CD14+ monocytes that were incidentally harvested from the DC culture. Given the diminished viability of CD14+ monocytes PR-1 (< 70%) after magnetic separation from donor PBMCs, it is logical that there would not be a high yield of viable iDCs (Table 5). In regard to PR-5, I also suspect that undifferentiated CD14+ monocytes were causing clumping during iDC flow analysis. It is important to note that iDCs were not assayed for classic molecular markers (e.g. CD86, CD80, and HLA-DR). Cell cultures were observed at Days 1, 3, and 5 for distinct morphological changes—indicative of iDC differentiation (data not shown).

Prior to CD14+ magnetic separation due to excessive cellular clumping, PR-1 and PR-5 PBMCs had to be treated with 40 μL of bovine pancreas DNAse. Despite nuclease treatment, persistent PBMC clumps from PR-1 and PR-5 had to be discarded because they would have significantly impeded column flow during separation.

There was no observed difference in protein expression levels between PRs and NPs, but PALLD SN donors were found to have statistically significant increased protein expression levels compared to NPs.

**Table 5. MACS CD14+ and iDC Cell Count and Viability**

| | CD14+ Monocytes | | Harvested iDCs | |
|---|---|---|---|---|
| Donor | Counts | Viability | Counts | Viability |
| PR-1 | 1.25 EE 6 | 69.70% | 2.70 EE 5 | 50% |
| PR-2 | 4.35 EE 6 | 91.30% | 9.00 EE 5 | 71% |
| PR-3 | 1.07 EE 7 | 90.90% | 2.52 EE 6 | 82% |
| PR-4 | 8.95 EE 6 | 96.70% | 5.40 EE 5 | 60% |
| PR-5 | 1.13 EE 7 | 94.60% | 6.30 EE 5 | 70% |
| NP-1 | 4.25 EE 6 | 96.80% | 6.30 EE 5 | 64% |
| NP-2 | 6.25 EE 6 | 87.60% | 5.85 EE 5 | 55% |
| NP-3 | 6.36 EE 6 | 97.50% | 7.80 EE 5 | 64% |
| NP-4 | 5.05 EE 6 | 98.20% | 6.30 EE 5 | 59% |
| NP-5 | 4.65 EE 6 | 97.10% | 5.40 EE 5 | 60% |

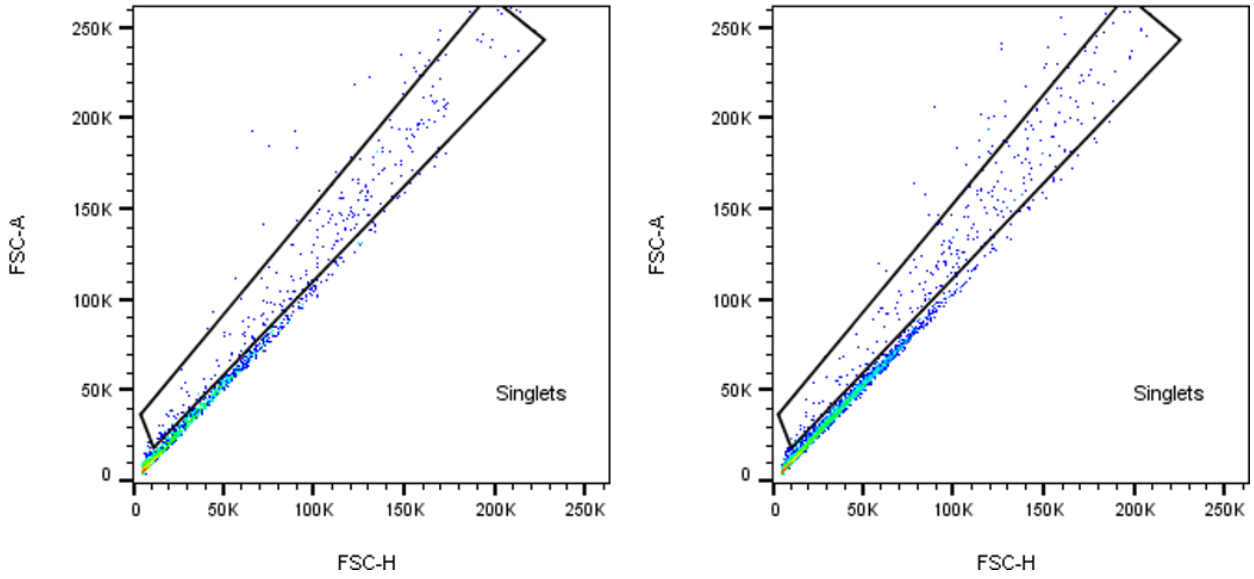


**Figure 6. Isotype Control Singlet Gating in PR-1 and PR-5**

## 4.3 CD1B SNP GENOTYPING REVEALS NO SIGNIFICANT TRENDS IN HAPLOTYPE ANALYSIS

All seven CD1b SNPs were analyzed in relation to a larger pool of MACS PR, NP, and SN DNA beyond the PR and NPs included in the qPCR and flow cytometry analysis. Haplotype analysis was conducted to investigate if there was a relationship between the frequencies of alleles at various loci for a target SNP within the CD1b gene. No significant differences were found.

## 4.4 MACS NONPROGRESSOR AND PROGRESSOR SAMPLES DISPLAY CHARACTERISTIC CD4 LYMPHOCYTE COUNTS AND PLASMA VIREMIA

NP and PR donors were assayed for CD4+ T-cell counts, HIV viremia, and HLA and CCR5 allelic variants prior to this thesis work. This figure was adapted with permission from Rappocciolo et al. *mBio* publication from 2014. Samples had been collected during bi-annual MACS visits. None of the donors exhibited homozygosity for HLA-57 or 27, nor did they exhibit homo- or heterozygosity for CCR5-Δ32 variants.

**Table 6. Immunological, Virological, and Genotypic Profiles of MACS Samples**

| SUBJECT | Duration of Infection pre-HAART | Mean CD4+ T cells per µL | Mean HIV RNA copies per mL plasma | HLA Genotype | CCR5-Δ32 |
|---|---|---|---|---|---|
| PR-1 | > 5 | 350 ± 57 | 147,660 ± 65,974 | 38:01/49:01 | WT/WT |
| PR-2 | 6.1 | 374 ± 28 | 117,151 ± 28,059 | 08:01/35:01 | WT/WT |
| PR-3 | 6.5 | 204 ± 30 | 429,049 ± 91,578 | 08:01/35:03 | WT/WT |
| PR-4 | 2.1 | 232 ± 49 | 396,572 ± 136,552 | 50:01/57:01 | WT/WT |
| PR-5 | 2.9 | 308 ± 51 | 63,159 ± 36,958 | 37:01/06:02 | WT/WT |
| | | | | | |
| NP-1 | > 25 | 1,259 ± 62 | 4,764 ± 4,432 | 14:02/47:01 | WT/WT |
| NP-2 | > 8 | 622 ± 29 | 67,006 ± 26,528 | 35:01/41:02 | WT/WT |
| NP-3 | > 10 | 1,007 ± 31 | 571 ± 90 | 15:01/42:01 | WT/WT |
| NP-4 | 19.5 | 759 ± 57 | 20,893 ± 7,585 | 15:01/44:02 | WT/WT |
| NP-5 | > 12 | 541 ± 31 | 1,014 ± 132 | 15:10/42:01 | WT/WT |

# 5.0 DISCUSSION

Advances in the treatment of HIV via antiretroviral therapy have increased survival rates by preventing progression to AIDS and improved overall quality of life for HIV+ individuals. In a unique subset of HIV+ individuals known as nonprogressors, these participants can innately maintain relatively stable CD4+ lymphocyte numbers and low levels of virus without the use of antiretroviral drugs. Previous data showed diminished rates of HIV infection in CD4 lymphocytes by iDCs during *trans* infection within HIV NPs. By identifying the molecular and genetic origins of viremic control by HIV+ NPs, there could be strong therapeutic applications.

## 5.1 TRANSCRIPTOME ANALYSIS REVEALS SIGNIFICANT GENE EXPRESSION TRENDS IN MACS NONPROGRESSORS THAT EXHIBIT DECREASED CELLULAR CHOLESTEROL LEVELS

When Rappocciolo et. al discerned that HIV nonprogression during *trans* infection is associated with lower levels of cellular cholesterol in APCs, they noted that none of the NP donors were homozygous for CCR5-Δ32 mutation or homozygous for HLA-B57 subtype. Noting that both mutations confer protection against HIV infection. Moreover, decreased *trans* infection efficiency was observed prior to antibody seroconversion. This implies the possibility of a novel genetic trait that drives HIV nonprogression. Transcriptome data using Illumina's HT-12 assay was generated on the same set of donors, and it revealed highly significant different gene expression profiles between NPs and PRs with $p < 0.0025$. Siglec-10, CD1b, and Palladin gene

and expression levels were observed to be differentially expressed between MACS NP and PR donors.

### 5.1.1 The functions of Siglec-10, CD1b, and Palladin expression are not interdependent during HIV infection

With the transcriptome data in mind, it was hypothesized that elevated Siglec-10, CD1b, and Palladin in HIV+ nonprogressors were contributing to decreased *trans* infection efficiency in NPs through independent immunological and molecular mechanisms. The purpose of this thesis was to provide preliminary data for the continued investigation of the molecular hypotheses for the target genes. Considering the functionality of each protein paired with recent observations, there is somewhat limited evidence within current literature to link these genes to viremic control via cholesterol metabolism, nor is there evidence of an interconnected relationship between Siglec-10, CD1b, and PALLD.

Siglec-10 functions as a mediator of oligosaccharide binding within cells. Its expression is limited within humans, and it can be found on the cellular surface within blood leukocytes. More specifically, increased Siglec-10 expression in NPs is potentially lessening *trans* infection efficiency by enhancing IL-10 production in iDCs and resultantly stabilizing T-cell counts.

CD1b is a cellular surface protein that is responsible for lipid antigen presentation through the endosomal pathway. During HIV infection, Nef has been linked to CD1b expression, endosomal trafficking of the virus, and NP state. It was hypothesized that elevated CD1b expression in NP is due to an upstream mutant Nef. While it is likely that Nef ultimately decreases trans infection efficiency by modulating cholesterol synthesis, upregulated CD1b potentially

correlates to increased activity within the endolysosomal system. This increased activity subsequently reduces viremia through lysosomal degradation.

However, given the role of the Palladin with actin cytoskeleton remodeling, there is the strongest potential for understanding HIV disease progression—specifically regarding viral trafficking and transmission. During HIV infection, it has been established that the cytoskeleton is remodeled in order to initiate viral and host envelope fusion[70,71]. More specifically, HIV has been found to exploit actin structures at the infectious synapse between antigen presenting cells and T cells during *trans* infection via membrane extensions and nanotubules[68,69,72]. It was hypothesized that Palladin is a molecular biomarker of upregulated actin production within iDCs during HIV infection, which results in alterations within the cytoskeleton. Because the virus cannot overcome the upregulated cortical actin cytoskeleton as efficiently, HIV transmission in NPs is reduced.

## 5.2 QUANTITATIVE PCR RESULTS YIELD SIGNIFICANT TRENDS, WHILE FLOW CYTOMETRY RESULTS PROVE CONTRADICTORY

Two of the primary aims were to analyze RNA expression data using qPCR and phenotype protein levels in the same subset of MACS NP and PR donors that were analyzed in the cholesterol metabolism investigation. It is important to emphasize that transcript levels do not necessarily equate to protein expression levels. PCR and protein analysis offers a deeper insight towards the relationship between the three target genes and HIV NP disease status.

Quantitative PCR results establish that there is a highly significant discrepancy in CD1b and Palladin gene expression levels between PRs and NPs (Figure 3). More specifically, NPs were found to have elevated gene expression when compared to PRs for CD1b and Palladin. When

compared exclusively to CD1b and PALLD transcript expression patterns from the HT-12 assay, there are similar trends. However, there is no statistically significant relationship observed for Siglec-10 between PRs and NPs.

Although RNA levels of Siglec-10 were relatively consistent between the two donor populations, there are several key factors to keep in mind. Twenty percent of nonprogressors were found to have decreased gene expression that were similar to progressors (Figure 1). It is also plausible that despite ideal A260/A280 ratios at around 2.1 for RNA, the inferred contamination of organic molecules (e.g. residual phenols) based on the low A260/A230 ratios could have affected cDNA synthesis. Thereby, potentially distorting Siglec-10 gene expression levels. More importantly, the Illumina HT-12 data utilizes a 3' IVT sequence. As a result, the assay does not detect any alternative splice isoforms that would be upstream of the poly-A tail. Because Siglec-10 has nine unique isoforms, there is a potential explanation for the contrasting transcriptome and qPCR data. That is, transcriptome data could potentially fail to detect alternative isoforms of Siglec-10 and Palladin, which would result in artificial transcript trends.

### 5.2.1 Flow cytometry analysis reveals unexpected trends

Protein expression of all target genes via flow cytometry staining did not equate with RNA transcript data from qPCR. More specifically, protein expression levels are relatively similar between NPs and PRs for all target genes—despite observation of significant trends in ΔCT values. It is important, however, to emphasize that assessment of flow cytometry staining for Siglec-10, CD1b, and Palladin is based on antibodies that bind surface and intracellular protein respectively. More specifically, monoclonal flow cytometry antibodies cannot necessarily recognize the same epitope expressed by protein isoforms due to extensive splicing like PALLD and Siglec-10[68].

Abcam anti-Palladin primary antibody that was utilized does not specify its ability to recognize multiple isoforms. The manufacturer had predicted it only could detect a band around 131kDa. Because of the antibody detection limitations, there could be an artificially lower amount of Palladin protein detected due to the presence of multiple isoforms. As a result, assessment of proteins by flow cytometry is limited by the antibody binding to the protein, which may not necessarily reflect total protein content. In a similar fashion, the antibody utilized for detection of Siglec-10 is not capable of recognizing all isoform variants and subsequent epitope variants.

Evaluation of total protein via western blot was the next step in protein analysis. Western blot analysis facilitates comparison of relative protein expression per target gene in relation to total protein content. Lysing of iDCs during WB would ensure that all available protein for Siglec-10, CD1b, and PALLD would be stained—not just surface or intracellular expression detected via flow cytometry. Due to the limited amounts of dendritic cells after conducting flow cytometry from cell compensation, all remaining cells had to be used for qPCR analysis

## 5.3 SNP GENOTYPING DOES NOT REVEAL POTENTIAL GENETIC LINKS TO HIV NONPROGRESSION

While the third aim was unrelated to protein and transcript analysis, it was pursued to investigate plausible genetic links to HIV nonprogression. More specifically, TaqMan SNP genotyping of CD1b was conducted to evaluate the possibility that SNPs could alter the bioavailability of the transcript, functionality of a protein, or association with NP disease state. Depending on the type of SNP, there could be a wide variety of effects on the regulation or splicing of genes, coding sequence and thus function of a protein product, or alterations to the ORF. Seven

CD1b SNPs that spanned across the entire gene had been evaluated in relation to a larger set of MACS PRs, NPs, and SNs genomic DNA. Haplotype analysis did not reveal any significant differences in allelic frequency data. Although results within this system did not reveal statistically significant relationships,

Palladin was omitted from SNP genotyping due to the extensive size of the gene (431,394 bp), multiple transcription initiation sites, and extensive splicing activity. Siglec-10 genotyping has been delayed until the availability of RNAseq data from an ongoing project.

## 5.4 EXPERIMENTAL LIMITATIONS OF IN VITRO SYSTEM

Although *in vitro* monocyte derived DCs share many functions with *in vivo* DCs—such as cross presentation to CD8 T cells, activation of CD4 T cells, and production of important cytokines like TNF-a and IL-1, 6, and 23, the majority of *in vivo* primary DCs are likely not derived from monocytes[16]. This *in vitro* monocyte-derived DC system only provides a conceptual framework for HIV *trans* infection. Furthermore, iDCs are not representative of all DCs within the human body. Susceptibility of iDCs to HIV infection is distinctly different from other DC subtypes, namely pDCs and mDCs. As a result, this system cannot be generalized across all DCs.

## 5.5 CONCLUSION AND FUTURE DIRECTIONS

Investigating the underlying mechanisms of HIV disease progression has a strong potential for therapeutic applications. Evaluation of Siglec-10, CD1b, and Palladin has provided a

foundation for future investigations of the molecular and immunological mechanism of nonprogression. Future directions include analysis of total protein between NPs and PRs via western blot, Siglec-10 SNP Genotyping, and further analysis into Palladin's role in actin remodeling and HIV disease progression.

# 6.0 PUBLIC HEALTH SIGNIFICANCE

Identifying the underlying genetic factors that drive HIV-1 disease progression could reveal potential therapeutic targets with the long term aims of improving HIV health outcomes.

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