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Christine Grech April 12, 2016

Global Transcriptional Analysis of Plasmacytoid Dendritic Cells (pDCs) in Pathogenic and Non-Pathogenic SIV Infection of Non-Human Primate Model Species

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Abstract

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Abstract:

In contrast to pathogenic HIV infections of humans and SIV rhesus macaques (RM), natural SIV infection of sooty mangabeys (SM) is typically non-pathogenic despite high viremia. Although it has been established that CD4 T cell loss is a direct result of over activation of immune system for pathogenic HIV/SIV infection as of yet, the mechanism by which SMs avoid SIV pathogenesis to AIDS is unknown. Previous studies have suggested that the modulated innate and adaptive immune response for SMs between acute and chronic infection is a result of the activation of immunoregulatory genes that arise during this period such that interferon-stimulated genes (ISGs) remain muted during chronic SIV infection in SMs. To investigate the possible mechanism by which SM avoid over activation of their immune system we sorted and RNA-sequenced (RNA-seq) plasmacytoid dendritic cells (pDCs) from blood in infected and uninfected SM and RM. pDCs are a small subset of peripheral blood mononuclear cells (PBMCs) that have been established as interferon (IFN) producing cells during acute SIV infection. To this end, we found significant up regulation ISGs in SIV-infected RM, but not in SMs. Therefore confirming the model that innate response to SIV is attenuated during chronic infection in SM. We found no expression of IFN across all four species, suggesting that pDCs in the blood are not the IFN producers during chronic infection. Thus, further exploration must be conducted in order to determine IFN producers during chronic infection.

Introduction:

In 1981, several healthy, young men were diagnosed with rare diseases associated with immune deficiency. As the case number grew the CDC officially diagnosed the patients with acquired immune deficiency syndrome (AIDS). Approximately two years after the first reported cases of AIDS the scientific community identified its cause: a retrovirus termed human immunodeficiency virus (HIV) (Murphey-Corb et al., 1986).

HIV pathogenesis

In the last 35 years, we have greatly furthered our understanding of HIV. Transmission of the virus occurs through direct body fluid contact between patients. Common transmission routes are infected needles, unprotected sexual contact, and mother-to-child during pregnancy or breast feeding (Sharp & Hahn, 2011).

The virus is extremely diverse even to the point of having multiple strains in one patient, but overall there are two main types: HIV-1 and HIV-2. HIV-1 has multiple subtypes: M, N, O, and P (Sharp & Hahn, 2011). HIV-1M is the most prevalent subtype among AIDS patients. HIV-2, on the other hand, is found mainly in Africa and is characterized by its rare progression to AIDS (Sharp & Hahn, 2011).

The CDC defines an AIDS diagnosis as a HIV infected individual with a high plasma viral load ($>10^5$ RNA copies/mL) and a CD4+ (helper) T cell count <200 cells/μL. A low CD4+ T cell count indicates a weakened immune system, therefore AIDS patients are open to opportunistic infections (OI), such as pneumonia, thrush, and toxoplasmosis (Mann, 1987).

We have developed regiments of drugs, antiretrovirals (ART), and life style changes that are able to suppress the virus to modest success (Yarchoan & Broder, 1987). HIV+ patients can live a relatively long life, yet we still lack many key pieces for exactly why it is one of the few examples of pathogenic infection in its family of retroviruses.

SIV infection is non-pathogenic in the majority of non-human primate models naturally infected in the wild; whereas pathogenic SIV infections only occur in experimental settings in Asian *Macaca* species

Simian immunodeficiency virus (SIV), a retrovirus highly related to HIV, is present in several non-human primates with approximately 40 distinct strains throughout Africa (Sharp & Hahn, 2011). The discovery of SIV provided a mechanism for how HIV entered into the human population. More specifically, the subtypes of HIV entered as a result of independent cross-species transmission events with the different strains of SIV found in chimpanzees (SIV_{cpz}), gorillas (SIV_{gor}), and monkeys (Janssens et al., 1994). HIV-1M is found to be related to the SIV_{cpz} strain, which is unique to other natural SIV infections as the host does develop AIDS like symptoms. On the other hand, the genome of HIV-2 is quite similar to SIV genome found in monkeys (Janssens et al., 1994).

The overwhelming majority of SIV infection of non-human primate species is non-pathogenic despite high viremia levels (>10^5 RNA copies/mL). One such species is the sooty mangabey (*Cercocebus atys)* that harbors the SIV_{smm} strain whose genome is over 90% identical to the HIV-2 strain, and therefore believed to be the origin of HIV-2 (**Fig 1**) (Sodora et al., 2009). Although, unlike HIV-2 and rare cases HIV-1 the lack of pathogenesis is not a result of viral suppression (**Fig 3**). High viral loads and absence of AIDS-like

symptoms indicate that the virus is not suppressed, but instead the immune system has learned how to "co-exist" with the virus (Steven E. Bosinger, 2021).

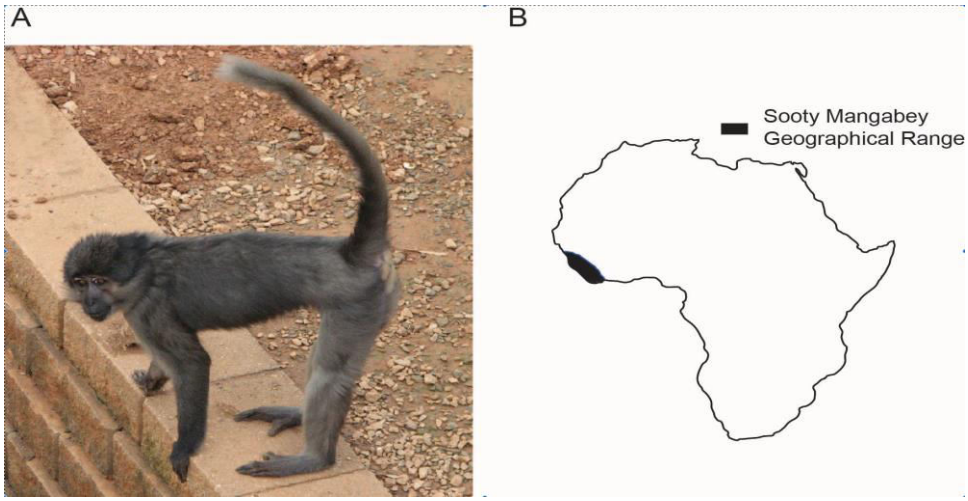

Figure 1 | Non-Human Primate Sooty mangabey (**A**) Photograph of sooty mangabey monkey at the Yerkes National Primate Research Center (**B**) Geographical range of sooty mangabeys in West Africa (Steven E. Bosinger, 2021)

SIV_{smm} not only is the origin of HIV-2, but also is the source of the SIV_{mac} strain of virus used to infect rhesus macaque (*Macaca mulatta*), the non-human primate model for pathogenic SIV infection (Sharp & Hahn, 2011). The rhesus macaque (RM) habitat is in Asia, and therefore they are not natural hosts to SIV (**Fig 2**). RM present similar infection pathogenesis to humans: loss of CD4+ T cells, disruption of lymph node architecture, weight loss, diarrhea, and susceptibility to opportunistic infections. If left untreated, infected RM have a time-to-death of 6 months - 2 years (Murphey-Corb et al., 1986). RM's shortened time table of SIV pathogenesis along with their similarity in AIDS progressions makes them good model species for HIV.

A **B**

Figure 2 | Non-Human Primate Rhesus Macaque (**A**) Photograph of rhesus macaque monkey at the Yerkes National Primate Research Center (**B**) Geographical range of rhesus macaque in South Asia (Tu, Pani, & Hampton, 2015).

Therefore, the development and the discovery of the two non-human primate models help understand pathogenic infection of HIV in humans. While RM mimic HIV pathogenesis, SM provide an appropriate contrast. How SMs are able to avoid AIDS is still under investigation because unlike in human cases where HIV does not progress to AIDS, SIV infection in SM retains high plasma loads similar to humans and to RM who develop AIDS like symptoms (**Fig 3**).

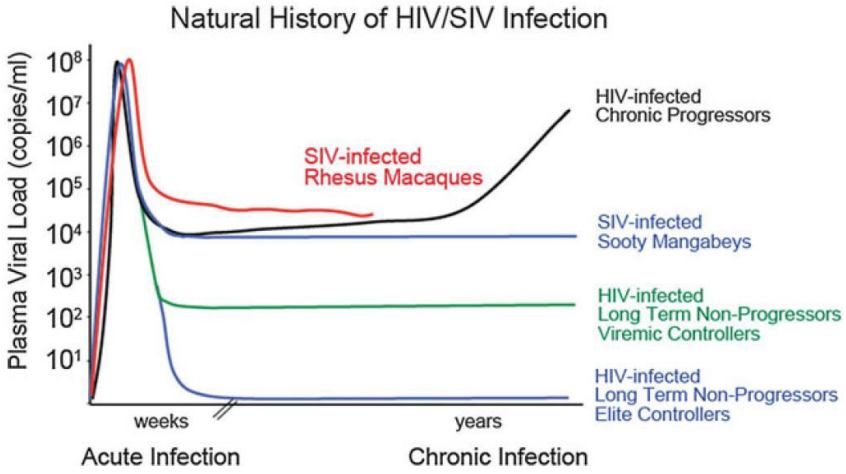

Figure 3 | Viral loads within models of HIV and SIV infection During the common course of HIV infection most patients will experience progressive disease, which, when left untreated, would result in AIDS overtime. These chronic progressors typically maintain viral loads in the plasma at 10^4-10^5 RNA copies/mL. A limited number of patients, long-term non-progressors, are able to control the virus (to varying degrees of success) and maintain stable CD4+ counts for several years. These patients are sub classified based on their plasma virus levels: viremic controllers and elite controllers. The former patients exhibit lowered, but detectible virus ~10^2, while the latter patients completely suppress the virus. In comparison, the plasma viral loads of rhesus macaques (RM), which develop the disease, and sooty mangabeys (SM) which remain AIDS-free, are similar to the virus levels in chronic progressors. This suggests that immune control is not the likely mechanism that SIV-infected SM avoid disease (Steven E. Bosinger, 2021)

SIV pathogenesis is the result of continued over activation of the immune system

The current accepted hypothesis for HIV and SIV pathogenesis is the "over-activation" of the immune system. It has been found that CD8+ (cytotoxic) T cells are at elevated levels even into the chronic stages of infection, while CD4+ (helper) T cells have an extremely rapid turnover leading to immune exhaustion (Silvestri et al., 2003). On the other hand, SM did not exhibit CD4+ exhaustion or CD8+ T cell activation into chronic infection (Silvestri et al., 2003). It was demonstrated however that SM have a robust innate and adaptive immune response to HIV that is quickly modulated (S. E. Bosinger et al., 2009). One hypothesis for this innate immune system regulation is that the response is

actively attenuated by immunoregulatory genes that act during the transitions between acute and chronic infection (**Fig 4**) (S. E. Bosinger et al., 2009).

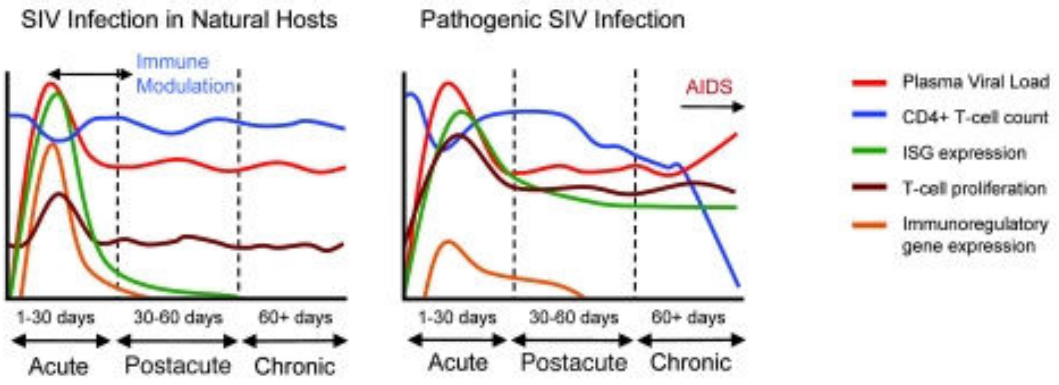

Figure 4| Model of immunomodulation during SIV infection of natural hosts and during pathogenic infection, demonstrating the differences in ISG induction and immunoregulatory gene expression (S. E. Bosinger et al., 2009)

SIV infection of SM drives a massive IFN response that rapidly resolves

Immune system activation is a vast subject of immunology, therefore we will only focus on innate immune activation. The innate response is a key component of protection against viral infections. Viruses are sensed by components of the innate immune system called pattern recognition receptors (PRRs) (S. E. Bosinger et al., 2013). PRRs recognize pathogens through their conserved chemical and structural features called pathogen-associated molecular patterns (PAMPs) and produce signaling cascades that result in the release of molecules, such as interferon (IFNs) (S. E. Bosinger et al., 2013).

In the case of HIV and SIV infection, the PRR is the endosome anchored toll-like receptors 7 (TLR7) (Saidi et al., 2016). TLR7 recognizes viral ssRNA, the binding between HIV/SIV's viral ssRNA with TLR7 triggers the recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor molecule (Cao & Liu, 2007). MyD88 creates a

complex with IL receptor-associated kinase 1 and 4 (IRAK1/4) to create what is called a cytoplasmic transductional-transcriptional processor. Activated IRAK1/4 can now associate with interferon regulator factor 7 and 5 (IRF7/5), tumor necrosis factor receptor-associate factors 6 and 3 (TRAF6/3), and IκB kinase-α (IKKα). The complex phosphorylates and activates IRF7. Activated IRF7 then translocates into the nuclei and initiates type I IFN transcription (**Fig 5**) (Cao & Liu, 2007)

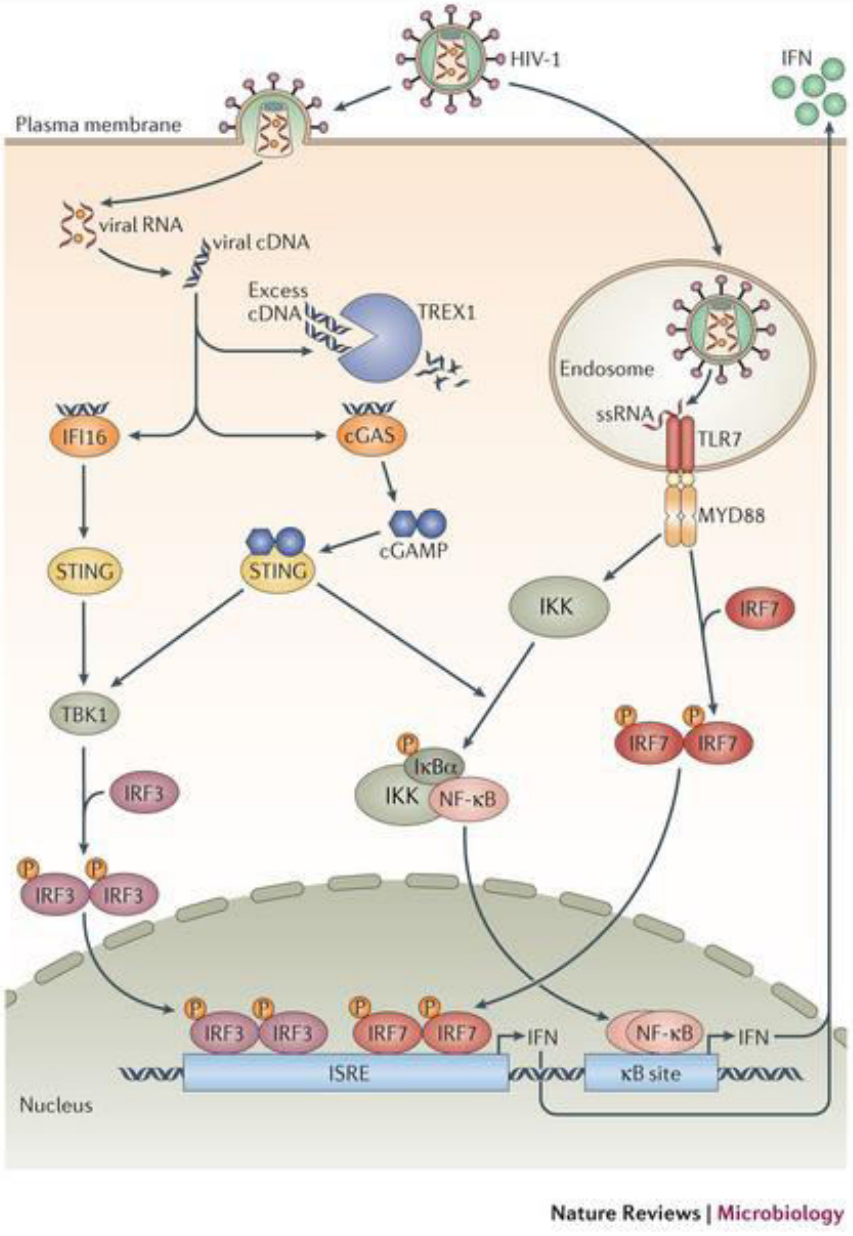

Figure 5| Pathway for IFN transcription TLR-mediated signaling triggers innate responses by pDCs. Engagement of TLR7 in the early endosome signals through a multicomponent complex that strongly activates IRF7, leading to IFN-α transcription. Simultaneous activation of IRF5, NF-κB and MAPKs promotes production of cytokines and chemokines and the maturation of pDCs. TLR triggering from the late endosome or lysosome preferentially stimulates the production of cytokines and the maturation of pDCs. (Doyle, Goujon, & Malim, 2015)

IFNs, especially type I, are pro-inflammatory and immune modulatory cytokines that induce antiviral immune response (S. E. Bosinger & Utay, 2015). Type I IFN-α is the most prominent in innate immune activation during HIV and SIV infection (Gringeri et al., 1996). IFNs activate natural killer (NK) cells, may contribute to CD4+ T cell loss, and up regulate interferon-stimulated genes (ISGs) (Schoggins et al., 2011). ISGs are varied in their jobs, but overall are responsible for innate antiviral response (G. Li et al., 2014). For example, ISGs enhance PAMP detection and amplify IFN response (Schoggins et al., 2011).

ISGs can also be restriction factors responsible for inhibition of viral replication. Two relatively common restriction factors that are important for HIV and SIV infection are APOBEC3, a protein that suppresses viral cDNA synthesis by interfering reverse transcription and TRIM5, a ligase that leads to reverse transcription fragmentation before cDNA synthesis (Fernandez-Oliva et al., 2015). Restriction factors have been mainly viewed as cross-species transmission barriers due to the fact they are produced early in infection and have little impact on HIV-1 transmission and replication (Doyle et al., 2015), and therefore may not be significant in stabilizing chronic infection.

Since IFN- α helps establish the antiviral environment and regulates the innate immune system, its response in pathogenic and non-pathogenic SIV infection may provide insight into how natural hosts modulate the innate immune system response (**Fig 4**). In pathogenic non-human primate models, IFN-α has both detrimental and beneficial effects (G. Li et al., 2014). Although production of IFN-α during acute activation contributes to the innate control of infection, it also provides target cells for the virus, impairs CD4 T cell

recovery during chronic infection, and is associated with disease progression (Sandler et al., 2014).

Our previous work has shown that SMs have a rapid control response for IFN-α after their robust IFN production during acute infection. RMs exhibit a more consistent level of IFN throughout acute and chronic infection (**Fig 6**) Therefore, IFN-α control follows the similar model of rapid modulation of the immune system in SM after acute infection (S. E. Bosinger et al., 2013).

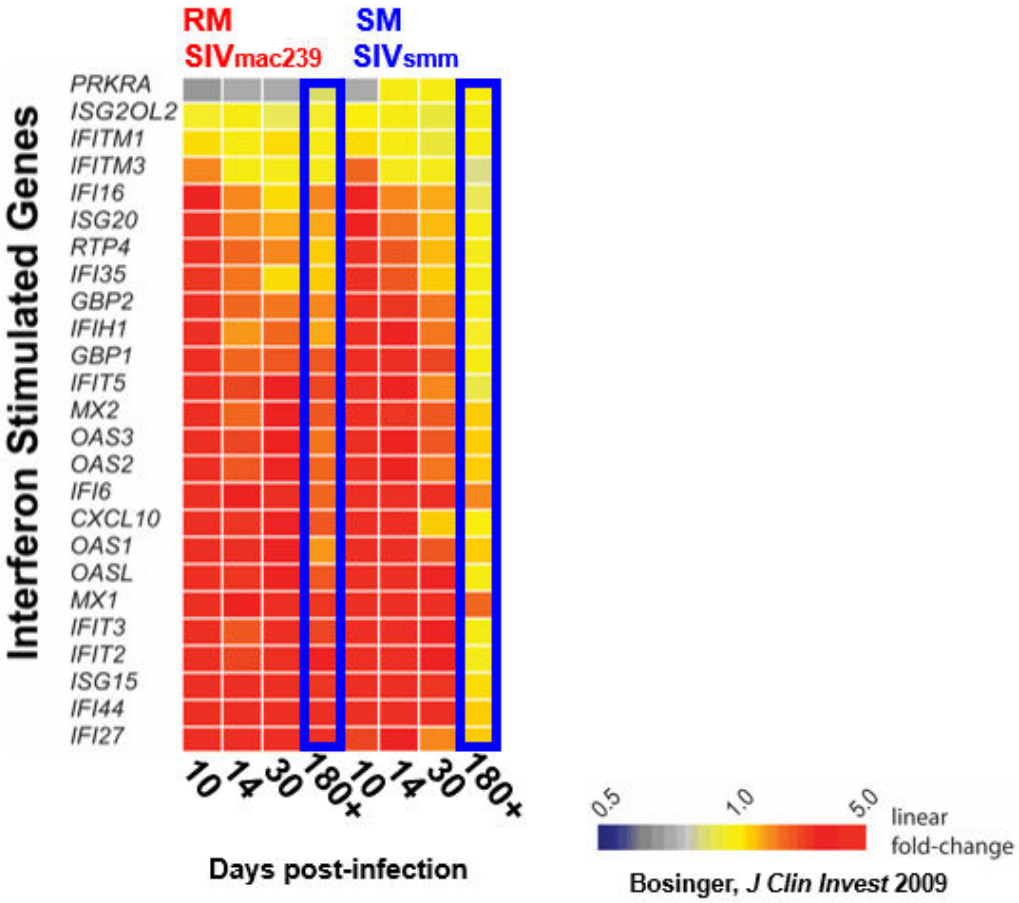

Figure 6 | SIV infection of SM drives a massive IFN response that rapidly resolves Heat map of ISG expression within the blood at 4 time points post SIV infection with time point 180+ as the chronic stage. Individual colored panels represent the fold change. The color scale is indicated at bottom. (S. E. Bosinger et al., 2009)

pDCs may cause pathogenesis in HIV and SIV infection

Until fairly recent, it was relatively unknown which cell subsets were producing the high levels IFN during viral infection.

A small subset, approximately 0.2-0.8%, of peripheral blood mononuclear cells (PBMCs) were identified to be the major IFN producers (Liu, 2005). They have been found in both humans and non-human primates. Originally called, interferon-producing cells (IPCs), the subset was thought to be of lymphoid origin and lacked any lineage surface cell markers. Later on, the IPCs would be receive the title of plasmacytoid dendritic cells (pDCs) (Liu, 2005).

pDCs are similar in size and granularity to lymphocytes and monocytes, but do not have the CD3, CD20, or CD14 surface cell proteins associated with T cells, B cells and monocytes respectively (Autissier, Soulas, Burdo, & Williams, 2010). pDCs do express CD4 and HLA-DR, a MHC class II cell surface receptor found only on professional antigen-presenting cells. It was believed that pDCs were similar to the previously identified antigen-presenting cell: myeloid dendritic cells (mDCs). mDCs, similar to pDCs do not have CD3, CD20 or CD14 surface markers and express HLA-DR(Autissier et al., 2010). However, pDCs and mDCs differ on CD123 and CD11c expression. pDCs express the protein CD123 and lack CD11c, while mDCs lack CD123 and express the protein CD11c (Autissier et al., 2010). Furthermore, pDCs and mDCs are distinct in their relative cell shape. pDCs are fairly spherical while mDCs have tendrils expanding from the center of the cell (**Fig 7**) (Klechevsky et al., 2005).

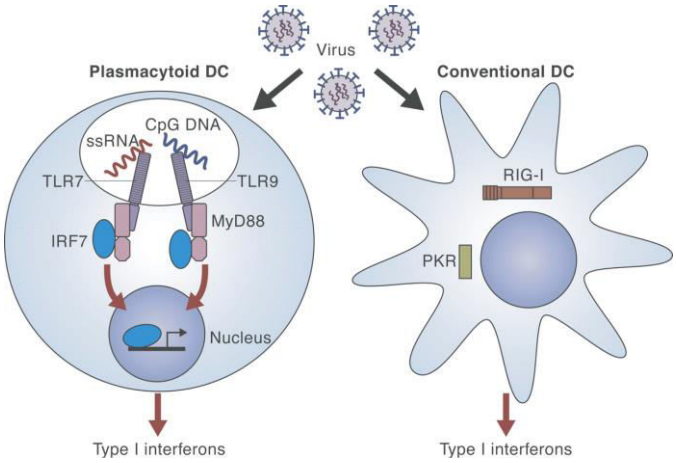

Figure 7 | Distinct structure between pDCs and mDCs Recognition of viral infection for induction of type I IFNs in conventional and plasmacytoid DCs. In pDCs, viral infection is recognized by TLRs, leading to type I IFN induction. Non-pDCs are suggested to utilize a TLR-independent system (Klechevsky, Kato, & Sponaas, 2005).

pDCs are the major IFN producers during acute viral infection as they are responsible for over 99% of the IFN produced (O'Brien, Manches, & Bhardwaj, 2013). pDCs' IFN response to viruses is hundreds of times more potent than any other cell type (Coccia et al., 2004). The potency of the pDC IFN response is a direct result of their rapid IFN production, which is because of their constitutively expressed IRF7 (**Fig 5**) (Dai, Megjugorac, Amrute, & Fitzgerald-Bocarsly, 2004). In other IFN producing cells IFN expression is regulated by autocrine feedback loop. They require a small amount of IFN to be produced by the slower IRF3 pathway in order to activate the IRF7 pathway, thus, in all other IFN producing cells there is a delay in activation that is not present in pDCs (Barchet et al., 2002).

There is a general consensus that pDCs are the primary producer of IFN-α during the acute phase of HIV/SIV infection, their role in chronic infection remains unclear (S. E. Bosinger & Utay, 2015). We have established that in chronic infection pDCs levels are

depleted in the blood, while they accumulate in rectal and vaginal mucosa after acute SIV infection (S. E. Bosinger & Utay, 2015). A recent study that looked at the depletion of pDCs in humanized mice was able to abolish IFN-I and ISG responses to HIV infection, therefore providing a basis for the hypothesis that pDCs are the primary IFN producers during chronic infection (G. Li et al., 2014). If pDCs are found to be the primary producers of IFN-α, they may be a major contributor to SIV and HIV pathogenesis.

Purpose

In this study, we used transcriptional analysis to further the understanding of pDCs role in chronic SIV-infection by comparing two NHP models: pathogenic and non-pathogenic. With the advancement of sequencing technology we are able to assess the gene expression of pDCs even with their low cell counts in the blood. We sorted pDCs, defined as lineage-HLA-DR+CD11c-CD123+, from 16 animals within four conditions: uninfected RM, chronically SIV-infected RM, uninfected SM, and chronically SIV-infected SM. We performed RNA sequencing (RNA-Seq) on the samples and analyzed their read counts in order to determine significant changes in gene expression due to infection status. Furthermore, we looked at differences in gene regulation between non-pathogenic infection, SM and pathogenic infection, RM in order to increase our understanding of the mechanisms by which chronically SIV-infected SMs are able to avoid SIV pathogenesis.

Materials and Methods:

Overall Experimental Design

In order to evaluate gene expression of plasmacytoid dendritic cells (pDCs) in relation to SIV status we performed a cross-sectional analysis on 16 animals. The animals were as follows: SIV- sooty mangabeys (SM), SIV+ SM, SIV- rhesus macaques, and SIV+ RM. These conditions allowed us to compare gene expression changes for non-pathogenic SIV infection (SM) to pathogenic SIV infection (RM). Blood was drawn from the animals and peripheral blood mononuclear cells (PBMCs) were isolated. The pDCs, a subset of PBMCs, were then sorted and sored for RNA extraction and library preparation for RNA-Seq. myeloid dendritic cells (mDCs) were processed alongside the pDCs as a comparative control in order to evaluate our data's accuracy through RNA-Seq (**Fig. 8**)

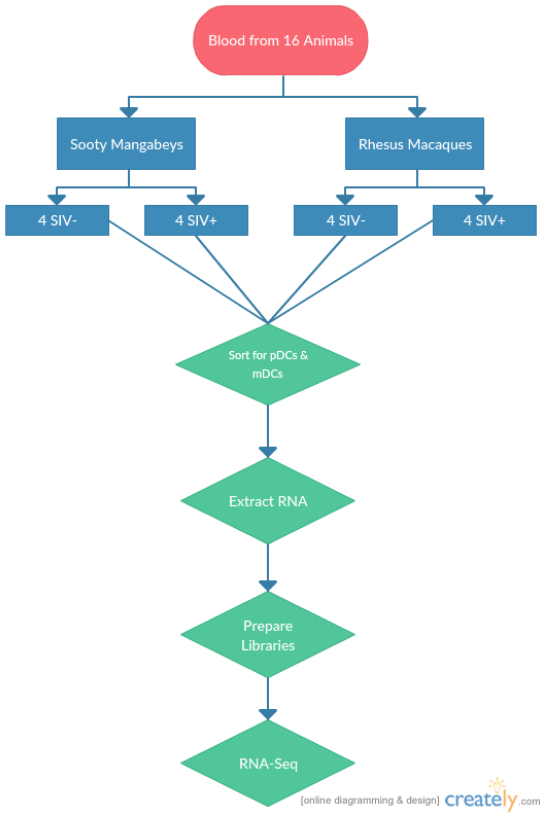

Figure 8 | Overview of Experimental Design
Blood was drawn from 16 animals from the four conditions: SIV+ RM, SIV- RM, SIV+ SM and SIV- SM. We then sorted pDCs from all 16 animals. The samples were sent to the Genomics Core at the Yerkes National Primate Research Center (YNPRC) for RNA extraction and library preparation. The libraries were pooled and sequenced on an Illumina HiSeq.

Animals

16 non-human primates (**Table 1**) were selected for blood draws. The animals were broken down into four conditions with four animals (n=4) in each: SIV- SM, SIV+ SM, SIV- RM, and SIV+ RM. The animals were housed at the Yerkes National Primate Research Center (YNPRC). All the animals were male. The SIV+ SM and RM were considered chronically infected with the SM infected via plasma transfer from a previously or naturally infected SM and the RMs infected intrarectally via SIVmac239, an isolated virus used in research.

Samples

Blood draws were obtained from sooty mangabeys (n=8) and rhesus macaques (n=8) housed at the YNPRC. Blood was collected in EDTA tubes. Then, PBMCs were isolated from the blood by Ficoll density centrifugation. At least 40 million PBMCs were collected from approximately 40 mL of blood (**Table 1**). Cells were stained with monoclonal antibodies and stored at 4°C until flow cytometry or cell sorting.

Table 1 | Animal Characteristics

Animal ID	Species	Date of Birth	Date of Sampling	Age	SIV status	Date of Infection	Duration of Infection	Virus Inoculum	PBMC Yield	pDC yield
FJb1	SM	5/22/11	12/15/15	4 years	SIV-	N/A	N/A	N/A	4.2E+07	2301
FKb1	SM	6/13/11	12/15/15	4 years	SIV-	N/A	N/A	N/A	4.2E+07	12,123
FRb1	SM	8/10/11	12/15/15	4 years	SIV-	N/A	N/A	N/A	4.0E+07	18,968
FTb1	SM	8/19/11	12/15/15	4 years	SIV-	N/A	N/A	N/A	5.0E+07	3357
FNy	SM	6/14/99	12/16/15	16 years	SIV+	11/2003	12 years	SIV+ plasma from FUo	5.0E+07	2002
FJy	SM	5/2/99	12/16/15	16 years	SIV+	12/2005	10 years	SIVssmE041	4.0E+07	2628
FTv	SM	5/15/97	12/16/15	18 years	SIV+	10/2006	9 years	SIV+ plasma from FBr	5.0E+07	3903
FUv	SM	5/30/97	12/16/15	18 years	SIV+	10/2000	15 years	SIV+ plasma from FUo	4.0E+07	13,113
RMt4	RM	4/20/94	12/17/15	21 years	SIV-	N/A	N/A	N/A	2.6E+07	10,000
RYa15	RM	3/8/12	12/17/15	3 years	SIV-	N/A	N/A	N/A	4.0E+07	26,716
RVp4	RM	3/25/94	10/23/15	21 years	SIV-	N/A	N/A	N/A	4.0E+07	12,000
RQn5	RM	3/12/96	1/21/16	19 years	SIV-	N/A	N/A	N/A	6.0E+07	28,793
102_11	RM	5/15/11	12/18/15	4 years	SIV+	4/29/15	8 months	Mac239	5.0E+07	20,000
97_11	RM	5/3/11	12/18/15	4 years	SIV+	5/29/15	7 months	Mac239	6.6E+07	12,519
79_11	RM	4/15/11	12/18/15	4 years	SIV+	6/22/15	6 months	Mac239	5.6E+07	15,000
112_11	RM	5/16/11	1/21/16	4 years	SIV+	5/29/15	8 months	Mac239	6.4E+07	3,348

Flow Cytometry and Surface Cell Staining

Flow cytometry uses antibodies linked to fluorophores to identify cells with specific surface proteins (markers). After cells are stained with said antibodies the cells are run singularly through a column where lasers shine onto the cells. The fluorescence emitted from the stained cells is detected and visualized through clustering on graphical representations. As the cells pass through the laser beam they scatter light and it is measured as forward and side scatter. Forward scatter correlates to the size of the cell. Side scatter is proportional to the complexity or granularity of the cell (Goetzman, 1993). We used flow cytometry to distinguish pDCs based upon their relative size, granularity and the presence or absence of surface markers.

After Ficoll, PBMCs were incubated with Aqua Live/Dead amine dye-AmCyan and then incubated with fluorescently labeled antibodies for specific surface markers. Cells were stained with monoclonal antibodies to the following proteins: anti-CD123 PE-Cy7 (clone 6H6), anti-CD20 Pacific Blue (clone 2H7), anti-CD14 PE-Texas Red (clone RMO52), anti-CD3 Pacific Blue (clone SP34-2), anti-CD11c APC (clone S-HCL-3), anti-CD16 BV650 (clone 3G8), and anti HLA-DR PerCP-Cy5.5 (clone G46-6) (Autissier et al., 2010). At least 1 million live lymphocyte events were acquired on a LSRII flow cytometer.

Analysis was performed with FlowJo 3; version 9.8.5 (TreeStar Inc, Oregon). Axes correspond to forward scatter (FSC, a measurement of the size of the cell), side scatter (SSC, a measurement of the granularity of the cell), or the specified antibody (populations above 10^3 indicate the presence of the antibody on the cells). Gating techniques looked at the separation between populations.

pDCs were classified through the following gates: lymphocyte, singlet, live cells, CD3-CD20-, HLA-DR+, CD14-, and CD11c-CD123+. mDCs were classified though the following gates: lymphocyte, singlet, live cells, CD3-CD20-, HLA-DR+, CD14-, and CD11c+CD123-. We specifically gated for the mDC subset identified as CD16+. Gating techniques are shown in (**Fig 9 + 10**)

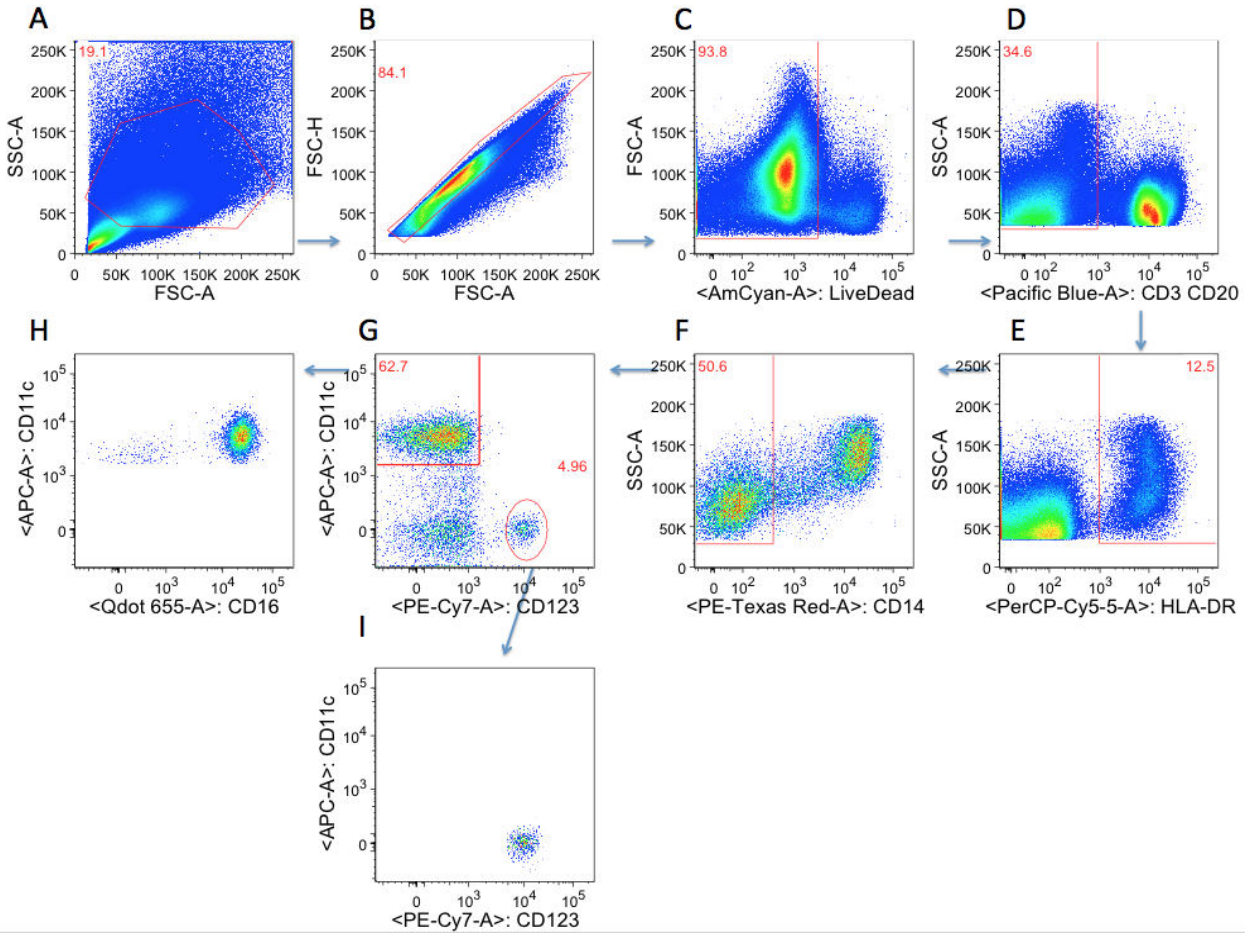

Figure 9 | Flow cytometry analysis of dendritic cells populations in Rhesus Macaques. DCs were gated on SSC and FSC to include both lymphocytes and monocytes (A). Doublets (B), dead cells (C), and Lin- (D) as defined by CD3+ T lymphocytes and CD20+ B lymphocytes were excluded. HLA-DR+ (E) cells were gated. CD14+ monocytes (F) were excluded. Two DC subsets as defined to be Lin-HLA-DR+ were distinguished by CD123 and CD11c expression (G). CD123+CD11c- (I) population defined as pDCs. CD123-CD11c+ (H) population defined as mDCs. Results presented here are from one animal and are representative of n=8. Numbers are percentages of each population within the same dot plot.

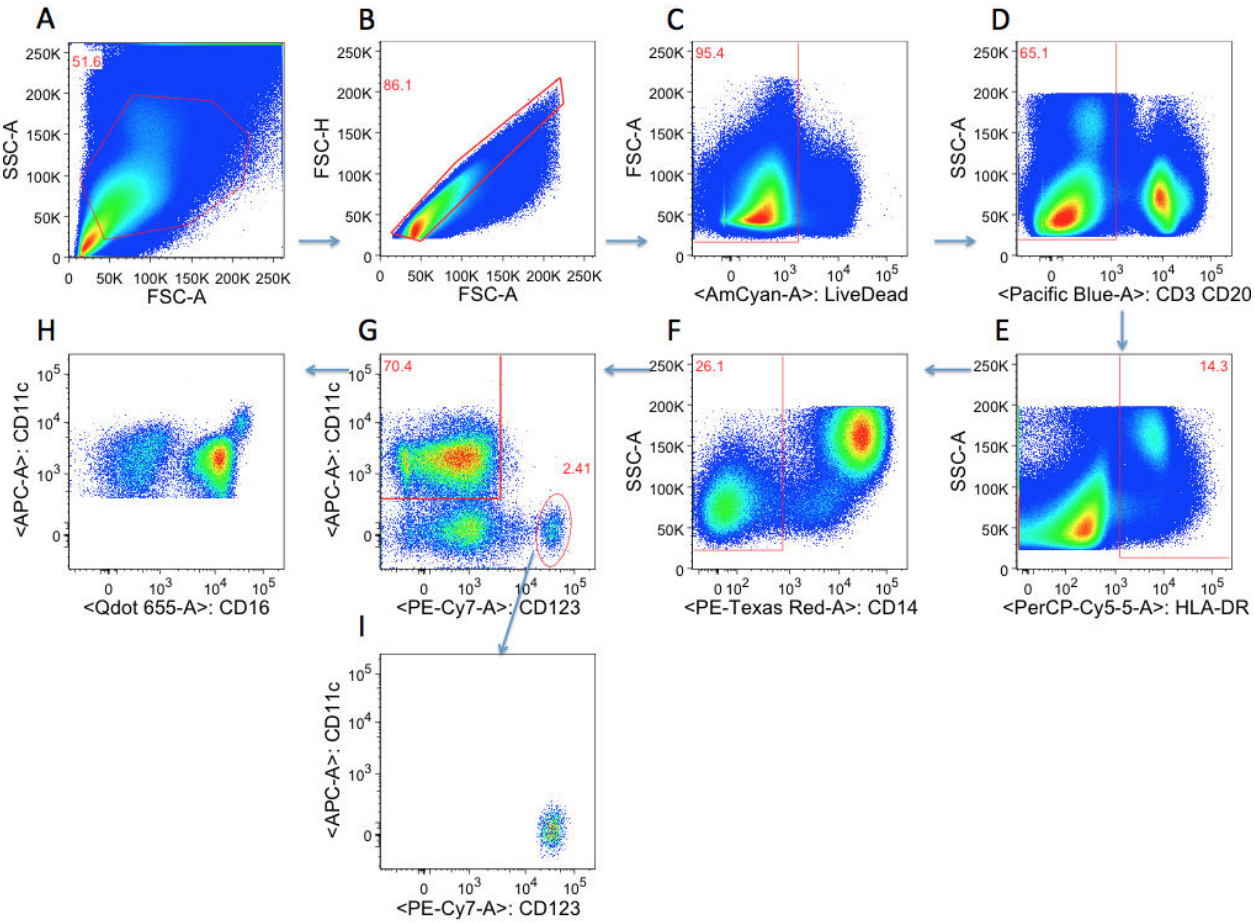

Figure 10 | Flow cytometry analysis of dendritic cells populations in Sooty Mangabeys. DCs were gated on SSC and FSC to include both lymphocytes and monocytes (A). Doublets (B), dead cells (C), and Lin- (D) as defined by Fig. 9 were excluded. HLA-DR+ (E) cells were gated. CD14+ monocytes (F) were excluded. Two DC subsets as defined to be Lin-HLA-DR+ were distinguished by CD123 and CD11c expression (G). Separation for SM was less distinct in comparison to RM, therefore contour maps and histograms were evaluated for gating. CD123+CD11c- (I) population defined as pDCs. CD123-CD11c+ (H) population defined as mDCs. Results presented here are from one animal and are representative of n=8. Numbers are percentages of each population within the same dot plot.

Sorting

Cells were stained with the monoclonal antibodies as previously discussed for flow cytometry. Then, pDCs and mDCs (CD16+) were sorted from PBMCs at the Flow Cytometry Core at YNPRCs on an Aria II instrument. Gating techniques developed on the LSRII flow cytometer were used to define the subsets. pDCs were collected in aliquots of up to 5,000 cells into media. mDCs were collected in aliquots of 5,000, 50,000 and 200,000 cells into media. mDC population purity was above 99% for all animals, but pDC purity could not be calculated due to low cell numbers. Aliquots were stored in RLT buffer, a lysis buffer for lysing cells and simultaneously isolating RNA, at -80° C until RNA extraction.

RNA Extraction and Library Preparation

Extraction and library preparation was performed in the Genomics Core at YNPRC. RNA was extracted from one aliquot of pDCs and one aliquot mDCs for each animal. Aliquots were at least 2000 cells and no more than 5000 cells.

Total RNA was isolated using the QIAGEN RNEasy Micro Kit. Libraries were generated using the CLONTECH SMARTer V4 kit, barcoding and sequencing primers were added using NexteraXT DNA kit (Freeman, 2013). Libraries were validated, quantified, pooled and clustered on Illumina TruSeq v3 flowcell (Bentley et al., 2008).

The CLONTECH SMARTer V4 kit is specifically used for low input samples and uses Switching Mechanism at 5' End of RNA Template (SMART). CLONTECH SMARTer kit allows for efficient incorporation of known sequences at both ends of cDNA during the first strand thesis, therefore greatly enhancing the ability to obtain the true 5' RNA end of a transcript (Freeman, 2013). Using the SMARTer technology along with the barcoding primers, we

were able sequence the libraries pooled based upon their conditions and still keep data to individual libraries (Bentley et al., 2008).

RNA-Sequencing (RNA-Seq) and Analysis

The flowcell with the clustered libraries was sequenced on an Illumina HiSeq 1000 in 100-base single-read reactions (Bentley et al., 2008). The reads were aligned to the *Macaca mulatta* (rhesus macaque) reference genome via Spliced Transcripts Alignment to a Reference (STAR) software in order to determine specified transcripts (Dobin et al., 2013). STAR software is a vast improvement of mapping speeds, sensitivity, and precision from previous software (Dobin et al., 2013). Data from the STAR software was processed with HTSeq-Count software for abundance estimations or counts of specified genes (Anders, Pyl, & Huber, 2015). Count, or gene expression, was analyzed using DESeq2 software for each gene. DESeq2 is a method for differential analysis of count data that calculates fold changes in normalized read counts, as well as p-values determined from the Wald test (Michael I. Love, Wolfgang Huber, & Simon Anders, 2014). Fold changes and p-values were calculated between SIV+ and SIV- RM/SM.

Results:

RNA-Seq of purified pDCs recapitulates lineage defining features

Our extensive sequencing reaffirms previous literature about lineage defining features of pDCs (Brown & Barratt-Boyes, 2009).

We applied the principal component analysis (PCA) on the sequencing data from all 32 samples (**Fig. 12**). PCA is an analytical technique that transforms gene expression data

into orthogonal vectors derived from co-variances of the data. Therefore, PCA depicts similarities between data sets through 2D spatial distance (Stratmann, Lakatos, & Albu-Schaffer, 2016). Animal, RMt4's mDC aliquot was dropped from the analysis due to lack of reads and poor mapping. Furthermore, it was a significant outlier (data not shown) to all other samples in the PCA. We found distinct clusters of pDCs and mDCs along the first principal component and SM and RM along the second principal component. Furthermore, there is a distinct separation of clusters between SIV- and SIV+ pDCs in RM, but not in SM, suggesting a greater variance in gene expression due to infection status in RM not present in SM.

In order to assess the accuracy of the RNA-Seq data to identify pDCs, we evaluated the gene expression specific to distinct immune subsets, we plotted expression of several pDC and mDC lineage defining markers. We evaluated the means of normalized read counts, or expression, of genes associated with pDCs and mDCs (**Fig. 11a**). Read counts of approximately 20 were defined as the baseline. pDCs expressed BDCA-2 a surface marker found to be distinct to the subset (Dzionek et al., 2000). pDCs expressed TLR7, TLR9, and IRF7, proteins involved in the IFN pathway, far greater than mDCs (Blasius & Beutler, 2010; Dai et al., 2004). mDCs expressed CDllc and RIG-I as per their definition (Brown & Barratt-Boyes, 2009). Both subsets expressed HLA-DR (Brown & Barratt-Boyes, 2009). Therefore, the genes found to be expressed on our data follows from previous studies.

We evaluated the expression of CD4 and chemokine receptor 5 (CCR5), in order to asses expression of HIV and SIV receptors and co-receptors on pDCs. Previous data has shown that CD4 is present on pDCs in both SM and RM species (Jochems et al., 2015). On the other hand, CCR5 has been found to be absent on the surface of SM cells, while present

on both RM and humans. This difference is due to a novel mutation in SM so CCR5 is not expressed on the cell surface (Riddick et al., 2010). DESeq2 found fold changes (log base2) greater than 1 between SIV- SM and RM as well as between SIV+ SM and RM (p values<0.05) (**Fig 11b)**.

Therefore, out data re-iterates what is known about pDCs. There are many technical factors that could have caused our assay to not provide the discussed results. By reaffirming previous data we have shown that our data can be relatively trusted to be accurate, and therefore further investigation of unknown expression changes can be understood as true changes and not a sign of technical errors.

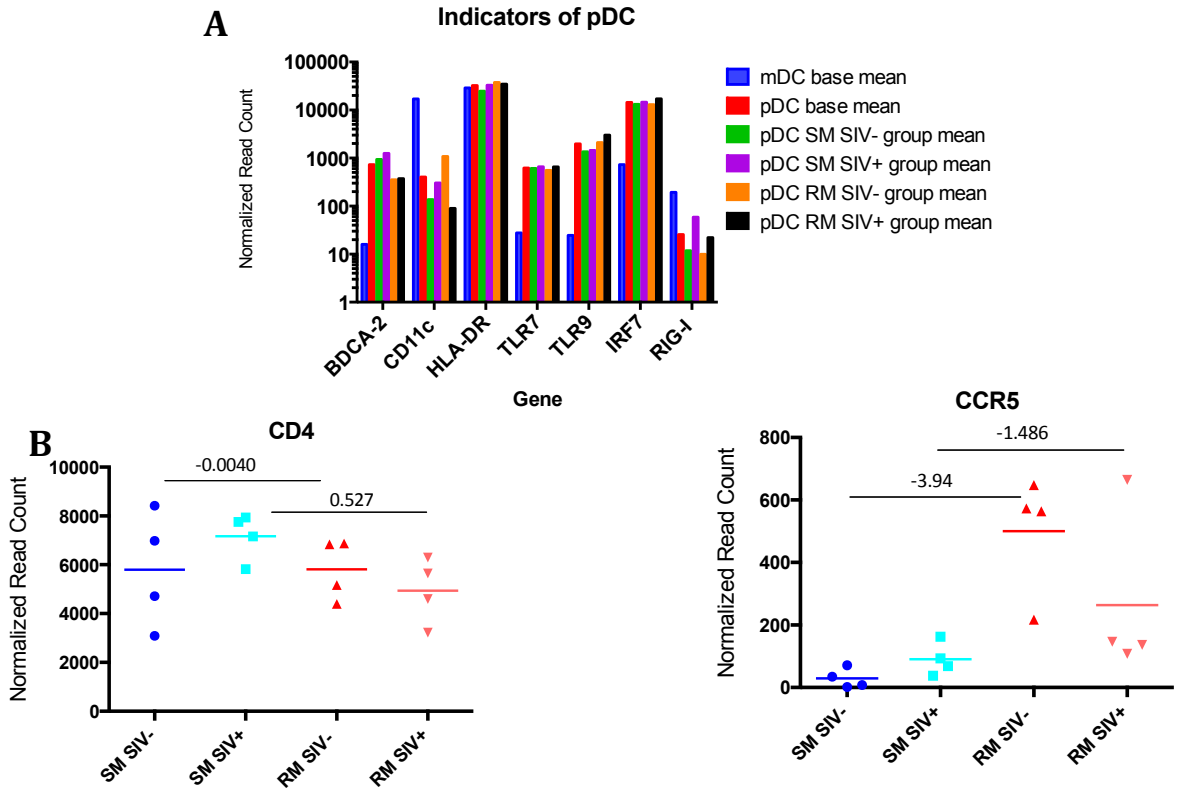

Figure 11 | RNA-Seq of purified pDCs recapitulates lineage defining features A. Expression assessed by RNA sequencing (RNA seq) of genes distinct to pDCs (BDCA-2, TLR7, TLR9, and IRF7, mDCs (CD11c and RIG-I), both cell types (HLA-DR) in mDCs (n=15) and pDCs (n=16). Expression graphed on a logarithmic scale. **B.** CCR5 and CD4 gene expression assessed by RNA-seq of pDCs in SIV- SM (n=4), SIV+ SM (n=4), SIV- RM (n=4), and SIV+ RM (n=4). Numbers on graphs depict log base 2 fold change between SM and RM.

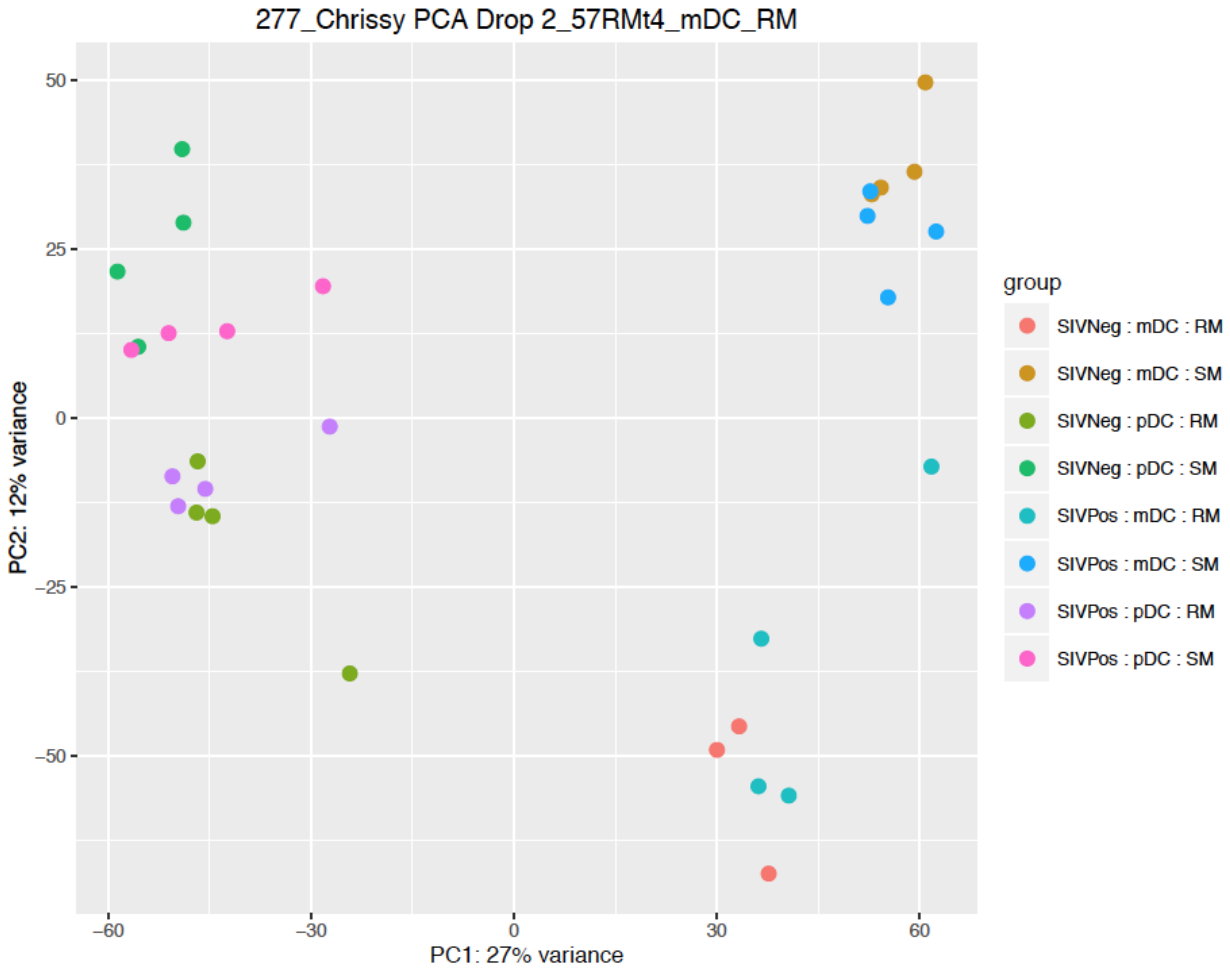

Figure 12 | RM Display variance in gene expression due to infection We applied principle component analysis (PCA) on the correlation matrix of the 31 samples (mDC sample collected from RMt4 was dropped from the analysis because it significantly out lied all other samples). The graph shows the relative position of the 31 samples in the 2D space formed by the first and second principle components. The plot shows good separation between pDCs and mDCs along the first principle component and between RM and SM along the second principle component.

Gene Expression Profiles of pDCS

We evaluated overall gene expression between SIV- and SIV+ animals (**Fig 13**).Genes were documented as having a significant fold change (p-value<0.05) between the uninfected and chronically infected animals of a particular species (calculated on DESeq2) and had a normalized read count base mean of at least 50. The genes with significant changes in expression were visualized through heat maps. Heat maps indicate fold change from the median through distinct color shading of a bi-lateral system. Furthermore, the familial hierarchy of genes can be indicated on the axis (Haghverdi, Buettner, & Theis, 2015). For our data, we indicated up or down regulation of genes from the median calculated by DESeq2 through a blue and red system.

Overall, we found a greater number of genes with significant expression changes due to infection status in RM in comparison to SM. Of the genes with expression changes in SM a greater percentage were down regulated in SIV+ animals (**Fig 13b**). On the other hand, approximately half of the changes in gene expression were down regulated in SIV+ RM (**Fig 13a**).

Of the genes that were significantly up or down regulated in SIV+ RM, only six had significant fold changes in SIV+ SM as well (when comparing to their respective SIV- condition), suggesting that majority of the genes that are changing expression levels due to infection status are unique to the species. One of the many family of genes found to have a change in gene expression is the interferon-stimulated genes (ISGs).

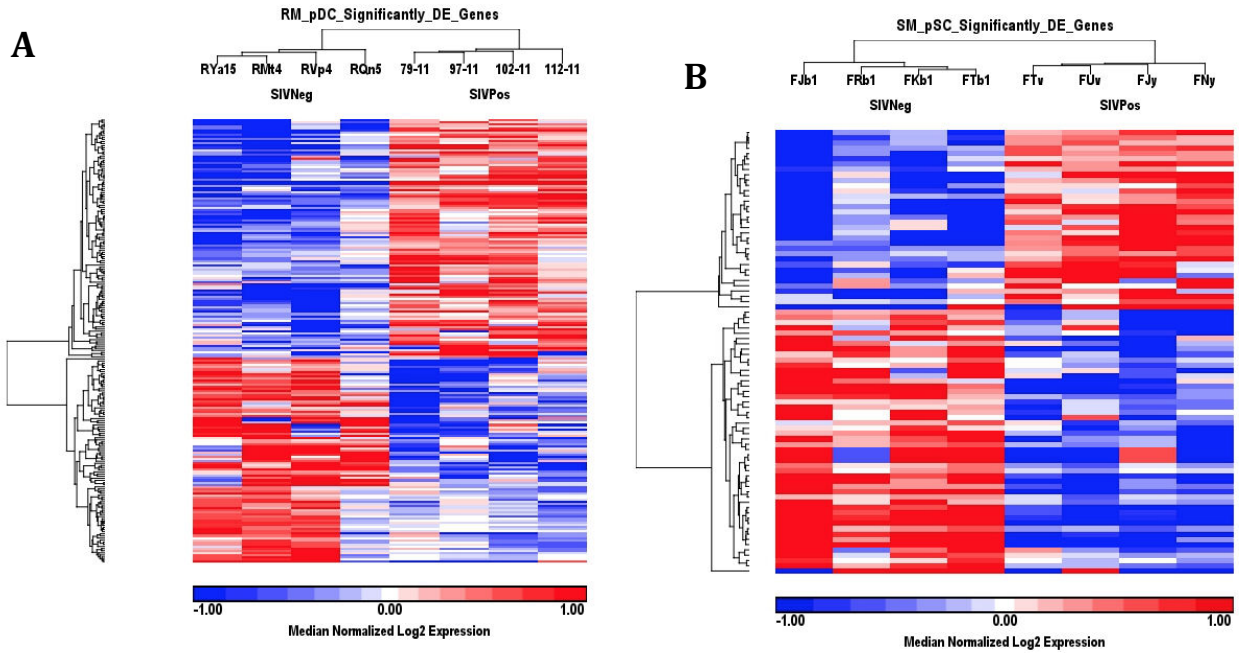

Figure 13 | Gene-expression profiles in pDCs of SIV infected and uninfected SMs and RMs. Heat map of genes up or down regulated in pDCs between SIV uninfected and chronically infected RM (**A**) and SMs(**B**). Significant changes in gene expression were defined by $p<0.05$ and normalized read count>50. Individual colored panels represent the fold change. The color scale is indicated at bottom.

Up regulation of ISGs in pDCs from RM, but not SM in chronic SIV-infection

We evaluated normalized read counts for several known ISGs in all four conditions and compared across infection status within species (**Fig 15**). Overall, we found the majority of ISGs were up regulated (positive fold changes) in SIV+ RM. On the other hand, the majority of ISGs expressed almost no up regulation (positive fold changes) in SIV+ SM. We found that SM gene expression remained relatively constant between SIV- animals and SIV+ animals.

Of the 19 ISGs, only four have a log base 2 fold change below 1.00 and eight have a fold change greater that 2.00 in RMs. While, only three genes have a fold change greater than 1.00 for SMs, suggesting a consistent up regulation across ISGs for SIV+ RM and relatively no up regulation for SIV+ SM. Of all the ISGs, interferon alpha-inducible protein

27 (IFI27) had the greatest up regulation in SIV+ RM with a fold change >7.00. IFI27 mediates IFN induced apoptosis via activation of caspase.

This coincides with previous studies that suggest that IFN-α responses decrease after acute infection in natural hosts (S. E. Bosinger et al., 2009). It suggests that during chronic infection ISGs are not activated for SM, while RMs have ISGs activated during chronic infection.

No Significance in expression of Inhibitory genes or Restriction factors

We evaluated normalized read counts for inhibitory genes: lymphocyte-activation gene 3 (LAG3) and programmed cell death (PDCD1). We compared counts across infection status within the same species. We found some down regulation in SIV+ SM, but it was not significant in accordance with the DESeq2 p-value (**Fig 14**). We found almost no change in gene expression in SIV+ RM. Overall; our data suggested no significance in expression of inhibitory genes due to infection status. In previous studies, the up regulation of inhibitory genes have been a major factor in HIV or SIV pathogenesis. Although, the up regulation shown in previous studies looks at overall leukocyte expression, which contains CD4+ T cells (Huson et al., 2016)

We evaluated normalized read counts for restriction factors as well: apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3) family (**Fig 16a**), bone marrow stromal cell antigen 2 (BST2) (**Fig 16b**), and tripartite motif containing 5 (TRIM5) (**Fig 16c**). We compared counts across infection status within the same species. We found no significant changes in gene expression across infection status in either SM or RM. We found no trend for up or down regulation in SIV+ animals across the APBEC3 family, BST2

or TRIM5. This is consistent with literature on pDC proteins during HIV-1 infection. Restriction factors, although stimulated by IFN-I, have been found to lack any upregulation in the blood of chronic HIV or SIV patients (Fernandez-Oliva et al., 2015; Zhu, Liu, Mu, Deng, & Zheng, 2016).

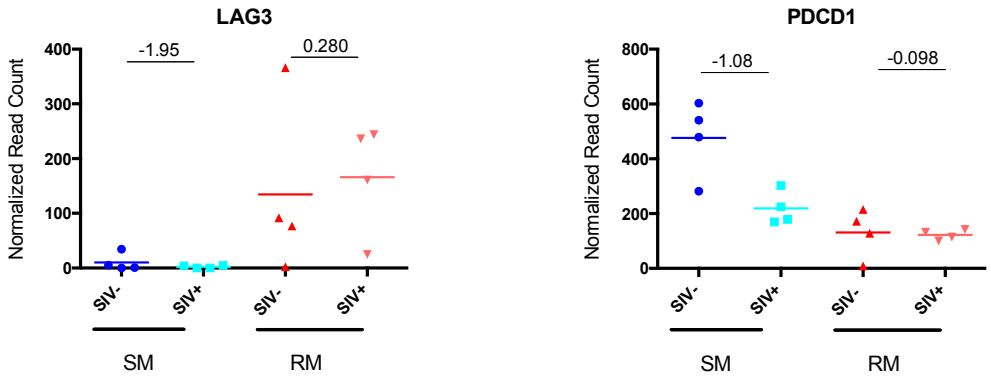

Figure 14 | No significance in expression of inhibitory genes between SIV+ and SIV- animals Expression assessed by RNA sequencing (RNA seq) of Inhibitory genes: LAG3 and RDCD1 in pDCs for SIV- SM, SIV+ SM, SIV- RM, SIV+ RM. Numbers on graphs correspond to log base 2 fold change between SIV- and SIV+ animals.

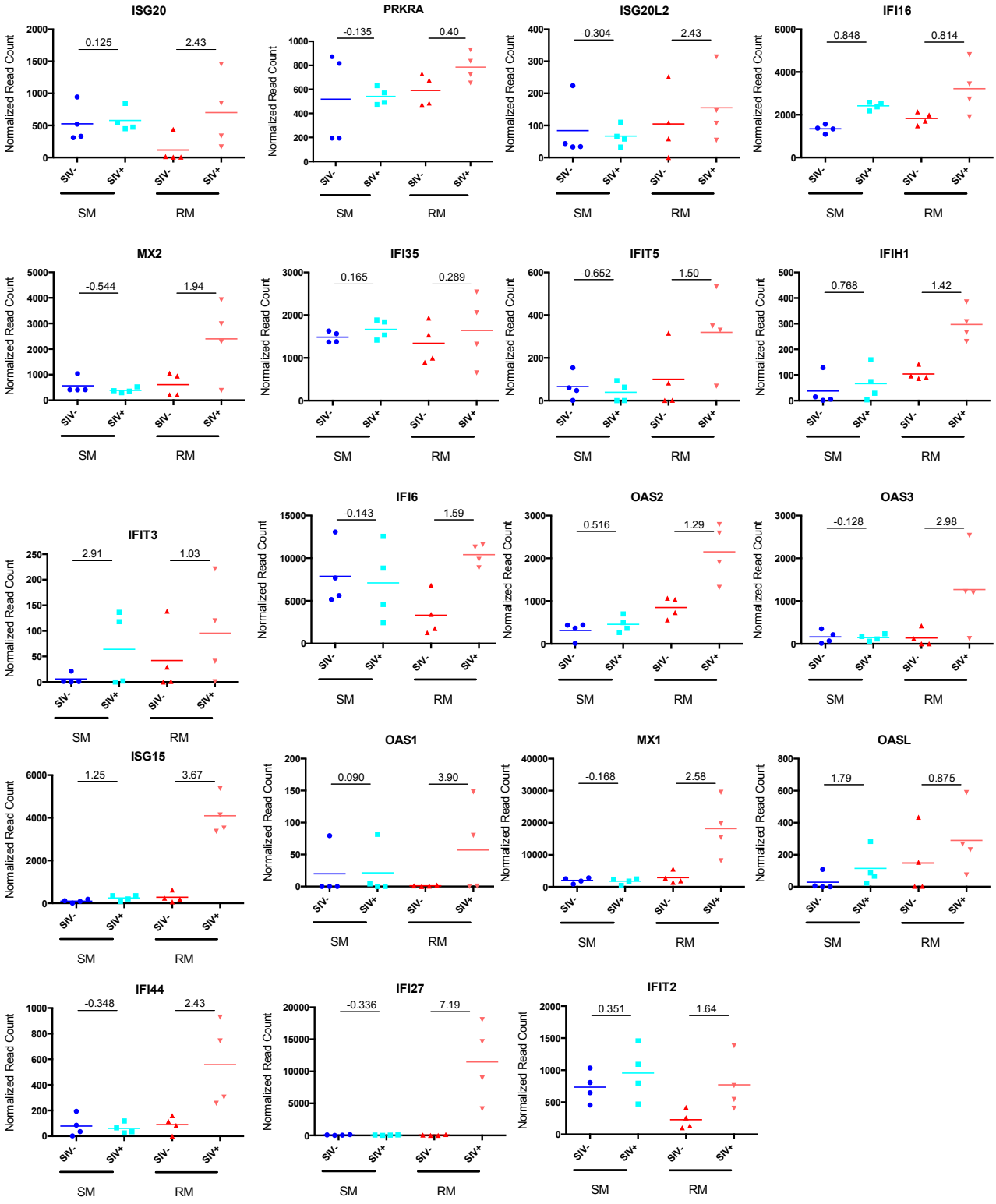

Figure 15 | Up regulation of ISGs in SIV+ Rhesus Macaques Expression assessed by RNA sequencing (RNA seq) of Interferon Stimulated Genes (ISGs) in pDCs for SIV- SM, SIV+ SM, SIV- RM, SIV+ RM. Numbers on graphs correspond to log base 2 fold change between SIV- and SIV+ animals.

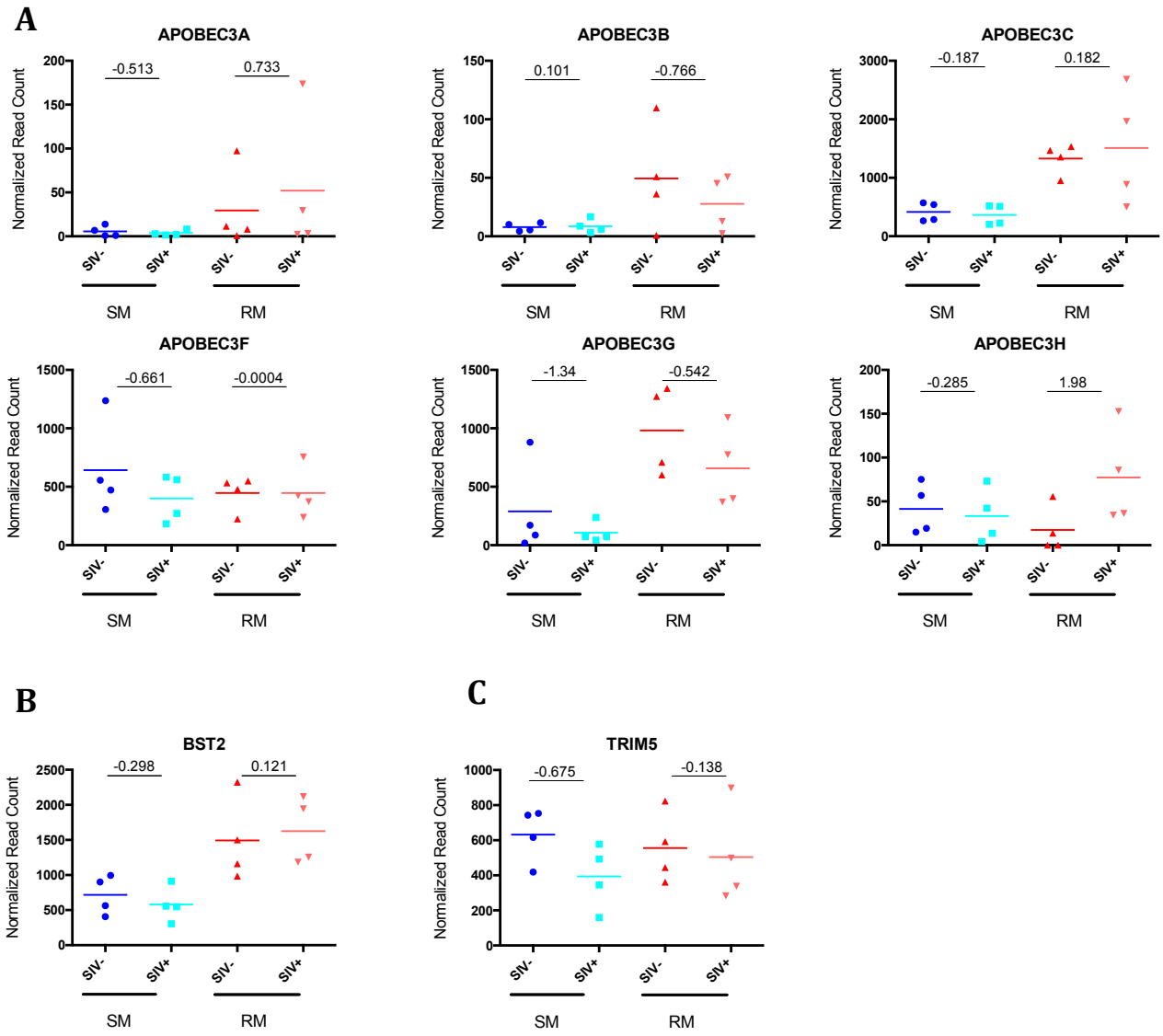

Figure 16 | Overall no significance in expression of restriction factors between SIV+ and SIV- animals
Expression assessed by RNA sequencing (RNA seq) of restriction factors: APOBEC3 family (**A**), BST2 (**B**), and TRIM5 (**C**) in pDCs for SIV- SM, SIV+ SM, SIV- RM, SIV+ RM. Numbers on graphs correspond to log base 2 fold change between SIV- and SIV+ animals.

pDCs from the blood lack IFN-α expression across species and infection status

We found no interferon-α (IFN-α) expression across samples. We did see high read counts for interferon receptors (IFNAR1/2). This suggests that pDCs in the blood do not express IFN-α and IFN-α production may occur somewhere else in body during infection.

The lack of IFN-α correlates a recent study of pDCs in the blood that found ISG upregulation in the pDCs, but not IFN transcripts (Sabado et al., 2010). Our data can neither confirm nor negate previous literature about pDC's IFN role during chronic infection as further work within all major tissues for the immune system must be evaluated (S. E. Bosinger et al., 2013; G. Li et al., 2014)

Discussion:

Since HIV was first discovered in humans, the scientific community has been trying to understand how HIV causes immunodeficiency and AIDS. SIV infection of rhesus macaques (RM) shares similar hallmarks with HIV infection and therefore has become the most common non-human primate model (Murphey-Corb et al., 1986). In contrast to HIV and SIV infection in humans and RM respectively, natural host species such as sooty mangabeys (SM) do not develop pathogenic SIV infection despite high viremia (Murphey-Corb et al., 1986). Therefore, comparative analysis between the two non-human primate models, RM and SM, has become a key component in understanding the differences in control mechanisms used during SIV-infection. Human HIV and RM SIV chronic infection are correlated to elevated CD8+ (cytotoxic) T cells levels and rapid turnover of CD4+ (helper) T cells causing immune system exhaustion (Silvestri et al., 2003). In contrast SM SIV chronic infection lacks immune activation (Silvestri et al., 2003). Thus, our current hypothesis is that SIV pathogenesis is a direct result of "over-activation" of the immune system that is not found in SM and other natural hosts (Silvestri et al., 2003). Since the publication 2003, the community has come to a general consensus to accept said model for

pathogenesis, but how the SM are able to regulate the immune system after infection and what cells are the cause of the over-activation in RM is still and issue.

The attenuated immune activation in chronically SIV-infected SM may occur as a result of rapid down-modulation of the robust innate immune activation observed during acute SIV-infection (S. E. Bosinger et al., 2009). Interferon-α (IFN-α) is a key antiviral mechanism of the innate immune system and its overall activation. The exact role of IFN-α in SIV-infection remains an issue. It has been found that during acute SIV-infection, the IFN-α contributes to the control of the initial infection (Sandler et al., 2014). On the other hand, during chronic infection consistently high IFN-α levels have been correlated to chronic immune activation that results in CD4+ T cells loss and immune exhaustion (Vanderford et al., 2012). Therefore, IFN-α has been correlated with pathogenesis of SIV-infection in non-natural hosts (Cao & Liu, 2007). During acute infection, it is likely that plasmacytoid dendritic cells (pDCs) are responsible for the production IFN-α, suggesting they may also be responsible for the IFN-α levels during chronic infection although, whether another population is responsible for IFN-α production as circulating pDC levels wane as the populations infiltrate mucosal and lymphoid tissues is still being investigated (S. E. Bosinger & Utay, 2015; G. Li et al., 2014).

Using a non-human primate model we investigated gene expression differences in pDCS from the blood between non-pathogenic (SM) and pathogenic (RM) SIV-infected animals. In order to search for possible mechanisms by which SM curb the innate immune system during chronic infection, we compared gene expression of pDCs between chronically SIV-infected animals to uninfected animals within the respective species.

Therefore we searched for significant regulation of gene expression as a result of infection status.

Critical analysis of gene expression regulation has been hampered by the difficulty of isolating enough pDCs from non-human primate models as well as finding cross-reactive antibodies in order to isolate the cells. With RNA sequencing (RNA-Seq) we were able to provide clear reads even from relatively low cell counts. BDCA-2, a protein uniquely expressed on pDCs has become a useful tool for sorting pDCs via flow cytometry in RM (Dzionek et al., 2000). Unfortunately the antibody anti-BDCA-2 has a poor cross reactivity with SM and therefore cannot be used to select for these cells. Our data shows high levels of BDCA-2 expression across species, while mDC expression levels remain at baseline. Therefore, our data allows us to define pDCs by the presence of BDCA-2 in both SM and RM and bypassing the need for cross-reactive antibodies.

We were able to stipulate the cell subsets and species via gene expression levels acquired from RNA-Seq. As depicted by the principal component analysis (PCA), we were able to find distinct clusters for SM pDCs and RM pDCs. The PCA also depicts separation, due to transcriptomic differences, between uninfected and chronically SIV-infected animals for RM, but not SM. This correlates with previous literature that states the up regulation in the immune system of natural host species returns to base line expression within the first weeks of infection (S. E. Bosinger et al., 2009). The separation seen between uninfected and infected RMs may be a direct result of chronic innate immune activation for pDCs in the case of pathogenic SIV-infection.

Not only were we able to distinguish species based upon the spatial separation in PCA, but through the identification of the presence of CCR5. In previous literature, CCR5

has been found to be down regulated in SM as a result of base pair mutations; suggesting SIV infection in SM relies on CCR5-independent entry pathway (Riddick et al., 2010). On the other hand, RM expresses CCR5 on pDCs as corroborated by our data (Jochems et al., 2015). Furthermore, CCR5 and CD4 are co-receptors for HIV and SIV infection and their presence on pDCs establish the conditions necessary for SIV infection (Tsao, Guo, Jeffrey, Hoxie, & Su, 2016). It still remains unknown whether pDCs are infected by SIV, but our data establishes the possibility for infection. Future work with the pDC population should examine the presence SIV DNA in pDCs and more specifically SIV DNA integration in order to establish whether or not pDC populations are infected.

When confirming the identity and purity of the pDC aliquots, interestingly we found no IFN-α gene expression in the sorted pDCs across species and infection status. This could be due technical error or it is accurate to the biology of pDCs in blood. If the lack of IFN-α expression is due to a technical error it may be that the IFN-α's were lost during the process or our mapping may have been missed them. In relation to the first idea, it is fairly improbable that across all samples only IFN-α transcripts were lost to processing, especially since all other gene transcripts we expected to find were present and fairly distinct to their population (**Fig 12a**). The algorithm mapping each gene is fairly complex, but we have used the algorithm for IFN transcripts in previous studies and were able to identify them (S. E. Bosinger et al., 2009). In a study in 2010, they looked at pDCs in the blood of chronically infected HIV patients and found ISG upregulation, but could not find IFN-α transcripts (Sabado et al., 2010). Therefore, it is more likely that the lack of IFN-α expression is a biologically true phenomenon of pDCs in the blood. There are two possible

biological explanations: pDCs are not the IFN-α producers during chronic infection or the pDC populations in other tissues are responsible for the IFN-α production during chronic infection. Although some cells have the capability to produce IFN-α, pDCs produce >99% of the IFN-α during acute infection (Doyle et al., 2015). In addition, during chronic SIV infection pDCs infiltrate mucosal and lymphoid tissues (H. Li, Gillis, Johnson, & Reeves, 2013). Therefore, it is possible that pDCs in gut or lymphoid tissues are the IFN-α producers during chronic infection instead of another cell subset. Further sorting and RNA sequencing (RNA-seq) studies in the gut and lymphoid tissues during chronic SIV infection will allow us to determine if pDCs are the IFN-α producers during chronic infection. Rectal mucosa collection of sequencing will be of utmost importance as pathogenic SIV-infection in RM has been associated with varying levels of damage to the gut, therefore it is more likely that IFN-α gene expression during chronic infection is located in the gut tissue of RM (Ponte et al., 2016).

Beyond identification of specific subsets we performed DESeq2, an analytical comparative analysis for read counts from RNA-Seq data that allowed us to identify significantly regulated genes between uninfected and chronically SIV-infected animals (M. I. Love, W. Huber, & S. Anders, 2014). We compared gene expression almost exclusively within species. We evaluated significant differences of between the species reactions to SIV infection through evaluating log base 2 fold changes. This was to eliminate any errors in significant expression changes between the species as a direct result of alignment issues for SM due to the reference genome being from a RM. Overall; we found a greater percentage of up regulation in gene expression between infection statuses for RM compared to SM. For

RM, DESeq2 found 215 genes with significant difference in gene expression between infection conditions. For SM, the analytical model only found 85 genes with significant difference between infection conditions. This suggests that there is a greater difference in gene expression due to infection status for pDCs in RM than those in SM. Further analysis of significant regulation needs to be conducted in determining specifically what kinds of genes are being up or down regulated in chronically SIV-infected animals. In addition, there were only six genes found to have significant changes in gene expression for both species. This suggests that the mechanisms used to regulate and/or activate the innate immune system due to chronic SIV-infection are distinct between species. Thereby, corroborating with much of the literature that natural host species are unique in their mechanisms for avoiding SIV pathogenesis in comparison to pathogenic infection (S. E. Bosinger et al., 2009). RNA-seq and DESeq2 analysis of other natural hosts, such as African green monkeys (AGMs) could provide us with insight on whether on the not the mechanisms for avoiding SIV pathogenesis is unique to each natural host. Such information will provide insight on whether or not we can create a broad treatment against the pathogenesis of HIV. If the innate immune system regulation is found to be distinct between SIV strains, this would suggest that treatment must be catered to the specific strain of HIV infecting the patient.

Several families of genes were looked at in closer detail for regulation of gene expression. Of most significance were the interferon-stimulated genes (ISGs). ISGs are primarily related by their antiviral activities and the fact that they are turned on by the IFN-α and β cytokines. ISGs are responsible for restricting the virus replication, enhancing pathogen associated molecular patter (PAMP) detection, and IFN signaling/amplification (Schoggins et al., 2011). ISG expression during acute infection combats the virus, but during

chronic infection the persistence of the innate response has cytopathic effects (G. Li et al., 2014). ISG expression has been detected in SIV-chronically infected RM, but not in SM after acute infection (Vanderford et al., 2012). Our data found that across all ISGs we have a trend of significant up regulation in chronically infected-SIV RM, but see no significant changes in gene expression for infected SM, therefore corroborating with the postulate that the natural hosts avoidance of SIV pathogenesis is a result of attenuated innate response. It is quite likely that the attenuated innate immune system stems from the ability of natural hosts to shut off the IFN production after the acute stage of infection (S. E. Bosinger et al., 2009). In other words, there is an IFN producer in chronically SIV-infected RM that continues to express IFN transcripts past the acute infection. Whether pDCs are the IFN producer remains to be seen until RNA-seq is performed on gut and lymphoid tissue. Our data helps solidify our current understanding of HIV/SIV pathogenesis that the immune system, in our case innate, is over activated during pathogenic infections. This may cause the exhaustion of the immune system that leaves it vulnerable to opportunistic infections (Silvestri et al., 2003).

Although we found significant up regulation in ISGs for RM, we found no significant change in gene expression due to infection status in either RM or SM for inhibitory genes or restriction factors, therefore suggesting the difference in chronic SIV-infection pathogenesis is not the result of either gene family. Restriction factors are induced early on in the infection and limit viral replication during entry, reverse transcription, nuclear entry/integration, transcription and budding (Stremlau et al., 2004; Van Damme et al., 2008). Although, we looked at several of the restriction factors, we have yet to search for significance in gene expression for SAMHD1, a protein that depletes nucleotides available

for reverse transcription or tetherin, a protein that interferes with virion release (Neil, Zang, & Bieniasz, 2008; Zhang, Bloch, Nguyen, Kim, & Landau, 2014). Therefore, we cannot fully state that restriction factors are not correlated to the pathogenesis of the virus, but that the pathogenesis may not be a direct result of over activated restriction factors (Doyle et al., 2015).

In conclusion, the data presented here demonstrates that pDCs may play role in the pathogenesis of SIV/HIV in non-natural hosts. Specifically, RM has an upregulation in ISGs in pDCs during chronic SIV-infection while SM do not despite similar viral loads. These data adds to the overall understanding that pathogenic SIV/HIV infection is a result of the constant activation of the innate immune system during chronic infection and that non-pathogenic natural hosts have developed mechanisms to shut off the innate immune system activation. Therefore, natural hosts are able to survive with high viral loads due to their ability to shut off the innate immune system. Furthermore our data, asks the question: are pDCs still the major producers of IFN-α during chronic infection? Since we were not able to find IFN-α transcripts in pDCs in the blood, the next step becomes sorting out and RNA-sequencing pDCs in other tissues, such as the gut during chronic infection. We believe that these findings provide a solid premise for future studies aimed at defining the molecular mechanisms by which the innate immune system responses to SIV are being down-modulated in natural host species despite the ongoing viral replication. Ultimately, it is hoped that these advances may help design interventions that target the chronic innate immune activation that is associated with HIV infection of humans.

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