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Plasmacytoid dendritic cell function in pathogenic vs. non-pathogenic HIV and SIV infection

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Plasmacytoid dendritic cell function in pathogenic vs. non-pathogenic HIV and SIV infection

by

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DISSERTATION

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DEDICATION

This manuscript is dedicated to my husband, James Botelho, whose unwavering love, support, and pride in my efforts was essential to completion of the work contained herein, and to my mother, Judith Cohen, who was my first and most important example of academic and professional success, and whose unwavering belief in my abilities was an invaluable resource during the last five years.

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Plasmacytoid dendritic cell function in pathogenic vs. non-pathogenic HIV and SIV infection

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ABSTRACT

The role of plasmacytoid dendritic cells (pDC) in HIV disease has been studied extensively but a clear consensus on the impact of pDC function on disease progression has not been reached. This thesis studied cases of pathogenic and non-pathogenic lentiviral infection in humans and non-human primates. Pathogenic HIV infection in humans and SIV infection in non-natural hosts is associated with a generalized, nonspecific immune activation and inflammation which persists during chronic infection and is associated with a progressive decline in CD4+ T cells, leading to immune deficiency and eventually development of AIDS. By contrast, nonpathogenic HIV and SIV infections are characterized by limited bystander immune activation and non-specific inflammation.

Plasmacytoid dendritic cells (pDCs) are an essential component of the innate immune response to viral infection and help to shape the innate and adaptive immune antiviral response. Multiple studies point to the possibility that pDC number and/or function may be different in the setting of pathogenic vs. non-pathogenic HIV and SIV infections. pDC functionality consists of maturation into antigen-presenting cells (APCs), secretion of type-I IFNs (most notably IFNα), and, as more recently characterized, production of the immunosuppressive enzyme, indoleamine-2,3-deoxygenase (IDO). All of these

functions have discreet effects on the development of the antiviral immune response, and all can be induced through stimulation across various receptors expressed by pDCs.

Because of the wide array of pDC functionality that exists, the potential for important differences in pDC function in pathogenic vs. nonpathogenic infections in humans and non-human primates are considerable. This thesis work examined whether intrinsic differences in pDC function (possibly influenced by genetics) might be associated with differences in HIV disease status. This work also examined whether intrinsic differences existed in pDC function between natural and non-natural hosts of SIV infection, who undergo non-pathogenic and pathogenic infection courses, respectively, and are thought to be a good model for pathogenic vs. non-pathogenic HIV infection in humans. Overall, this work concludes that while significant differences exist in pDC function between natural and non-natural SIV hosts, few, if any, significant differences exist in pDC function between HIV+ human patients with different disease outcomes.

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INTRODUCTION

The human immunodeficiency virus (HIV) is a significant source of morbidity and mortality throughout the world. After infection with this virus, most patients experience a loss of CD4+ T cells, leading eventually to the development of the acquired immunodeficiency syndrome (AIDS). Notably, however, some HIV-infected patients do not show loss of CD4+ T cells and do not progress to AIDS, and thus can be characterized as having a non-progressive/non-pathogenic infection. One group of individuals, identified in the 1980s and known as long-term non-progressors (LTNPs), is defined immunologically and clinically: as having been infected with HIV for more than ten years without developing AIDS, in the absence of highly-active antiretroviral therapy (HAART).[1] Another group of individuals identified more recently and known as elite controllers, is defined virologically; as having been infected with HIV but able to suppress circulating virus to undetectable levels in the absence of therapy. Although LTNP and elite controllers are relatively rare, individuals in both groups have been found within the SCOPE cohort (established by Drs. Steve Deeks and Jeff Martin) at San Francisco General Hospital, and are available for analysis. It is important to note that because elite controllers are defined virologically rather than immunologically or clinically, there is a small percentage of these individuals who do experience CD4+ T cell decline and who even progress to AIDS, despite their effective suppression of viral loads. Further complication is introduced by the fact that heterogeneity exists between individuals in each group. For example, elite controllers fall into two groups based primarily on their levels of HIV-specific polyfunctional (IL-2+IFNγ+) CD8+ T cells: "T cell controllers" with high levels of such cells, and "non-T cell controllers" with levels

that are low to undetectable. Individuals with so-called "protective" HLA alleles (primarily HLA-B57) tend to be significantly enriched amongst individuals who are classified as T Cell controllers within the elite controller cohort.[2]

Parallels can be seen in the commonly-used non-human primate models of simian immunodeficiency virus (SIV) infection. SIV infections in natural hosts, such as the Sooty Mangabey (SM), African Green Monkey (AGM), and Chimpanzee are generally non-pathogenic and non-progressive despite high levels of viral replication. In these animals, bystander immune activation and generalized inflammation tend to be limited. By contrast, SIV infection in non-natural hosts, such as the Rhesus Macaque (RM) and Pigtail Macaque (PM) is pathogenic and generally leads to progressive loss of CD4+ T cells and development of a syndrome that closely resembles AIDS in humans.[3]

In both HIV infection in progressor humans (vs. LTNP) and SIV infection in non-natural (vs. natural) hosts of SIV, we see disease which is pathogenic and hence progressive towards immune depletion and the eventual development of AIDS, and that which is non-pathogenic and allows the host to not progress to AIDS. The former, pathogenic case, is associated with progressive loss of CD4+ T cells, one of the hallmarks of AIDS, whereas in the latter nonpathogenic case, CD4+ T cells are maintained. A great deal of study has been devoted in the field of HIV and SIV research to attempting to characterize the differences in the immune responses associated with these two very different disease courses. While a great deal remains to be resolved in characterizing the differences in these two outcomes, several important factors have become clear.

One key determinant of HIV or SIV disease progression and CD4+ T cell loss is immune activation.[4] The exact mechanism by which CD4+ T cells are lost as a result of immune activation is not entirely clear, although several hypotheses have been proposed, including higher levels of apoptosis in CD4+ T cells through activation-induced cell death (AICD) or other forms of so-called "bystander activation." This bystander activation is further thought to perturb the steady states of resting and naïve T cell populations, leading to a disruption of the homeostatic processes by which depleted T cell populations would otherwise be lost.[5] Interestingly, the effects of T cell activation on CD4+ T cell levels seem to persist even in the setting of effective viral suppression via HAART. A recent study found that higher levels of T cell activation, as measured by CD38 expression on T cells, corresponded with lower levels of CD4+ T cells in individuals undergoing treatment, and also corresponded with a slower rate of CD4+ T cell recovery during treatment, indicating that whatever mechanism links T cell activation and CD4+ T cell depletion is still active in HAART-suppressed patients and therefore is not dependent on the presence of high viral loads.[6]

Another hypothesis suggests that loss of CD4+ T cells in the circulation may be due to recruitment of these cells to secondary lymphoid tissues (especially the gut), e.g. as a result of upregulation of CCR5 on their surfaces. Recruitment to lymphoid tissues, in turn, places cells in close contact with large amounts of virus when they are most susceptible to infection, leading to massive depletion, either through bystander activation and apoptosis in these tissues or through direct infection of activated CD4+ T cells, as

well as significant damage to these tissues and their functionality.[7] Studies have demonstrated that damage to lymphoid tissues in HIV-1 infected humans includes collagen deposition and fibrosis in these tissues which disrupts their functional architecture, and that the degree of fibrosis is associated with loss of CD4+ T cells and most strongly with loss of naïve CD4+ T cells, suggesting that destruction of lymphoid tissue architecture may be another contributing factor in CD4+ T cell loss and HIV pathogenesis.[8] There is also evidence to suggest that T cell production is impaired during HIV infection, e.g. as a result of HIV infection or indirect destruction of multilineage and lineage-restricted hematopoietic prgenitor cells in the bone marrow, thymus, and lymph node.[9] None of these hypotheses are mutually exclusive, and indeed, all probably contribute to the depletion of CD4+ T cells as a result of immune activation in HIV and SIV infection.

However it occurs, the profound loss of CD4+ T cells during pathogenic HIV and SIV infections is clearly an important correlate of progression to AIDS, and clinical data supports the idea that the overall level of immune activation is determinative of the rate of CD4+ T cell loss. During primary infection with HIV, an immunologic activation set-point, as determined by CD38+ expression on CD8+ T cells, has been shown to be established. This set point varies considerably among different patients, but remains fairly stable and constant within a given patient after about week 12 of infection. The level of activation represented by this set point is predictive of CD4+ T cell loss over time, independently of the level of viral load set-point.[10] Even in patients who effectively control viral replication to low or undetectable levels, relatively higher levels of immune

activation, again as measured by expression of CD38 on T cells, are associated with lower CD4+ T cell counts.[11] Further support of this paradigm is given by studies showing that activation levels on peripheral monocytes during primary HIV infection are also predictive of CD4+ T cell apoptosis levels and CD4+ T cell depletion in HIV infection.[12] Immune activation during the chronic stage of HIV infection is also associated with CD4+ T cell depletion and clinical progression, and is a better predictor of survival time than plasma viral RNA levels.[13]

In non-pathogenic infections of humans with HIV, both in the setting of treatment with anti-retroviral drugs leading to partial control of viremia,[14,15] or in the setting without anti-retroviral treatment where patients naturally do not progress clinically to AIDS (e.g. elite controllers and long-term non-progressors), a strong, polyfunctional T cell response to HIV seems to be important. These individuals have been shown to have higher levels of HIV Gag-specific IL-2+IFNγ+ CD4+ T cell responses and simultaneously lower levels of non-specific CD8+ T cell activation, as measured by CD38 expression on CD8+ T cells, compared to individuals with progressive disease.[16,17] Other work has further showed that polyfunctional CD8+ T cell responses are associated with lack of disease progression, independently of absolute CD8+ T cell numbers.[18] African species of non-human primates – natural hosts of SIV – such as the African Green Monkey (AGM) and Sooty Mangabey (SM) also undergo non-pathogenic infections with their native simian immunodeficiency (SIV) viruses. As is seen in nonpathogenic HIV infections in humans, these infections are associated with significantly lower levels of non-specific bystander

immune activation when compared to pathogenic infections seen in non-African primate species such as the rhesus macaque.[19]

While much remains to be determined about the differences in T cell responses and other parameters of the adaptive immune response in pathogenic vs. nonpathogenic HIV and SIV infections, still less is known about the role of innate immunity on disease progression in these two different types of infections. One specific aspect of the innate immune response is of particular interest to us: the role of the plasmacytoid dendritic cell or pDC. These cells have a unique role in bridging the innate and adaptive immune response against viruses. They are the principal producers of type I IFNs (including IFNα) within the immune system, and the only cells that produce significant amounts of IFNα under normal physiologic conditions. IFNα has powerful direct antiviral effects, serves as a positive feedback signal for further pDC activation, and also activates other cells of the innate immune system. pDCs, by example, are known to activate NK cells, myeloid dendritic cells (mDCs) and monocytes through a variety of effects mediated by their production of soluble factors (including IFNα) and also through direct cell contact-dependent mechanisms.[20]

Another important aspect of pDC functionality is that, upon appropriate stimulation, these cells can differentiate into mature dendritic cells with antigen-presenting capabilities. Activated pDC migrate to secondary lymphoid tissues, upregulate co-stimulatory molecules, and present antigens to CD4+, CD8+ and Treg cells. The relative capacity of pDCs to present to these different cell types can be strongly infuenced by the

cytokine milieu, thus allowing pDCs to have a profound effect in shaping the adaptive immune response to viral infection – pDC are known to induce either Th1 and/or Th2 responses, depending on the cytokine signals they receive.[21] pDCs also have the capacity to induce tolerance and Treg cells, and have been shown recently to mediate the induction of Treg cells and tolerance to vascularized grafts through presentation of alloantigens in the LN environment.[22] This tolerogenic functionality may be due in part to the recently-described capacity of pDCs to produce the enzyme indoleamine-2,3-deoxygenase (IDO).[23] IDO is fundamentally linked with Treg cell induction, in that Treg cells induce the expression of IDO in antigen-presenting cells (APCs), and those IDO+ APCs, in turn, promote the development of naïve T cells into Treg cells.[24,25,26]

Two of the most important receptors involved in pDC sensing of viral infection and subsequent determination of the pDC response to viruses are Toll-like receptors (TLR) 7 and 9. These receptors are the only two TLRs that are expressed by the pDC, and they recognize ssRNA (TLR7) and unmethylated CpG motifs (TLR9). It is important to note that while each of the functions above that are attributed to pDCs (IFNα production, APC functionality, and IDO production) can be induced by signaling through these TLRs, the pathway that leads to each is distinct and involves different signaling molecules within the cell. These signaling pathways are summarized in Figure 1.[27]

Due to the broad array of influences that pDCs can exert on the development of an anti-viral immune response, their impact on the host response to lentiviral infection is of interest. It remains unclear, however, whether their function contributes to a protective

response or, alternatively, to one that might drive disease progression. It was the intent of this thesis work to shed some light on this question.

Multiple lines of evidence suggest that pDC function can be "protective" against HIV disease progression. Certainly, it seems likely that pDCs could and probably do have profound effects on the levels of generalized immune activation as well as the development of specific anti-viral responses, the balance of which has been shown to be so important in dictating HIV and SIV disease progression. Levy and others have shown that LTNPs maintain higher levels of pDCs in their peripheral blood compared to patients that progress to AIDS, as well as compared to uninfected controls.[28] Reduced levels of peripheral blood pDCs have also been documented in primary HIV infection.[29] pDCs in the peripheral circulation of HIV-infected subjects with progressive disease were shown to have a reduced IFNα response to viral stimulation compared with those from healthy HIV-uninfected controls.[30] One important role of pDCs in HIV infection may be their ability to promote lysis of HIV-infected CD4+ T cells by NK cells: a recent study showed that pDCs and pDC-derived IFNα are essential for NK cell lysis of autologous HIV-1 infected CD4+ T cells. Impairment of pDC number and/or function may impair the effectiveness of an immune response to HIV by impairing the ability of NK cells to effectively lyse HIV-infected target cells.[31] This last is particularly interesting in light of recent literature showing that NK cells may play an important determinative role in disease progression, including via variable expression levels of various NK cell inhibitory and activating KIR receptors.[32] Most of the literature suggests that pDC number and

function are both able to recover, at least partially, following treatment with HAART.[33,34,35]

Counterposing the above data, there are also indications that IFNα secretion by pDCs and possibly activation and maturation of pDCs may not be beneficial in preventing HIV disease progression. Work in our laboratory showed that IFNα produced by pDCs in the thymus helped to promote the selection of dysfunctional CD8low T cells, which may contribute to the overall immune suppression usually seen in HIV infection.[36] Additional recent work has shown that production of IFNα in the Thy/Liv implants in SCID-hu Thy/Liv mice increases levels of CCR5 expression on Intrathymic T Progenitor Cells (ITTPs), which increases their vulnerability to R5-tropic HIV infection (Cheryl Stoddart, personal communication). IFNα has also been shown to induce the expression of CD38 on CD8+ T cells from HIV-infected individuals.[37] One recent study showed that pDCs isolated from the periphery of HIV-infected with progressive disease individuals had *higher* levels of intracellular IFNα compared to those with nonprogressive disease and to uninfected controls. Furthermore, levels of IFNα in pDCs in the lymphoid tonsillar tissue of chronically HIV-infected individuals with progressive disease were found to be significantly higher than those in pDCs in the lymphoid tonsillar tissue in chronically HIV-infected non-progressors and in uninfected controls. Based on information provided in this paper, the "non-progressors" appear to have been defined primarily based on virologic criteria and can therefore be considered elite, or at least viremic, controllers.[38]

The role of pDCs and specific aspects of pDC functionality in pathogenic vs. nonpathogenic SIV infection is also not clear, but some interesting lines of evidence have emerged. Several studies suggests that IFNα levels do not correlate with suppression of viral replication in lymphoid tissues of pathogenically infected macaques. Rather, higher levels of type I IFNs appear to be correlated with higher levels of viral mRNA in the macaque model of chronic pathogenic SIV infection.[39] By contrast, another study showed that during early acute SIV infection in the macaque, upregulation of IFNα was delayed at the mucosal site of infection and was minimal in the colon, which experienced dramatic depletion of CD4+ T cells during this time, while other proinflammatory cytokines such as IL-6 and TNFα were upregulated in mucosal lymphoid tissues, and extremely high levels of IL-8 and IFNγ were found to persist in the colon, the major site of CD4+ T cell depletion, while IFNα was never significantly induced in this tissue.[40] Furthermore, IFNγ-mediated inflammation in lymphoid tissues was found to be strongly associated with pathogenic but not nonpathogenic SIV infection in rhesus macaques.[41] On the other hand, IFNα mRNA levels in PBMCs in RMs undergoing non-pathogenic acute SIV infection were higher than those in RMs undergoing pathogenic acute infection.[42]

More recent work suggests pronounced intrinsic differences in the ability of pDCs to secrete IFNα between species that undergo pathogenic vs non-pathogenic SIV infections. This study found that pDCs from Sooty Mangabeys (a natural host of SIV) failed to secrete high levels of IFNα in response to *in vitro* stimulation with TLR7 and TLR9 ligands, while cells from rhesus macaques and humans did secrete large amounts of

IFNα.[43] This work suggests an intrinsic difference in the activation capacity of pDCs from natural and non-natural SIV host species, with average humans more closely resembling the latter. Indeed, this work reflected our own in this vein, and begged the question, which we tried to examine here, of whether those rare humans who do not undergo progression to AIDS as a result of HIV infection might resemble the natural hosts of SIV in certain key aspects of their immune function, particularly the capacity of the pDC to secrete IFNα.

It has been suggested that chronic hyper-activation of pDCs may contribute to the chronic immune activation seen in pathogenic HIV and SIV infections.[44] It is not entirely clear whether direct viral stimulation or other factors would cause this chronic stimulation of pDCs, but studies in which TLR9 ligands (CpG ODNs) were administered repeatedly to mice led to the development of symptoms in both the lymphoid tissues and the periphery of these mice which strongly resembled what is seen in pathogenic HIV infection, including disruption of lymphoid architecture, depletion of CD4+ and CD8+T cells, and upregulation of activation markers on T cells. Interestingly, this effect was at least partially mediated by IFNα, because the effects of the repeated CpG treatment were significantly more mild in mice lacking the IFNα receptor.[45]

The tolerogenic potential of pDCs may also play an important role in determining their effect on pathogenic vs. non-pathogenic disease outcomes. One component of pDC tolerogenic function that has been examined in the context of HIV infection is pDC expression of the enzyme indoleamine-2,3-deoxygenase (IDO). One study found that the

level of IDO mRNA was increased in pDCs from peripheral blood of HIV-infected individuals as compared to healthy controls. This study also showed that *in vitro* stimulation of pDCs with inactivated HIV induced IDO expression in the pDCs and inhibited CD4+ T cell proliferation. Blockade of IDO activity in this system increased the proliferative capacity of CD4+ T cells, suggesting that the IDO expressed by the pDCs was active and had a regulatory effect. In the setting of stimulation with either replication-competent or aldrithiol-2 (AT-2) inactived HIV, pDCs were the primary source of IDO mediating these effects.[46] IDO has also been shown to be associated with pathogenic SIV infection in non-natural hosts – in one study, IDO was increased in the tissues of rhesus macaques with higher vs. lower plasma viral loads during progressive SIV infection, although pDCs may not be the main source of this IDO.[47] Additionally, recent work showed that HIV-activated pDCs not only express IDO, but that they induce Tregs through an IDO-dependent mechanism, possibly pointing to a way in which pDCs may influence the Treg/Th17 balance in HIV infection.[48] The tolerogenic potential of pDCs is an important factor to consider in infections such as HIV, where the infection occurs through mucosal surfaces, as pDCs have recently been shown to mediate antigen tolerance via a CD4+ T cell-dependent mechanism in the oral mucosa.[49]

Despite these intriguing preliminary results the association of varying levels of IDO expression by pDCs on HIV and SIV disease progression has not been characterized. On the one hand, pDC-derived IDO might dysregulate T cell function and impair the development of an effective HIV- or SIV-specific adaptive immune response. On the other hand, the inhibition of CD4+ T cell proliferation shown to be induced by pDC-

derived IDO might be beneficial to the host because it might inhibit non-specific T cell proliferation, which is associated with worse disease outcomes.[50]

Due to the importance of the T cell response in determining HIV disease progression, it is also important to consider whether pDC functionality is directly correlated with T cell activation and/or HIV-specific polyfunctional T cell responses. Evidence on this point in the specific context of HIV infection is scarce, however, considerable literature exists which suggests that various aspects of pDC functionality can have profound effects on T cell responses. One study examined the direct effect that HIV-activated pDCs might have on T cell function, and showed that among other effects, Type I IFN secreted by HIV-activated pDCs causes upregulation of CD38 on CD4+ and CD8+ T cells.[51] Studies looking at other viral infection models have shown that pDCs are capable of priming influenza- and HBV-specific T cell responses, among others.[52,53] Furthermore, pDCs stimulated by TLR7 or -9 or viral ligands have been shown to effectively cross-prime naïve T cells in an *in vivo* context, which would be one likely mechanism for pDC control of T cell responses in HIV infection.[54] Another recent study definitively showed that pDCs interact directly with T cells in the traditional manner of antigen-presenting cells.[55] Specifically, these authors were able to show that immature pDCs form more transient interactions with naïve T cells while more mature pDCs form more stable contacts, thus additionally highlighting the importance of assessing pDC maturation/activation status when analyzing the potential affect of pDCs within the immune response.

OVERVIEW OF THESIS WORK

The above discussion and data underscore the counterposing effects that pDC may exert upon lentiviral infection. On the one hand, some reports indicate that pDC number and possibly function may be lower in progressive HIV disease and higher in a setting of non-progressive disease (Soumelis *et al* 2001 *Blood*). But some reports have shown elevated levels of peripheral and tissue-associated IFNα to be associated with more rapid HIV disease progression (Herbeuval *et al* 2006 *PNAS*). On the other hand, pDC functionality has been shown to be intrinsically reduced in species of non-human primates that undergo nonpathogenic SIV infections (Mandl *et al* 2008 *Nat Med*). Higher levels of IFNα mRNA expression were associated with protection from pathogenicity in at least one RM model (Abel K *et al* 2003 *J Virol*), but higher levels of IFNα expression in lymphoid tissues did not correspond with viral suppression and in fact seem to be associated with pathogenicity (Abel K *et al* 2002 *J Virol*).

Based on the above background, we hypothesized that strong pDC functionality, and particularly production of IFNα, is likely to contribute to pathogenic infection in humans and non-human primates, and that intrinsic differences in pDC function were likely to be associated with differences in disease outcome. A number of studies in the lab pointed to the possibility that intrinsic differences in pDC function could contribute to HIV and SIV disease progression:

1. IFNα production in the thymus results in upregulation of MHC I and the generation of dysfunctional CD8low T cells. (Keir *et al* 2002 *J Immunol* 169(5):2788-96, Keir *et al* 2002 *J Immunol* 168(1):325-31)

2. IFNα production in the thymus results in upregulation of CCR5 on intrathymic T progenitor cells, extending the infective range of HIV to this key progenitor cell population. (Cheryl Stoddart, personal communication)

3. pDC from LTNP fail to upregulate CD40 upon TLR9 stimulation. We observed this from work by the post-doctoral fellow Tim Beaumont looking at pDC activation in whole blood from HIV-infected individuals

4. Certain polymorphisms in the TLR9 locus appeared, in preliminary studies by David Williamson, to segregate disproportionately with progressors vs. elite controllers.

This thesis examined whether these preliminary findings could be confirmed and extended to evaluate whether intrinsic differences in pDC function are associated with HIV and SIV disease progression. To do this, I first optimized a number of assays and tools to carry out this work, which included protocols for *in vitro* stimulation of pDCs with TLR7 and -9 ligands, as well as other stimuli, as well as flow cytometry panels to identify pDCs and evaluate their upregulation of activation/maturation markers in both humans and non-human primates.

Optimization of pDC stimulation and detection protocols was especially challenging. Two members of the lab had worked on this problem before me – Post-Doctoral Fellow Tim Beaumont, who generated the intriguing preliminary results discussed above, and Clinical Fellow Brinda Emu. Unfortunately, Brinda and I were both unable to duplicate Tim's original, promising results, and in fact, found that using the methods he originally developed led to significant variability in our data. Thus, my first task as part of my thesis work was to develop a reliable and reproducible pDC activation assay and a corresponding flow cytometry panel that could be used to evaluate pDC function in large numbers of humans and non-human primates. This assay is described in the Materials and Methods sections of this thesis.

Once the methods had been optimized, I used them to evaluate pDC number and function in selected cohorts of elite controllers and patients with progressive disease whose viral loads have been suppressed by HAART ("suppressed"). HAART-suppressed patients, rather than those with progressive HIV disease were used for this study to eliminate the potential effects of the presence of virus that could confound any apparent intrinsic differences in pDC function. Peripheral blood from each of these groups was analyzed to examine the number and function of pDCs, in terms of expression of the activation/maturation markers CD40, CD86, and HLA-DR, as well as production of IFNα, and to determine if intrinsic differences in pDC number and function were associated with disease progression status. It appears, based on these studies, that this is not the case in humans, i.e. that intrinsic differences in pDC function do not differentiate

elite controllers from other HIV+ patients when controlling for the effect of virus in the periphery.

Additionally, expression of IDO by pDCs was evaluated in these two patient groups using intracellular flow cytometry. Similar to the functional parameters described above, IDO expression by pDCs was not found to be significantly different between the two patient groups. Additionally, significant heterogeneity did exist in pDC activation within each group, and so I also examined whether, despite the lack of difference in pDC function between the two groups studied, other factors might be associated with differences in pDC function, including treatment with specific anti-retroviral drugs, and specific parameters of T cell activation.

While I was conducting these studies, David Williamson was extending his findings with regards to genetics in overlapping patient cohorts, which was especially pertinent given the focus of this work on intrinsic differences in pDC functionality. This work led to the eventual characterization of genetic polymorphisms in two proteins known to be expressed by pDCs – TLR9 and the signaling molecule interferon regulatory factor 5 (IRF5). Analysis of elite controllers vs. non-controller HIV+ patients identified two single-nucleotide polymorphisms (SNPs), rs352139 G>A and rs352140 A>G, which were associated with non-control of viremia. These two SNPs in combination with two others, define three TLR9 haplotypes (haplotypes 1-3) in Caucasians. Of these, TLR9 haplotype 1 is associated with high viremia and increased loss of CD4+ T cells in HIV-1+ Caucasians, leading to more rapid HIV disease progression. These findings have

since been confirmed in the literature, in a study looking at a large cohort of HIV-infected Caucasians in Switzerland.[56,57] Thus, I evaluated whether expression of either of these TLR9 SNPs and/or expression of TLR9 haplotype 1 in Caucasian patients were associated with any difference in pDC functionality in HIV+ patients. This evaluation showed that no association existed between either the individual TLR9 SNPs or TLR9 Haplotype 1 and differences in pDC functionality.

Finally, I examined whether there might be intrinsic differences in pDC function between natural and non-natural hosts of SIV infection. Other recent work, completed while our studies were on-going, has suggested that this is the case, particularly with regards to IFNα secretion following TLR7 and TLR9 stimulation (Mandl *et al*). Using similar assays for pDC function as those described above in humans, my work showed that indeed, differences do appear to exist in intrinsic pDC functionality between natural and non-natural host species. Specifically, pDCs from non-natural hosts appear to have a higher capacity for activation, as measured both by IFNα secretion (as previously shown), and as measured by upregulation of activation/maturation markers. In my hands, this effect was particularly pronounced following stimulation across TLR9.

Given the above data, I also evaluated whether B Cells, the only other cell type known to express the same TLRs as pDCs, TLR9 and TLR7, would be similarly different between the two species, and indeed, B Cells from non-natural host species also showed stronger upregulation of activation markers following stimulation across TLR9. In these animals, I also looked at IDO expression in various cell types via intracellular flow cytometry, as

in the human samples. Consistent with other results obtained in our lab, IDO activity and expression in non-natural hosts appears to be much higher than that seen in natural hosts in the context of SIV infection. These results are described in detail in Chapter 3 (cite paper).

Taken together, this thesis then demonstrates that intrinsic differences in pDC function between HIV+ humans with differing disease outcomes appear subtle, if they exist at all, which suggests that they are unlikely to play a major role in determining disease progression. Additionally, genetic polymorphisms in human TLR9 and IRF5, while they are weakly linked with viral control status in HIV-infected humans, do not appear to be associated with significant differences in pDC function. By contrast, it appears, from this work and others, that pronounced intrinsic differences do exist in pDC functionality between species of non-human primates that are natural vs. non-natural hosts of SIV. Additionally, B Cell function appears to be significantly different in these two types of species, especially with regards to stimulation across TLR9.

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CHAPTER 2

PROLOGUE

As discussed in Chapter 1, the nature of the relationship between pDC function and HIV disease progression in HIV-infected human patients remains unclear. As part of this thesis, I attempted to shed additional light on this complex issue by comparing the number and function of pDCs between two HIV-seropositive patient subsets – the elite controllers, who naturally control HIV viremia in the absence of anti-retroviral therapy, and HAART-suppressed patients who had previously shown signs of disease progression but now had their viremia controlled through treatment with antiretroviral drugs. Our primary hypothesis was that these two patient groups would display intrinsic differences in the functionality of their pDCs. This hypothesis was based on both functional and genetic data from our lab and others, as outlined above. This work was submitted for publication as the attached manuscript, entitled "HIV-Seropositive 'Elite Controllers' Have Apparently Normal Plasmacytoid Dendritic Cell Function."

In addition to the published data, I examined one additional parameter of pDC functionality: the expression, and upregulation upon stimulation, of the immunosuppressive enzyme indoleamine-2,3-deoxygenase (IDO) by pDCs. IDO expression by pDCs was detected via intracellular flow cytometry. As with other measures of pDC functionality, I compared two HIV-seropositive patient groups, the elite controllers, and the HAART-suppressed. Similar to what was seen with other parameters of pDC function, no differences were observed between the two groups.

Given the considerable heterogeneity within the two patient groups with regards to pDC function, I also examined whether there might be other factors that might be associated with pDC function, that could either mask or explain any differences (or lack thereof) between and within the two groups. Within the HAART-suppressed group, I examined the possible effect that treatment with certain anti-retroviral drugs might have. This analysis showed that no significant differences in pDC number or function were associated with either treatment with a protease inhibitor or with the drug abacavir, both thought to cause increased immune activation and inflammation.[1,2]

The second analysis was designed to evaluate possible differences in pDC functionality within the elite controller cohort. As others have shown, significant heterogeneity exists in immune parameters measured in elite controllers, implying that there may be a wide range of mechanisms for durable control of viremia amongst these patients.[3] Recent work has shown that immune parameters such as nonspecific T cell activation can affect CD4+ T cell counts in HIV elite controllers,[4] and so I evaluated whether pDC function was associated with CD4+ T cell counts amongst the elite controllers and found that it was not. Next, I wanted to test the hypothesis that within the elite controllers, the "T Cell Controllers" might have lower pDC activation, while those who did not appear to control viral replication with a durable anti-viral T cell response might have higher pDC activation. To test this hypothesis, I looked at both expression of the "protective" HLA-B57 allele, enriched in T cell controllers, as well as polyfunctional CD8+ T cell responses to HIV, which had been previously shown to be associated with T cell control of viral

replication.[5] pDC activation status was not found to be associated with expression of HLA-B57 or with the presence of higher numbers of polyfunctional.

Next I looked at whether pDC function was associated with nonspecific T cell activation in elite controllers as well as in the entire patient cohort. This analysis was based on reports showing that various pDC functions can influence the activation of T cells, as discussed in Chapter 1. In a few cases, discussed in this chapter, higher pDC activation capacity was found to be correlated with lower levels of non-specific T Cell activation. While it is important to be cautious about reading too much into such results, this correlation does suggest that perhaps more active pDC function in the periphery helps to control nonspecific T cell activation.

See attached - manuscript entitled “HIV-Seropositive “Elite Controllers” Have Apparently Normal Plasmacytoid Dendritic Cell Function”

SUPPLEMENTARY MATERIALS & METHODS

Intracellular flow cytometry staining for IDO

Cells were stained as described above in the manuscript entitled "HIV-Seropositive 'Elite Controllers' Have Apparently Normal Plasmacytoid Dendritic Cell Function" for extracellular pDC markers. Following this staining, cells were washed twice, and then permeabilized using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Cells were then stained with intracellular antibodies as follows: rabbit anti-IDO primary antibody (Axxora, San Diego, CA) and Alexa-700 conjugated goat anti-rabbit (Molecular Probes). Samples were resuspended in PBS-plus medium following staining, and run on an LSR II flow cytometer. All data was analyzed using FlowJo software (Treestar).

SUPPLEMENTARY RESULTS

IDO expression not significantly different between elite controllers and HAART-suppressed patients.

IDO expression by pDCs was measured by intracellular flow cytometry. To compare the two patient groups, the baseline expression of IDO as well as the fold-change in expression upon stimulation were all examined. As seen in Figure S1, the expression of IDO, whether measured by the percentage of pDCs positive for IDO or by the mean fluorescence intensity, was not significantly different between the two groups. This was the case at baseline (Figure S1a-b) as well as following stimulation with various TLR and viral ligands (Figure S1c-d), suggesting that IDO expression by pDCs was not significantly different between the two groups. The fold-change measured in Figures S1e-f shows the change over baseline expression of IDO following stimulation with the indicated ligands, a measure of the activation capacity of the pDCs, which was also not significantly different between the two groups.

No differences in pDC number or function associated with anti-retroviral treatment

Given the evidence that anti-retroviral drugs, and especially protease inhibitors and the nucleoside reverse transcriptase inhibitor (NRTI) abcavir, are associated with generalized inflammation, we thought it made sense to examine whether HAART-suppressed patients within the cohort receiving one or both of these treatments might have differential pDC function. If true, such an effect could have masked any intrinsic differences in pDC function that existed between the two groups in the study, the elite controllers and the

HAART-suppressed, by altering the activation status and/or capacity of peripheral pDCs in some of the HAART-suppressed patients.

As shown in Figures S2 and S3, patients undergoing treatment with a protease inhibitor did not show differences in the number of pDCs per μL of blood (S2a), the production of IFNα (S2b), the baseline expression of activation/maturation markers (S2c), or the upregulation of such markers (S3a-e). Similarly, patients who were being treated with abacavir also showed no such differences compared to HAART-suppressed patients who were not being treated with abacavir (Figures S4-S5).

Elite controllers show no differences in pDC activation based on CD4+ T Cell count

Given recent work showing a correlation between immune parameters linked to disease progression – such as nonspecific T cell activation – and CD4+ T Cell count amongst the elite controllers, it made sense to ask whether pDC functionality might be associated with CD4+ T cell count in the elites included in this study. There were no significant correlations found between parameters of pDC function, including baseline expression and fold-change upregulation of activation markers, and CD4+ T Cell Count in the elite controller cohort. This data suggests that pDC function is unlikely to play a strong role in controlling CD4+ T Cell count in these patients.

Within elite controllers, "T Cell controllers" do not show differences in pDC function

Recent work has shown that polyfunctional CD8+ T Cell responses to HIV antigens, which in turn are associated with expression of the so-called "protective" HLA-B57 allele, can be used to define a subset of elite controllers who can be considered "T Cell controllers," in that they appear to use T cell responses to help control HIV viremia.[6,7,8] Given the apparent importance of T cell responses in these patients, it was logical to examine whether either the presence of the B57 allele and/or of polyfunctional CD8+ T cell responses specific for HIV might be correlated with differences in pDC function.

As shown in Figures S6 and S7, expression of B57 was not associated with significant differences in pDC number or function, including production of IFNα. Additionally, as summarized in Table S1, no trend in correlation was found between parameters of pDC function and the number of polyfunctional HIVgag-specific CD4+ and CD8+ T cells. These data suggest that pDCs are unlikely to play an important role in the apparent protection and T cell control of viremia that exists in the T Cell controllers within the elite controller cohort.

In some cases in elite controllers, higher pDC activation is associated with lower nonspecific T cell activation.

Since nonspecific T cell activation, traditionally characterized using the expression of CD38 on peripheral T cells, as well as more recently, Ki67,[9,10] has been shown to be associated with HIV disease progression, and HIV-activated pDCs have been shown to cause the upregulation of CD38 on T cells,[11] I decided to examine whether a correlation existed between pDC activation and measures of nonspecific T cell activation. Of note,

this association between nonspecific T cell activation and measures of disease progression has been shown in elite controllers (Hunt PW *et al*, 2008, *JID*), as well as in the general population of HIV-infected individuals. Therefore, I examined both the entire patient cohort as well as elite controllers in isolation.

As summarized in Table S2, the entire patient cohort in this study showed significant correlations between measures of nonspecific T cell activation and pDC functionality. However, as further summarized in Table S3, and illustrated in Figure S8, several statistically significant correlations did exist between measures of pDC activation, specifically across TLRs -7 and -9, and measures of nonspecific T cell activation amongst the elite controllers. Surprisingly, and contrary to our original hypothesis, higher capacity for pDC activation appeared to be associated with lower levels of nonspecific T cell activation in the periphery. This would seem to imply that perhaps having more highly functional pDCs in the periphery could help to control nonspecific T cell activation and thus, disease progression, at least in the elite controllers. This preliminary result would need to be confirmed in a much larger cohort to truly assess its significance, but this initial analysis indicates that such a line of inquiry might be worthwhile at some point in the future.

No apparent association between selected TLR9 and IRF5 genetic variations and pDC function

Neither of the two TLR9 SNPs discussed above (rs352139 G>A and rs352140 A>G) nor TLR9 haplotype 1 correlated with apparent differences in pDC functionality in White

American patients from the cohort included in this study. We searched for such correlations on both an individual SNP basis for rs352139A>G and rs352140G>A (Figure S9a) and as a function of TLR9 haplotype, where TLR9 haplotype 1 had been shown to be associated with more rapid disease progression in White Americans (Figure S9b). In both cases, we examined the fold-change in upregulation of activation/maturation markers on pDCs in response to stimulation across TLR9, with the expectation that if genetic differences in TLR9 drove functional differences, they would be seen following stimulation with TLR9 ligands. No such functional differences were observed, although that may have been due to relatively small sample size (only 29 of the total 52 patients included in the functional studies were classified as White Americans and thereby included in the analyses shown in Figure S9).

DISCUSSION

While the results of these analyses, taken together with the published work included in this chapter, seem to indicate that intrinsic differences in pDC number or function probably do not have a strong effect on control of HIV viremia, several important factors should be taken into account before drawing that conclusion. The first is the issue of the location of the pDCs in question. Some evidence suggests that when they are faced with HIV infection, pDCs may upregulate CCR7 and migrate to the lymphoid tissues,[12] where they may secrete large amounts of IFNα.[13] Recent evidence suggests that this migration is exactly what happens in a primate model of primary pathogenic SIV infection in a non-natural host species, with pDCs migrating to lymphoid tissues and expressing much of their functionality in those tissues.[14] If pDCs migrate to lymphoid tissues upon activation rather than remaining in the periphery, our results may be reflecting only a small percentage of the pDC population in one or the other of these patient groups and therefore may not capture underlying differences.

Another parameter to consider is the time during infection that was examined in this study. By definition, pDCs are part of the innate immune system, which acts early in infection. Therefore, the crucial time of pDC influence on the outcome and pathogenesis of HIV infection may, in fact, be very early on, at time points not captured in this study. One study examined HIV-infected inviduals during primary infection did conclude that pDC production of Type I IFNs is significantly impaired early in infection, although these authors did not examine the effects of such an impairment on eventual disease

progression and prognosis.[15] Recent work by Malleret *et al* suggests that this is certainly the case in SIV infection – these authors showed that pDCs played a critical role in shaping the immune response very early in a pathogenic SIV infection. Given the relative difficulty of studying the early events in human HIV infection, *in vitro* models of the relevant mucosal surfaces such as that recently developed by Bouschbacher *et al* may provide an important tool for examining the early events in HIV infection and the roles that pDCs might play in these events.[16]

Another possibility is that pDCs may exert their influence on disease progression more indirectly, and in a more heterogeneous manner than we originally hypothesized here. Evidence does exist that pDCs can activate virus-specific CD4+ and CD8+ T cells.[17] This suggests that perhaps not only might pDCs exert their influence earlier in infection than the time points examined here, but perhaps the effects are indirect, and are seen in the form of T cell activation. T cell activation has been definitely shown to correspond with HIV disease progression[18] and it is possible that pDC activation in turn corresponds with T cell activation. This correspondence could exist across a spectrum, rather than being strictly divided into two patient groups as described here, because, as Hunt *et al* clearly demonstrated, T cell activation levels vary considerably even within the elite controller cohort but higher levels are still linked to disease progression (Hunt *et al*, 2008, *JID*).

It is also important to note that the primate model upon which part of the original hypothesis was based is not a perfect reflection of HIV+ elite controllers in one crucial

respect: viral load. While natural hosts for SIV infection maintain relatively high viral loads,[19] HIV+ elite controllers suppress their virus. This suggests that these two examples may, in fact, be distinct immune paradigms, the one which achieves a kind of homeostasis with significant amounts of virus present in the body, and the other which avoids pathogenesis by suppressing viral loads by some mechanism that has not been fully characterized yet.

Additionally, it is possible that the functionality of pDCs themselves has not yet been fully characterized yet. Just recently, the capacity of pDCs to produce the immunosuppressive enzyme indoleamine-2,3-deoxygenase (IDO) when stimulated with AT-2 inactivated HIV was described by Boasso *et al*.[20] Perhaps this IDO-producing capacity, or some other, as-yet undescribed functionality of pDCs may be influential in determining disease outcome. It is also possible that some other cell type that also expresses TLR9 and/or IRF5 may drive differences in disease progression between these two patient groups. Finally, it is possible that anti-retroviral drugs themselves may exert a direct influence on pDC function. While such an effect has not been described in the literature, neither have such tests been conducted in a rigorous fashion, to the best of our knowledge. Such an effect could mask or suppress any differences that might otherwise exist between the two groups in this study.

The lack of correlation of pDC function with most T cell-related factors indicates that pDCs are unlikely to play an important role, at least during chronic infection, in dictating the formation and preservation of strong, polyfunctional, HIV-specific CD8+ T cell

responses. However, this study did produce some evidence to suggest that, contrary to the original hypothesis, higher activation capacity in pDCs of the periphery may correlate with lower levels of nonspecific T cell activation and thus with less progressive disease. This effect was restricted to elite controllers, however, it may point towards a role in disease progression for certain pDC functions, namely the IRF5-mediated upregulation of maturation/activation markers, including CD86, in controlling nonspecific immune activation during chronic HIV infection.

While this study did not show significant differences between elite controllers and HAART-suppressed HIV+ patients with regards to pDC number or function that might explain the genetic differences in TLR9 and IRF5 between these two groups, it is impossible to rule out the idea that pDC function may have some influence on disease progression. Due to all of the potentially confounding factors outlined above, in combination with the genetic data, we feel that further studies of pDC functionality in HIV-infected patients may be warranted, particularly if additional functionalities of pDCs are brought to light.

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Table S1. HIV-Specific Polyfunctional T Cell Correlation with pDC Activation - Elite Controllers

	pDC Baseline Parameters			
HIVgag-Specific T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+IL\text{-}2^+IFN\gamma^+$	NS	NS	NS	NS
$CD8^+IL\text{-}2^+IFN\gamma^+$	NS	NS	NS	NS
	pDC Fold-Change Following CpG-C Stimulation			
$CD4^+IL\text{-}2^+IFN\gamma^+$	NS	NS	NS	NS
$CD8^+IL\text{-}2^+IFN\gamma^+$	NS	NS	NS	$p = 0.0062$
	pDC Fold-Change Following imiquimod Stimulation			
$CD4^+IL\text{-}2^+IFN\gamma^+$	NS	NS	NS	NS
$CD8^+IL\text{-}2^+IFN\gamma^+$	NS	NS	NS	NS

NS = no significant correlation found between the indicated parameters

Table S2. T Cell Activation Correlation with pDC Activation - All Patients

	pDC Baseline Parameters			
T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD8^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS

	pDC Fold-Change Following CpG-C Stimulation			
T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD8^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS

	pDC Fold-Change Following imiquimod Stimulation			
T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD8^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS

NS = no significant correlation found between the indicated parameters

Table S3. T Cell Activation Correlation with pDC Activation - Elite Controllers

	pDC Baseline Parameters			
T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD8^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS

	pDC Fold-Change Following CpG-C Stimulation			
T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD8^+CD38^+HLA\text{-}DR^+$	NS	NS	$p = 0.0253$	$p = 0.0157$
$CD4^+Ki67^+$	$p = 0.0215$	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS

	pDC Fold-Change Following imiquimod Stimulation			
T Cell Parameters	%IDO+	%CD40+	%CD86+	%HLA-DRhi
$CD4^+CD38^+HLA\text{-}DR^+$	NS	NS	NS	NS
$CD8^+CD38^+HLA\text{-}DR^+$	NS	NS	$p = 0.031$	NS
$CD4^+Ki67^+$	$p = 0.0371$	NS	NS	NS
$CD4^+Ki67^+$	NS	NS	NS	NS

NS = no significant correlation found between the indicated parameters

Figure S1.

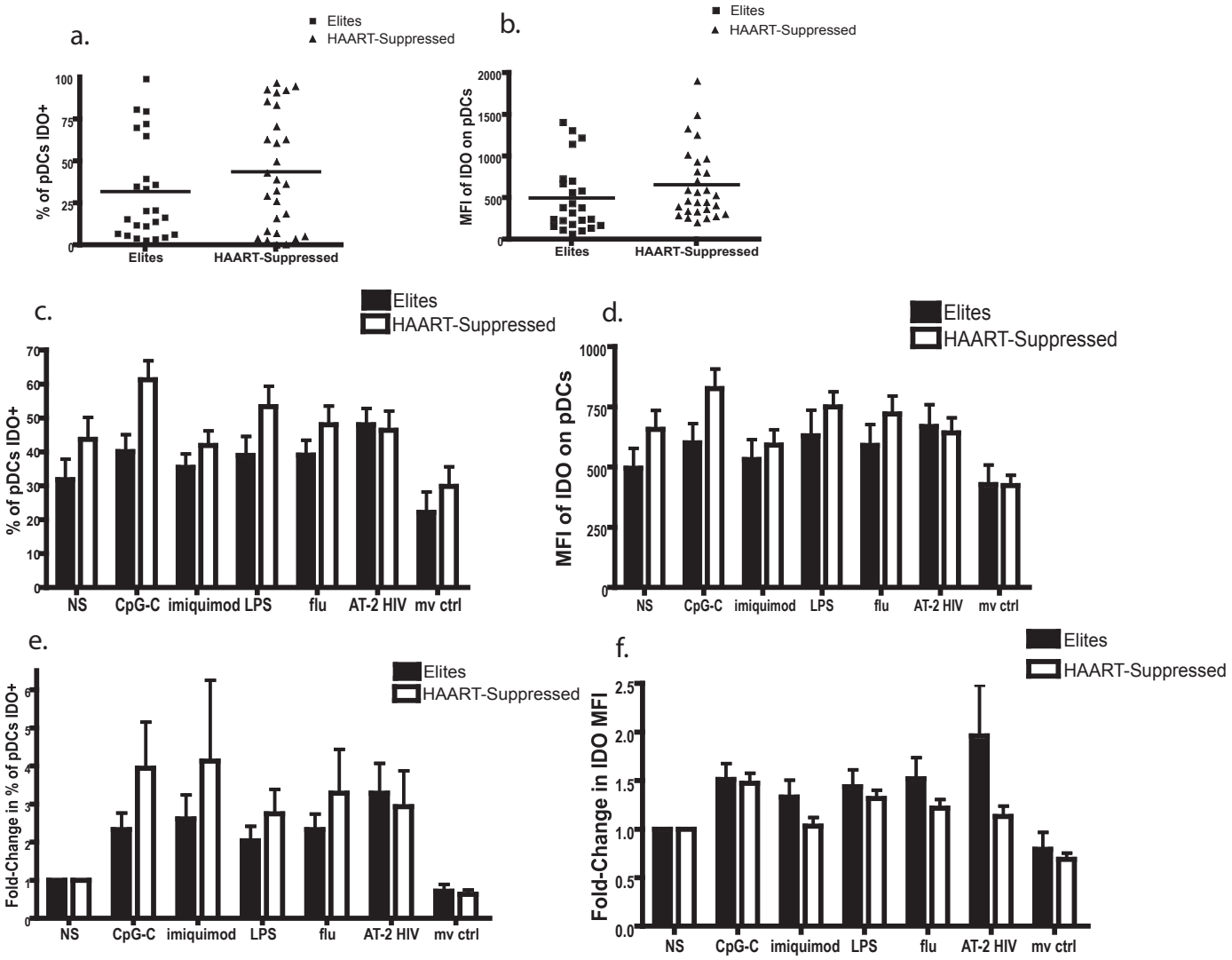

a.
Elites
HAART-Suppressed
% of pDCs IDO+
100
75
50
25
0
Elites
HAART-Suppressed
b.
Elites
HAART-Suppressed
MFI of IDO on pDCs
2000
1500
1000
500
0
Elites
HAART-Suppressed
c.
Elites
HAART-Suppressed
% of pDCs IDO+
70
60
50
40
30
20
10
0
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl
d.
Elites
HAART-Suppressed
MFI of IDO on pDCs
1000
750
500
250
0
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl
e.
Elites
HAART-Suppressed
Fold-Change in % of pDCs IDO+
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl
f.
Elites
HAART-Suppressed
Fold-Change in IDO MFI
2.5
2.0
1.5
1.0
0.5
0.0
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl

Figure S2.

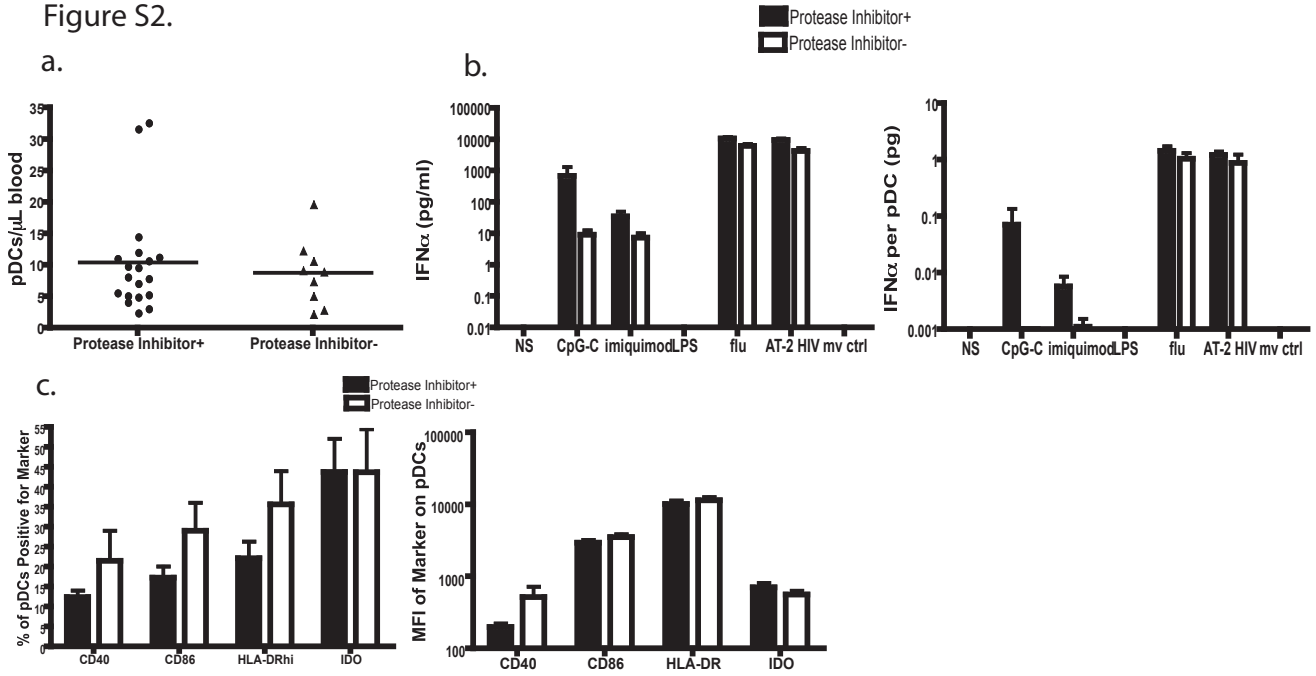

a.
b.
c.
Protease Inhibitor+
Protease Inhibitor-
pDCs/μL blood
IFNα (pg/ml)
IFNα per pDC (pg)
% of pDCs Positive for Marker
MFI of Marker on pDCs
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl
CD40
CD86
HLA-DRhi
HLA-DR
IDO

Figure S3.

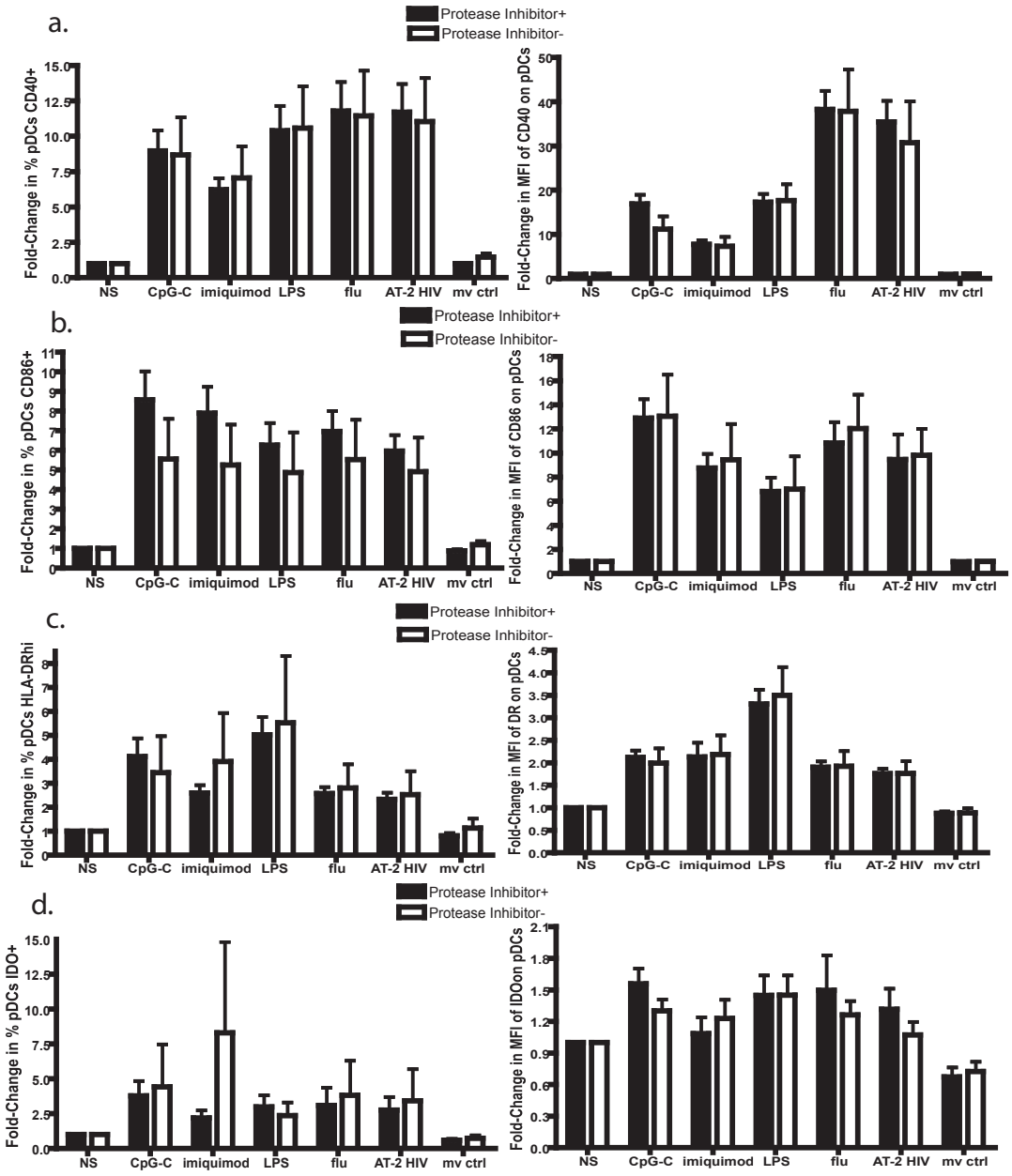

a.
Protease Inhibitor+
Protease Inhibitor-
Fold-Change in % pDCs CD40+
Fold-Change in MFI of CD40 on pDCs
b.
Fold-Change in % pDCs CD86+
Fold-Change in MFI of CD86 on pDCs
c.
Fold-Change in % pDCs HLA-DRhi
Fold-Change in MFI of DR on pDCs
d.
Fold-Change in % pDCs IDO+
Fold-Change in MFI of IDOon pDCs
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl

Figure S4.

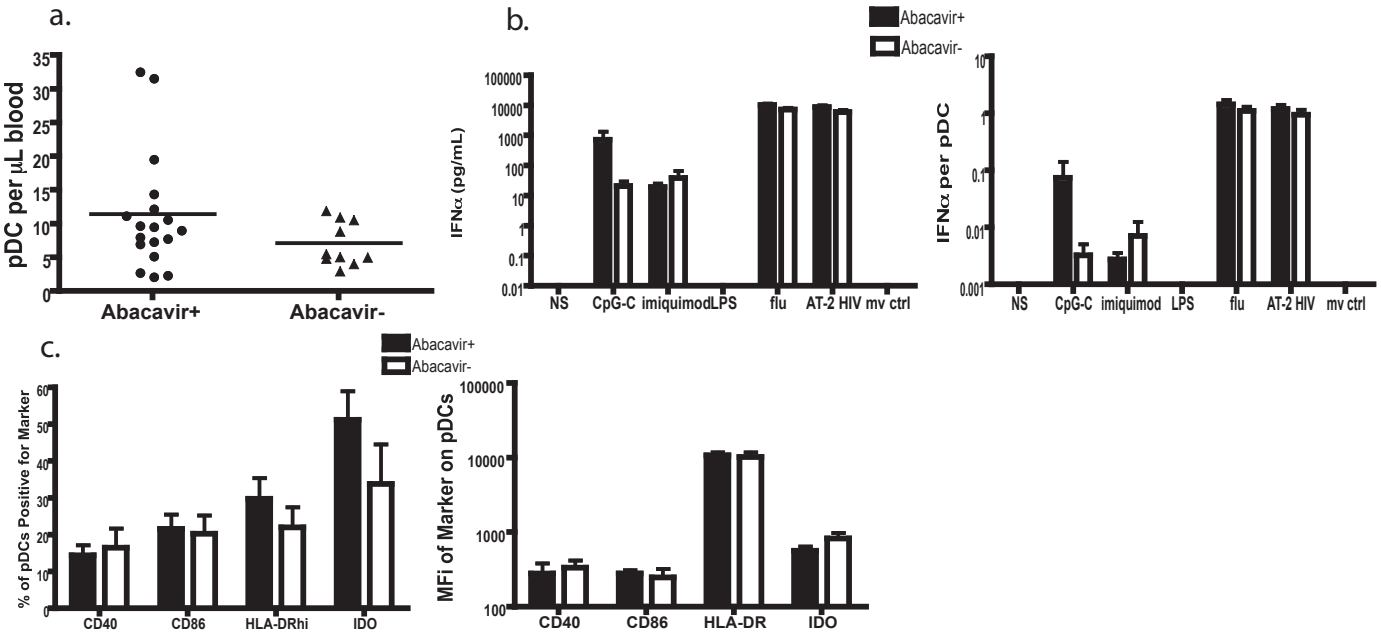

a.
pDC per μL blood
Abacavir+
Abacavir-
b.
Abacavir+
Abacavir-
IFNα (pg/mL)
IFNα per pDC
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl
c.
% of pDCs Positive for Marker
CD40
CD86
HLA-DRhi
IDO
MFi of Marker on pDCs
HLA-DR

Figure S5.

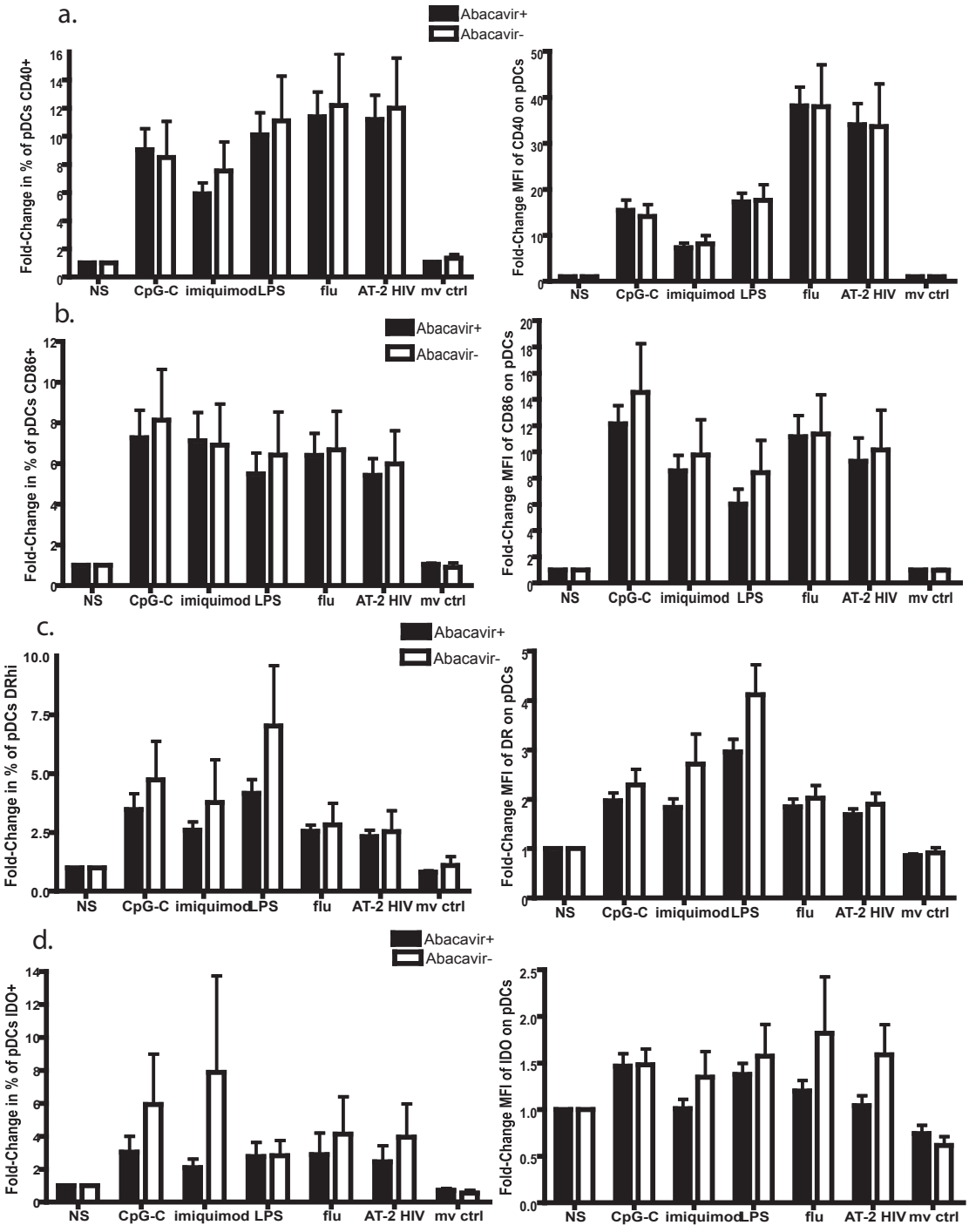

a.
Abacavir+
Abacavir-
Fold-Change in % of pDCs CD40+
Fold-Change MFI of CD40 on pDCs
b.
Fold-Change in % of pDCs CD86+
Fold-Change MFI of CD86 on pDCs
c.
Fold-Change in % of pDCs DRhi
Fold-Change MFI of DR on pDCs
d.
Fold-Change in % of pDCs IDO+
Fold-Change MFI of IDO on pDCs
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl

Figure S6.

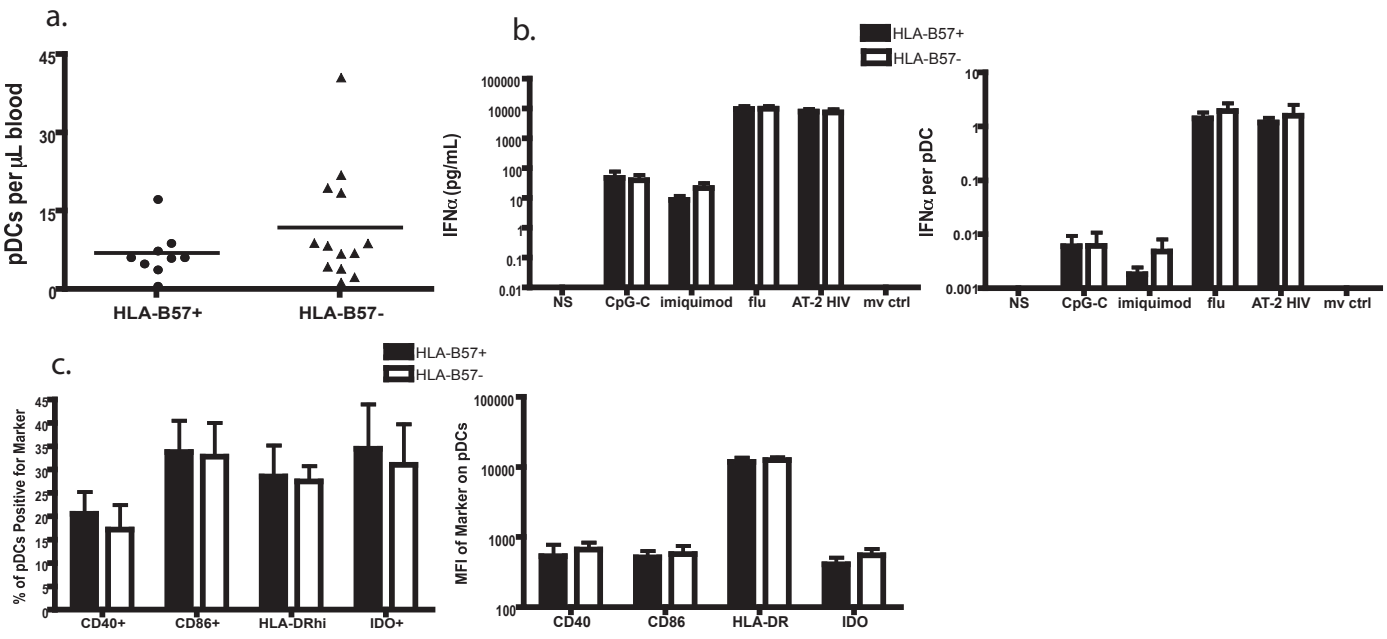

a.
pDCs per μL blood
45
30
15
0
HLA-B57+
HLA-B57-
b.
HLA-B57+
HLA-B57-
IFNα (pg/mL)
100000
10000
1000
100
10
1
0.1
0.01
NS
CpG-C
imiquimod
flu
AT-2 HIV
mv ctrl
IFNα per pDC
10
1
0.1
0.01
0.001
NS
CpG-C
imiquimod
flu
AT-2 HIV
mv ctrl
c.
HLA-B57+
HLA-B57-
% of pDCs Positive for Marker
45
40
35
30
25
20
15
10
5
0
CD40+
CD86+
HLA-DRhi
IDO+
MFI of Marker on pDCs
100000
10000
1000
100
CD40
CD86
HLA-DR
IDO

Figure S7.

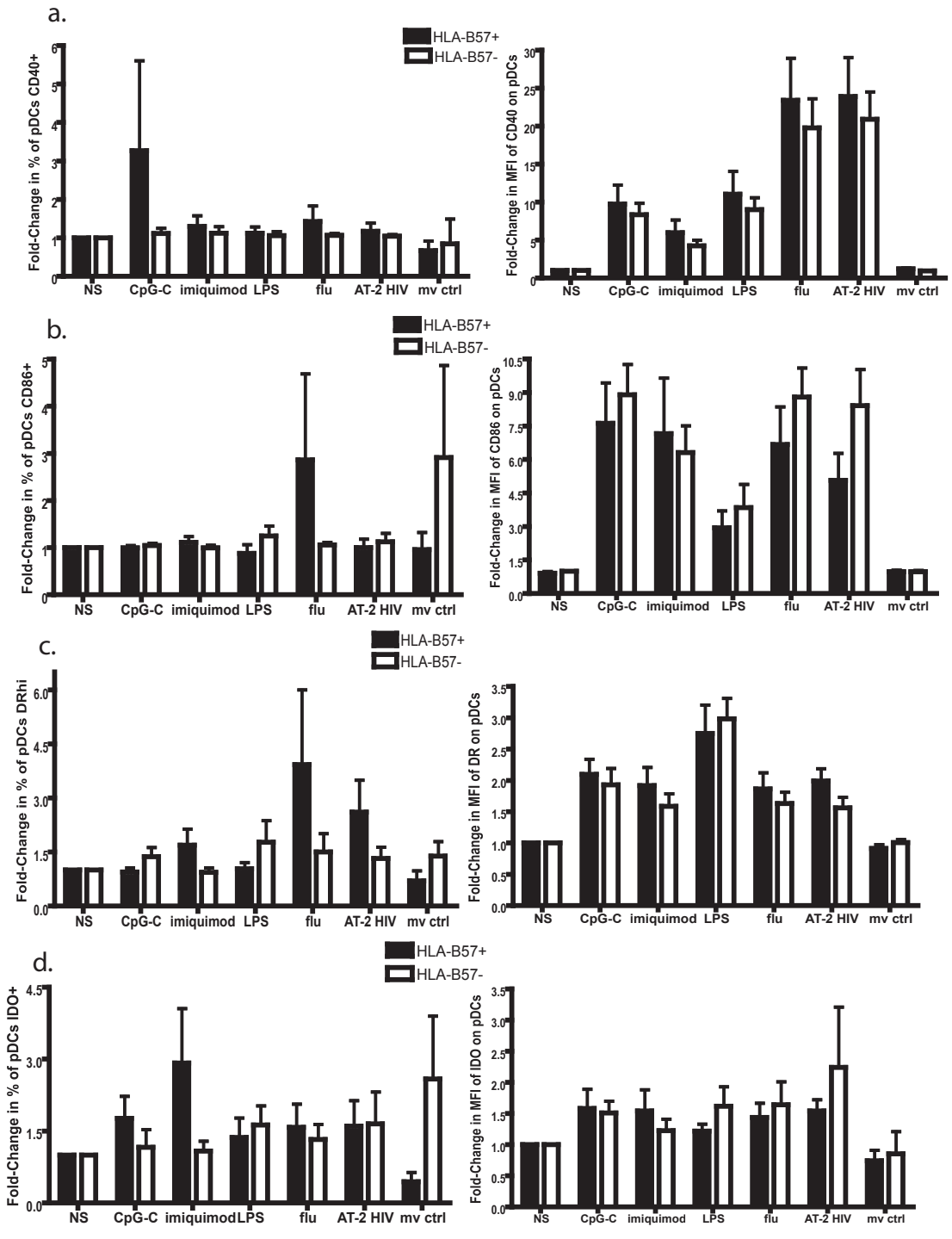
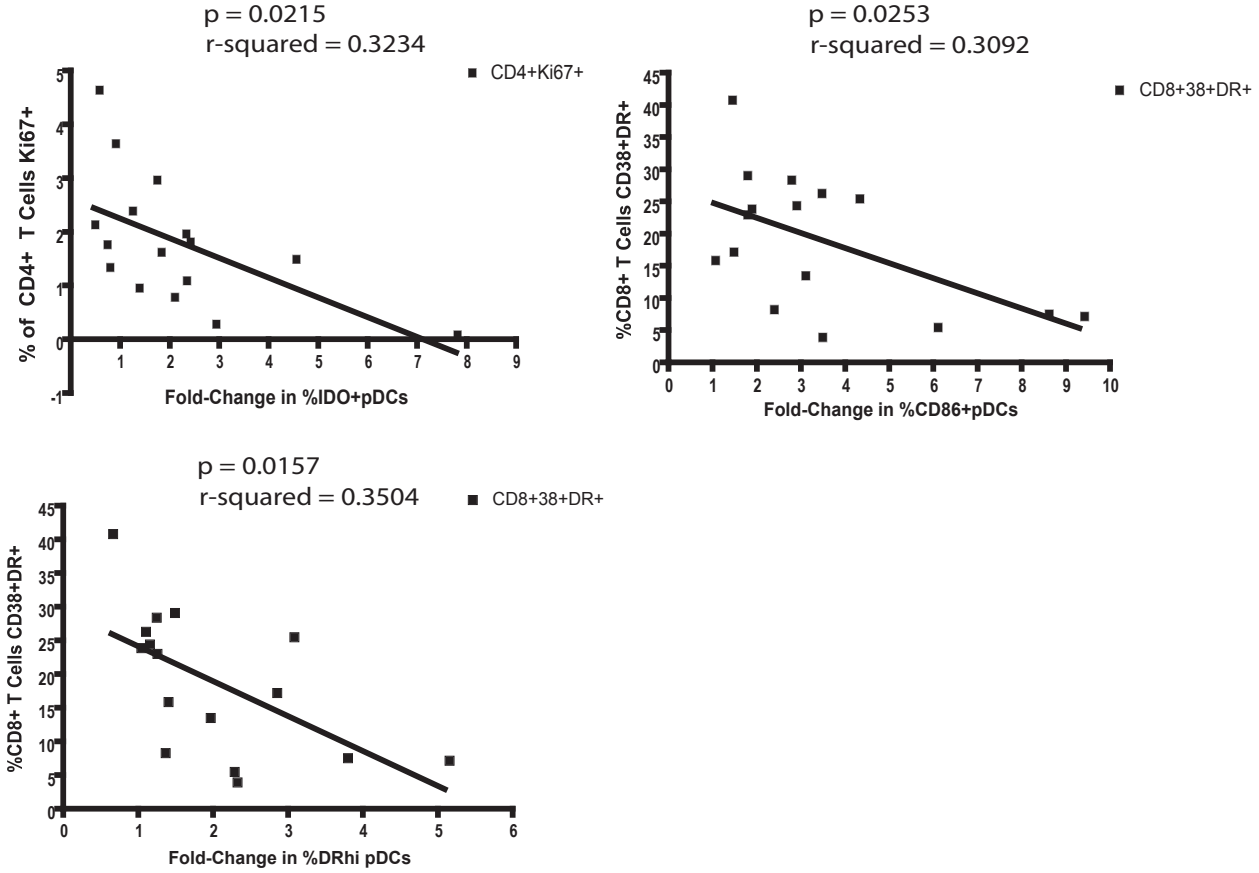

a.
HLA-B57+
HLA-B57-
Fold-Change in % of pDCs CD40+
Fold-Change in MFI of CD40 on pDCs
b.
Fold-Change in % of pDCs CD86+
Fold-Change in MFI of CD86 on pDCs
c.
Fold-Change in % of pDCs DRhi
Fold-Change in MFI of DR on pDCs
d.
Fold-Change in % of pDCs IDO+
Fold-Change in MFI of IDO on pDCs
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl

Figure S8.

a. pDC parameter changes following CpG-C stimulation



b. pDC parameter changes following imiquimod stimulation

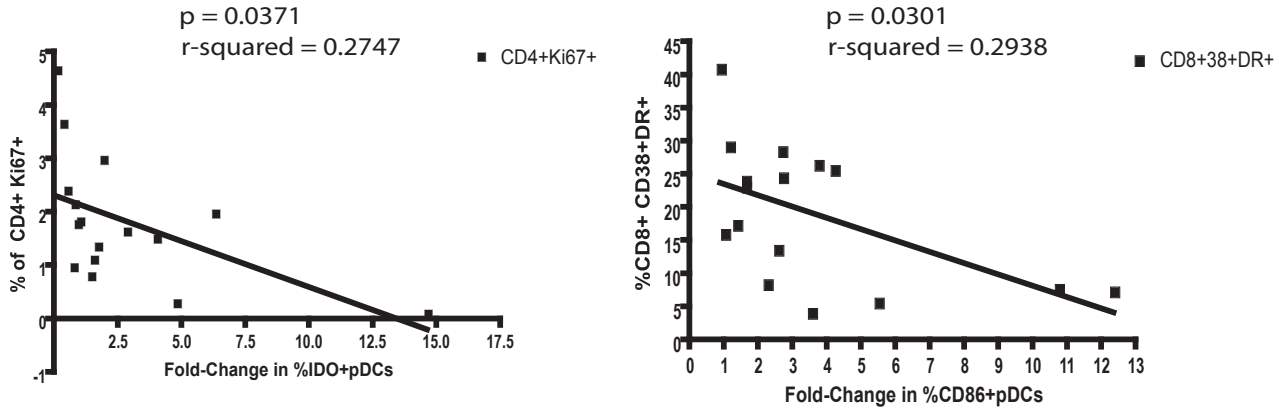
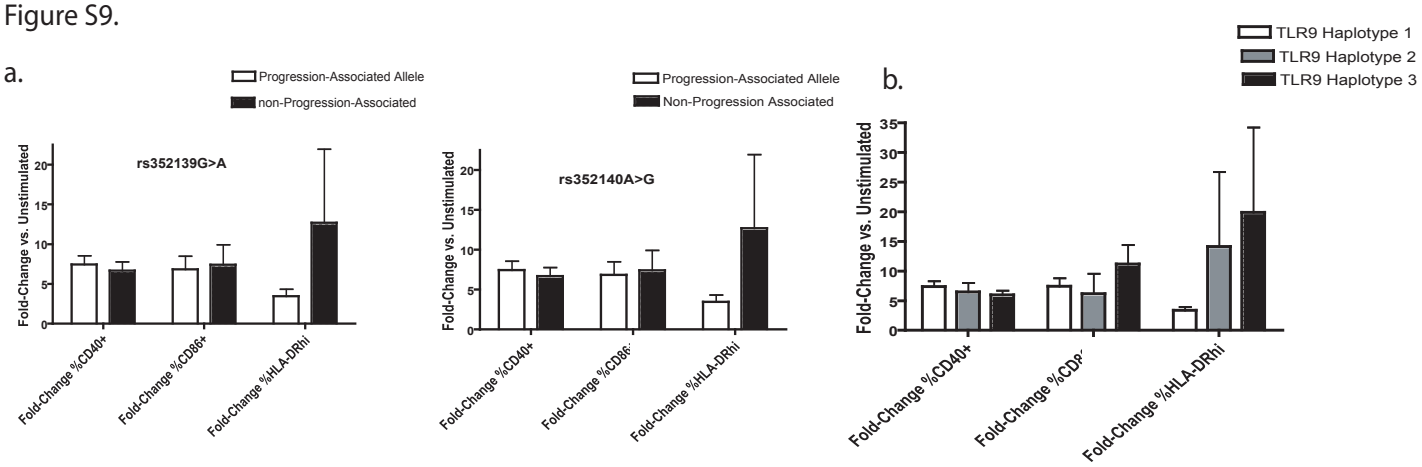


Figure S9.


a.
Progression-Associated Allele
non-Progression-Associated
rs352139G>A
Fold-Change vs. Unstimulated
Fold-Change %CD40+
Fold-Change %CD86+
Fold-Change %HLA-DRhi
Progression-Associated Allele
Non-Progression Associated
rs352140A>G
Fold-Change vs. Unstimulated
Fold-Change %CD40+
Fold-Change %HLA-DRhi
b.
TLR9 Haplotype 1
TLR9 Haplotype 2
TLR9 Haplotype 3
Fold-Change vs. Unstimulated
Fold-Change %CD40+
Fold-Change %HLA-DRhi

HIV-Seropositive "Elite Controllers" Have Apparently Normal Plasmacytoid Dendritic Cell Function

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ABSTRACT

Conflicting reports have linked varying levels of plasmacytoid dendritic cell (pDC) function with lentiviral pathogenesis. On the one hand, the production of interferon alpha (IFNα) and indoleamine-2,3-deoxygenase (IDO) from pDCs has been associated with disease progression; conversely, pDC function has been found to be deficient in the context of nonpathogenic SIV infection of sooty mangabeys. To determine whether pDC function might be altered in nonpathogenic HIV infection in humans, we evaluated the number and function of pDCs in HIV-seropositive human patients who are able to control HIV in the absence of therapy ("elite controllers") and HIV-seropositive individuals who had progressive disease. We observed no significant difference in the number of circulating pDCs or, after stimulation with HIV and with TLR7 and 9 agonists, the function of such cells *in vitro*. These observations suggest that differences in pDC functionality are not likely to account for differential disease outcomes between those who are able to control virus absent therapy and those who cannot. (Word Count: 161)

Keywords: human, AIDS, plasmacytoid dendritic cells

INTRODUCTION

Human immunodeficiency virus (HIV) disease is a significant source of morbidity and mortality throughout the world. After infection with this virus, most patients experience a loss of $CD4^+$ T cells, leading eventually to the development of the acquired immunodeficiency syndrome (AIDS). Notably, however, some HIV-infected patients ("elite controllers") do not show loss of $CD4^+$ T cells and do not progress to AIDS, and thus can be characterized as having a non-pathogenic infection. The mechanism of control in these rare individuals is the focus of intense research (1,2) and emerging data suggest that, although strong HIV-specific $CD8^+$ T cell responses may be partially responsible for virus control in some patients, such responses are neither necessary nor sufficient. Moreover, there is a sizeable subset of elite controllers (about 40-50%) that shows no evidence of T cell control at all (3-6).

We and others have considered the possibility that protective immunity to HIV may be conferred by elements of the innate immune system (7-9). Amongst these, the plasmacytoid dendritic cell (pDC) is an attractive candidate (10, 11). HIV can induce this cell population to produce large amounts of IFNα, a cytokine with potent antiviral properties (12). Additionally, pDCs activated by viruses such as HIV migrate into lymphoid organs and mature into antigen presenting cells, thereby initiating virus-specific T and B cell responses (13, 14). In this manner, the pDC stands at a critical junction linking the innate and adaptive immune responses.

Observations linking pDC number and function to lentiviral disease progression have been contradictory. Some reports have associated this compartment with a protective role against disease progression. Thus, patients who do not progress immunologically ("long-term nonprogressors") were found to have higher levels of pDCs in their peripheral blood compared to those who progressed (15, 16), and pDCs in the peripheral circulation of HIV-infected patients with pathogenic disease apparently produce less IFNα compared to those from HIV-uninfected controls (17). By contrast, other reports have linked IFNα production and robust pDC function with lentiviral disease progression (13, 18-22). Thus, higher levels of IFNα are associated with higher levels of immune activation, inflammation, and pathogenic SIV disease in macaques (21, 22). Upon initial infection of the vaginal mucosa, a signaling cascade dependent on pDCs has been shown to be essential for propagating viral expansion (23). Herbeuval *et al.* have found that pDCs isolated from the periphery of HIV-seropositive individuals with pathogenic disease had *higher* levels of intracellular IFNα compared to those with non-pathogenic disease (24). IFNα secretion by pDCs in the thymus has been associated with the selection of dysfunctional $CD8^{low}$ T cells, which may contribute to the overall immune suppression seen in HIV infection (25). Finally, recent studies have shown that pDCs from sooty mangabeys with non-pathogenic SIV infection produce markedly less IFNα in response to SIV and to other TLR7 and TLR9 ligands *in vitro* than do pDCs from rhesus macaques with progressive disease (20). Possibly, chronic stimulation of the innate immune system, including pDCs, by SIV and HIV in non-natural hosts may drive persistent immune activation and dysfunction, leading to AIDS (20, 26).

In light of the above findings, we examined pDC function in HIV-infected patients who were elite controllers. We wished to know: do pDCs from such patients manifest differences compared to those from HIV^+ patients with progressive disease? To avoid possible confounding effects of high level viremia (27), pDCs from subjects in the latter group were studied after effective viral suppression had been attained using highly effective antiretroviral therapy (HAART). We examined pDC numbers in peripheral blood, levels of pDC activation and maturation *in vitro* before and after stimulation with TLR7 and TLR9 ligands, and IFNα secretion in response to stimulation. Our data, in aggregate, show that pDCs in elite controllers are numerically and functionally similar to those found in HAART-suppressed patients with progressive disease.

MATERIALS AND METHODS

Cohort characteristics: For our studies focused on pDC number and function, freshly collected blood was obtained from individuals enrolled in SCOPE, a prospective observational cohort study based at the University of California, San Francisco. Individuals were routinely seen at 4-month intervals, at which time HIV plasma RNA levels and $CD4^+$ T-cell counts were obtained. From this cohort, participants were selected who met the criteria for one of the following two treatment groups: "elite controllers," defined as untreated individuals who are HIV seropositive (with an initial HIV antibody test >2 years prior to study entry) and three documented viral loads (VL) of <75 copies/ml in the previous 12 months, or "HAART-suppressed," defined as individuals with a prior history of progressive disease (VL > 10,000 copies/mL and median CD4 nadir of 120 cells/μL) and whose viral load had been effectively suppressed with HAART.

In studies aimed at defining possible associations between disease status and genetic polymorphisms in TLR9, common tagging single nucleotide polymorphisms (SNPs) were genotyped in 96 white American elite and viremic controllers (the latter with viral loads of 2000 copies/mL or less in the absence of therapy) and 202 white American non-controllers (with a steady state HIV viral loads of >10,000 copies/mL). Samples were contributed by clinicians from San Francisco, Boston, and Tennessee, and all subjects provided informed consent. Genomic DNA was isolated from the thawed blood samples using the Qiagen QIAamp DNA Blood Mini Kit protocol (2003, Qiagen, Valencia, CA).

Patient sample collection and stimulation: Human peripheral blood samples were collected by venipuncture from elite controllers and HAART-suppressed HIV-positive subjects under the auspices of a protocol approved by the UCSF Committee on Human Research. All pDC counts and functional studies were done on the samples obtained from the SCOPE cohort at San Francisco General Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated immediately using Ficoll Histopaque (Sigma Aldrich, St Louis, MO), according to the manufacturer's instructions, resuspended in RPMI-based media containing fetal calf serum (FCS), L-glutamine, and penicillin-streptomycin, and then incubated 20-24 hours at 37° C. The following stimulations were applied during the incubation period: CpG-C (ODN M362) and lipopolysaccharide (LPS) (both from Invivogen, San Diego, CA) were used at 5 μM; imiquimod (Invivogen, San Diego, CA) was used at 20 μM; heat-inactivated influenza virus (VR-95, ATCC) was used at 2 MOI (2 pfu/cell); aldrithiol-2 (AT-2) inactivated HIV and a corresponding microvesicle control were kindly supplied by Dr. Jeffrey D. Lifson (AIDS Vaccine Program, SAIC Frederick, NCI-Frederick, Frederick, MD) and were used at 1.96 μg/mL p24-equivalent final protein concentration. Following stimulation, supernatants were collected and stored at -20 C for later analysis, and cells were stained for analysis via flow cytometry. Trucount (BD Biosciences, San Jose, CA) staining was performed separately on whole blood collected at the same time into EDTA-containing tubes.

Flow cytometry: Following stimulation, cells were washed with a "PBS-plus" buffer [containing phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2.5 mM EDTA]. The same buffer was used to prepare cocktails of fluoresceinated antibodies

against surface markers, including CD123 PE-Cy5.5, CD11c PE, CD4 ECD, HLA-DR PE-Cy7, and "Lin markers" (CD3, CD14, CD20) in APC-Cy7. An amine dye (Invitrogen, Carlsbad, CA) was used to discriminate live from dead cells, according to the manufacturer's instructions. Cells were stained for 30 minutes at 4° C, and then washed twice with PBS-plus. Samples were resuspended in PBS-plus buffer following staining and run on an LSR II flow cytometer (BD Biosciences, San Jose, CA). All data were analyzed using FlowJo software (Treestar).

Quantitative cell counts were obtained using Trucount tubes (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, carefully calibrated quantities of antibodies (the same as those described above) and whole blood were added to tubes containing precise quantities of fluorescently labeled beads. Following incubation, red blood cells were lysed and the staining reaction was stopped by addition of a lysing buffer (BD FACS Lyse, BD Biosciences, San Jose, CA). Samples were then analyzed on a flow cytometer and the precise number of cells of a particular type was determined by calculation based on the number of beads present in a particular gate.

ELISA for IFNα: ELISAs to detect IFNα secretion were performed using the *Verikine*™ Human Interferon-Alpha Serum Sample ELISA kit (PBL Biomedical Labs, NJ), according to the manufacturer's directions. Briefly, supernatants were diluted as necessary, and incubated in antibody-coated plates for 1 hour at 26° C. Plates were washed and then a biotin-conjugated secondary detection antibody was added. Following secondary antibody incubation, the plates were washed and then incubated with a

streptavidin-conjugated horseradish peroxidase (HRP) enzyme. Finally, a 3,3' 5,5'-tetramethylbenzidine (TMB) substrate for the HRP was added, causing the development of a color which was analyzed using a plate reader to measure the optical density of each sample. The concentration of IFNα was then calculated based on known standards included with each test.

Genotyping of patient samples for TLR9: To explore the possibility that polymorphisms in TLR9 might be associated with levels of viremia, four common tagging TLR9 SNPs (rs187084 T>C, rs5743836 T>C, rs352139 G>A, rs352140 A>G) (28) were genotyped in 96 White American elite and viremic controllers and 202 White American non-controllers. TLR9 genotyping assays were performed using restriction fragment length polymorphism (RFLP) analysis.

Statistical analysis: Statistical analyses of flow cytometry and ELISA data were performed using Prism software (Graphpad, Inc, La Jolla, CA). Differences between elite controller and HAART-suppressed HIV-infected patients were assessed using a nonparametric 2-tailed Mann-Whitney *U* Test. Analysis of genetic data was performed using Haploview 4.0 software, and all permuted p-values were calculated using 1×10^6 permutations.

RESULTS

Pilot studies and expansion into larger patient cohort

This study was motivated by intriguing preliminary observations of pDC function in a small group (n=9) of "long term nonprogressors" (LTNPs), individuals defined as having maintained a normal $CD4^+$ T cell count over 10 or more years of documented HIV infection. Four of these individuals had viral loads of <75 copies/mL and would thus now also qualify as elite controllers; the remaining individuals had viral loads that were substantially higher. When stimulated *in vitro* with the TLR9 agonist, CpG, it was noted that pDCs from these individuals showed little if any activation (as measured by upregulation of CD40) compared to pDCs from HIV-seronegative controls or from those with progressive HIV disease (data not shown).

To confirm and extend these observations, we studied a larger number of subjects classified as either elite controllers (n=24) or HAART-suppressed (n=28), based on virological criteria (1). The HAART-suppressed individuals previously had progressive HIV disease (viral loads of >10,000 and median CD4 count of 120 cells/μL) with subsequent treatment-mediated suppression of viral load to undetectable levels (see Table 1). The median $CD4^+$ T cell counts for the controllers and HAART-suppressed patients were similar (median 734 cells/μL in the former and 626 cells/μL in the latter).

Optimization of flow cytometric analysis of pDC numbers and function

Methodology was first optimized so that pDCs could be reliably identified by flow cytometry and stimulated consistently with five unique ligands: (1) CpG-C, a TLR9

ligand which should stimulate both IFNα secretion and the upregulation of activation/maturation markers on pDCs, (2) imiquimod, a ligand for TLR7, (3) lipopolysaccharide (LPS), a TLR4 ligand that may serve to enhance activation in the setting of pathogenic HIV disease (29), (4) heat-inactivated influenza virus as a positive control for both pDC activation/maturation and IFNα secretion, and (5) aldrithiol-2 (AT-2) inactivated HIV to determine the response of pDCs to HIV. The representative flow plots in Figure 1a illustrate the markers found to be the most consistent and reliable for analysis of pDCs from PBMCs: Lin^-(CD3, CD14, CD20)$CD123^+HLA\text{-}DR^+$. Additionally, Figure 1b shows representative examples of gating of activation/maturation markers on pDCs after stimulation with the above ligands. These gates were established and uniformly applied to all specimens in a blinded fashion, i.e., before the patient status was decoded. Activation gates were based on this blinded analysis of the complete data set and, where possible, reflected a bi-modal distribution of the data on the flow plots. Target values were set automatically based on the use of cytometer setup and tracking beads (BD Biosciences, San Jose, CA) for standardization with each run, which ensured the comparability of fluorescence intensities over the length of the study.

No significant difference in absolute number of pDCs/μL or baseline activation between elite controllers and HAART-suppressed patients

The absolute number of pDCs/μL in the peripheral blood of the elite controllers and the HAART-suppressed patients was determined using Trucount tubes (30) along with the staining and gating strategy described in Figure 1. As shown in Figure 2a, the number of phenotypically defined pDCs was not significantly different between the two groups.

To determine whether baseline levels of pDC activation might differ between the two groups, we interrogated the compartment for the fractional and absolute level of expression of CD40, CD86, and HLA-DR (all of which are markers associated with activation/maturation of pDCs) (10,11,31). Since unstimulated pDCs express detectable levels of HLA-DR, we focused on the percentage of cells that expressed this marker at a higher level after stimulation. As shown in Figures 2b and 2c, pDCs from the two groups were generally similar with respect to the percentage positive for a given marker and for the mean fluorescence intensity (MFI) of that marker on positive cells. The only exception was the presence of a marginally significant ($p = 0.047$) elevation of the baseline percentage of pDCs positive for CD86 in the elite controllers as compared to the HAART-suppressed patients.

No significant differences in pDC activation between elite controllers and HAART-suppressed patients

We next examined the expression of activation/maturation markers on pDCs in the two patient groups following stimulation with CpG-C, imiquimod, LPS, heat-inactivated influenza virus and AT-2 HIV. To determine the capacity of pDCs from each patient group to become activated and/or to mature in response to the various stimuli, we evaluated the actual percentage positive for each activation/maturation marker, the MFI of these markers, and the fold-change in these markers following stimulation. The fold-change in the MFI and percentage of pDCs positive for the different activation/maturation markers were determined based on the baseline values shown

above in Figures 2b and 2c. As shown in Figures 3a-3d, most of the stimulations showed no significant and consistent differences in activation of pDCs between the two patient groups. The exception was again CD86, the expression of which was upregulated significantly more in the HAART-suppressed patients upon stimulation with CpG, imiquimod, LPS, flu, and AT-2 than in the elite controllers ($p=0.0079$; shown in Figure 3b). In all other respects, pDCs appeared to be functionally similar in elite controllers and in the HAART-suppressed patients with respect to stimulus-induced up-regulation of CD40 CD86, and HLA-DR.

No significant differences in pDC secretion of IFNα between elite controllers and HAART-suppressed

To determine whether pDCs from elite controllers might have a reduced capacity to produce IFNα, cells from each patient group were stimulated with a variety of TLR and viral ligands, including CpG-C, imiquimod, heat-inactivated influenza virus, and AT-2 inactivated HIV. No significant differences in IFNα secretion were observed between the two groups, either on a per-volume basis (Figure 4a) or on a per-cell basis (Figure 4b).

DISCUSSION

The role of pDCs in determining the course of disease progression in SIV and HIV infection is the subject of intense investigation. Although there are emerging data that suggest a critical role in SIV pathogenesis (20), we did not find any consistent differences in pDC number or function in patients who are able to control viremia in the absence of therapy via host mechanisms (elite controllers) as compared to those who had experienced progressive disease. We also found no apparent differences in the baseline levels of pDC expression of various activation/maturation markers, with the single exception of CD86, and no differences in the ability of these cells to upregulate these markers in response to stimulation by agonists of TLR7 and TLR9 (again, with the sole exception of CD86). Finally, we found no difference in the ability of pDCs from the two patient groups to secrete IFNα on a per-volume or per cell basis. In summary, although it is possible that pDC function may be associated with control of HIV replication and disease progression *in vivo*, no such relationship could be discerned using standard phenotypic and functional assays on circulating pDCs from well-defined cohorts of HIV-seropositive subjects *in vitro*.

As is the case in most studies of this type, these are several caveats to bear in mind. First, it is clear that the group of patients studied are heterogeneous with respect to a number of non-controlled parameters and that there may be subsets of patients within each that harbor pDC compartments with unique characteristics. If so, it is possible that enrollment of a larger number of patients into each group would have revealed true differences in the number and/or function of pDCs within and between the two groups of patients. In

addition, it is possible that other assays, directed at pDC functions such as their migration and/or response kinetics *in vivo*, might have revealed differential pDC function between elite controllers and those who had progressive disease. While we cannot rule out or assess the relative influence of each of the above caveats, we can nonetheless state that readily apparent distinctions between the pDC compartments of these two groups are not evident using standard tools of assessment *in vitro*.

In some subsets of patients *in vivo*, it is possible that certain pDC functions may play an important role in disease progression. Thus, the capacity of pDCs to produce the immunosuppressive enzyme indoleamine-2,3-deoxygenase (IDO) was recently discovered and might play a role in influencing HIV disease progression (32). Additionally, there may be other functional parameters of pDCs that have not been characterized to date. In particular, the role of pDCs following viral stimulation and subsequent migration to lymphoid tissues has not been as well studied as their functionality in the periphery. Further evaluation of pDC function in patients with HIV disease may later reveal distinctions such as these.

Given our preliminary observation that CD40 upregulation in response to TLR9 stimulation was significantly reduced in pDCs from long-term nonprogressors, it is worth noting that we examined known single nucleotide polymorphisms (SNPs) in TLR9 to determine whether some might be enriched in HIV-infected patients who can control viremia without therapy. To do so, we surveyed a large cohort of HIV-seropositive individuals and found that certain polymorphisms in the TLR9 locus segregated

disproportionately among white Americans with individuals who were not controlling HIV (steady-state plasma HIV RNA level > 10,000; non-controllers n=202) compared to a group of white Americans who were controlling HIV replication (controllers; n=96). This effect was not present in other ethnic groups included in the study. In particular, alleles of two SNPs (rs352139 G>A and rs352140 A>G) were enriched in patients with high viremia and more progressive disease (data not shown, permuted p = 0.0026 and 0.0357, respectively). Using these two SNPs in combination with two other SNPs (rs187084 T>C and rs5743836 T>C), three TLR9 haplotypes (haplotypes 1-3) were defined in white Americans. As has been reported (33) TLR9 haplotype 1 was found to be associated with more pathogenic disease in HIV-seropositive white Americans (data not shown, permuted p = 0.0082). When related to the various functional parameters examined in this study, however, neither of the two above TLR9 SNPs (rs352139 G>A and rs352140 A>G) or TLR9 haplotype 1 were found to be correlated with apparent differences in pDC functionality. Further information on such relationships must await more complete analysis of the haplotype map of a larger group of elite controllers.

In summary, the results of this study underscore the fact that there are few if any functional differences in pDCs from individuals who control their virus via host mechanisms and from individuals who once had progressive disease but were then effectively treated. In future studies, it would be of interest to focus on other measures of pDC function, on tissue rather than blood samples, and on other disease stages, e.g., in the context of those exceedingly rare individuals who do not lose $CD4^+$ T cells despite

high level viremia (and who are accordingly are more analogous with the non-progressor phenotype observed in sooty mangabeys) (20, 34).

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FIGURE LEGENDS

Figure 1. Flow cytometry gating strategies. (a) The strategy chosen to identify pDCs in PBMC suspensions: cells found within the indicated regions of the forward vs. side scatter plot to the left; dead cells were eliminated using an amine dye (Amine gate); cells negative for the "lin markers" CD3, CD14, and CD20 were selected and then those that were $Lin^{-}CD123^{+}HLA\text{-}DR^{+}$ (circled in the right-most plot) were defined as pDCs. (b) Representative examples of gating for the activation markers CD40, CD86, and HLA-DR on pDCs after stimulation with nothing (NS), CpG-C, imiquimod, LPS, heat-inactived influenza (flu), AT-2 inactivated HIV, and the microvesicle control (mv ctrl) for AT-2 inactivated HIV.

Figure 2. No significant difference in absolute number of pDCs or baseline activation between elite controllers and HAART-suppressed. (a) Number of pDCs per μL of whole blood, as measured by Trucount analysis. (b) The percentage of pDCs positive for CD40, CD86, or HLA-DR. (c) The mean fluorescence intensity (MFI) of CD40, CD86, or HLA-DR on pDCs *$p<0.05$

Figure 3. No significant differences in pDC expression of activation markers following stimulation. (a) The percentage of pDCs positive for CD40, CD86, or HLA-DR. (b) The fold-change in percentage of pDCs positive for CD40, CD86, or HLA-DR. (c) The mean fluorescence intensity of CD40, CD86, or HLA-DR on pDCs. (d) The fold-change in mean fluorescence intensity of CD40, CD86, or HLA-DR on pDCs. Abbreviations: NS-no stimulation, flu - heat-inactivated influenza virus, AT-2 - AT-2 inactivated HIV, mv

ctrl - microvesicle control for AT-2 inactivated HIV, MFI - mean fluorescence intensity. *p <0.05, **p<0.01

Figure 4. No significant differences in pDC secretion of IFNα between the two patient groups. (a) The total secretion of IFNα, in pg/mL. (b) The secretion of IFNα on a per-pDC basis (calculated based on Trucount pDC quantification shown in Figure 1). Note that no detectable IFNα secretion was observed in the case of no stimulation (NS) or stimulation with the microvesicle control for AT-2 HIV (mv ctrl) (data not shown).

Table I. Patient Cohort Characteristics

	Elite Controllers	HAART-Suppressed
Total No. of Patients	24	28
Age		
Mean Age	47	52
Median Age	48	52
Gender		
Male	18	23
Female	4	4
Male to Female Transgender	2	1
Ethnicity		
African American	6	7
Asian	0	2
Caucasian	14	15
Latino	1	3
Native American	2	0
Pacific Islander	1	0
Latino/Nat Am	0	1
Viral Load (copies/ml)		
Mean VL	91*	48*
Median VL	61*	39*
$CD4^+$ T Cell Count (cells/μL)		
Mean $CD4^+$	798	676
Median $CD4^+$	734	626

* note: viral load was below the limit of detection for many patients, including all of the HAART-suppressed patients. When this was the case, the number nearest the limit of detection of the assay used for that patient's viral load test was used to calculate mean/median viral load.

Figure 1.

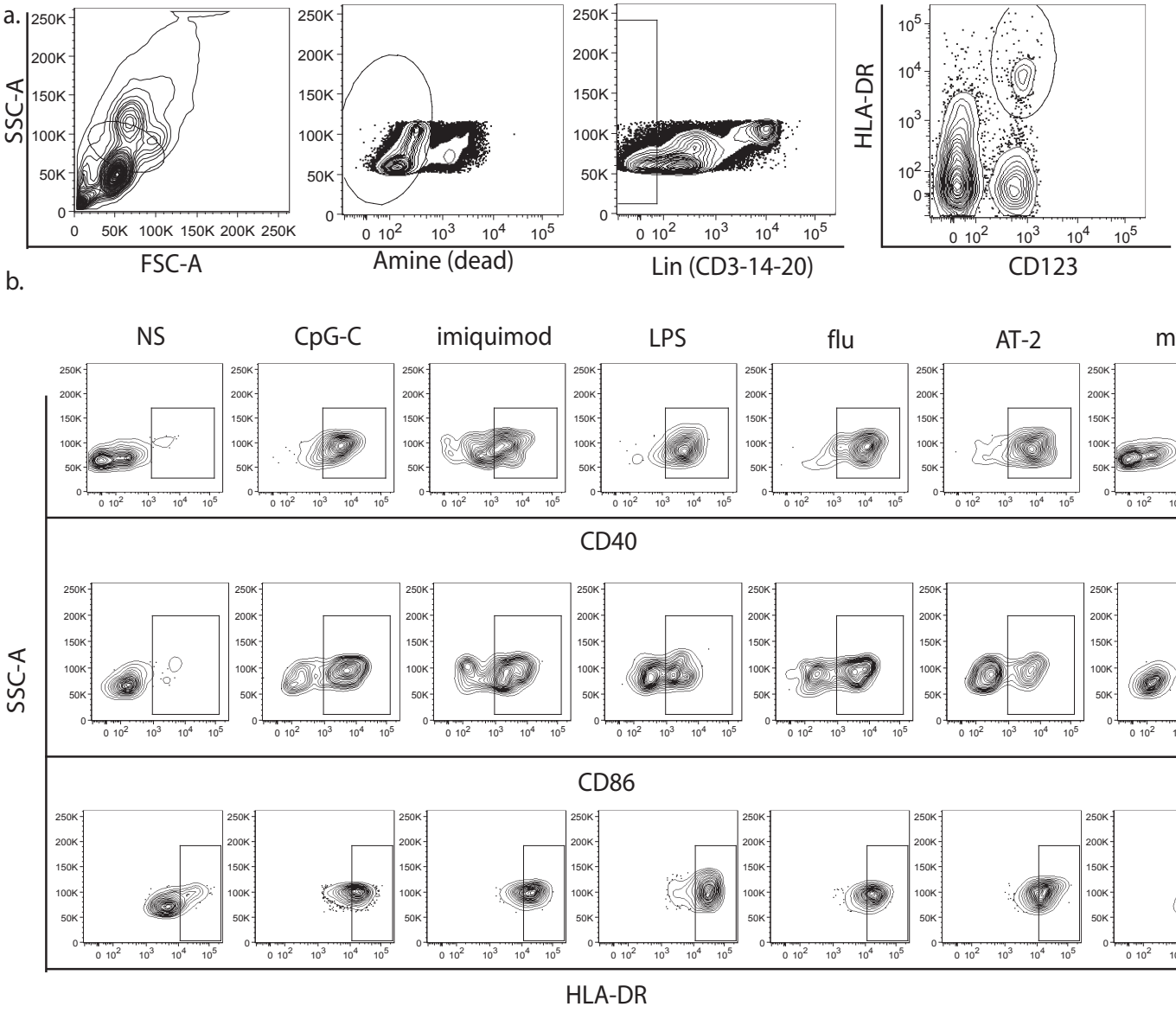
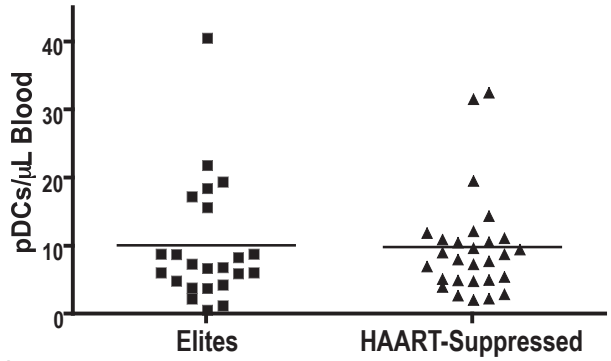
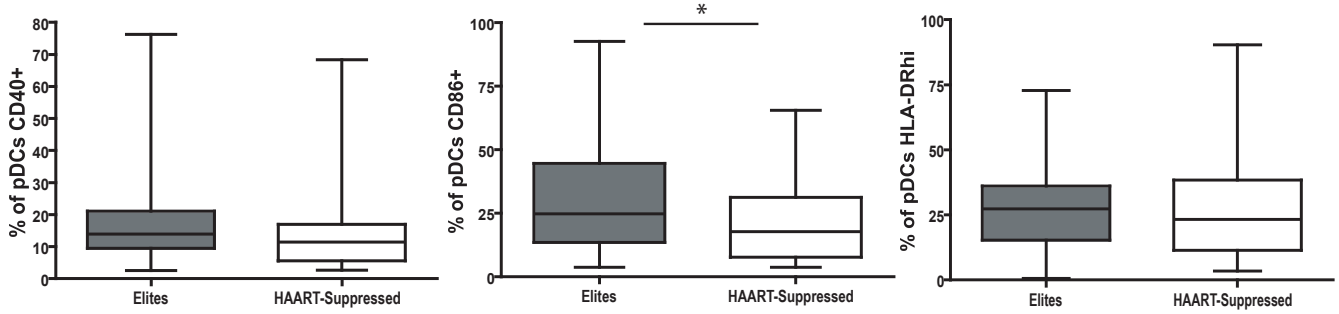

a.
SSC-A
FSC-A
Amine (dead)
Lin (CD3-14-20)
HLA-DR
CD123
b.
NS
CpG-C
imiquimod
LPS
flu
AT-2
mv ctrl
CD40
SSC-A
CD86
HLA-DR

Figure 2.

a.


pDCs/μL Blood
40
30
20
10
0
Elites
HAART-Suppressed

b.


% of pDCs CD40+
% of pDCs CD86+
% of pDCs HLA-DRhi
*
Elites
HAART-Suppressed

c.

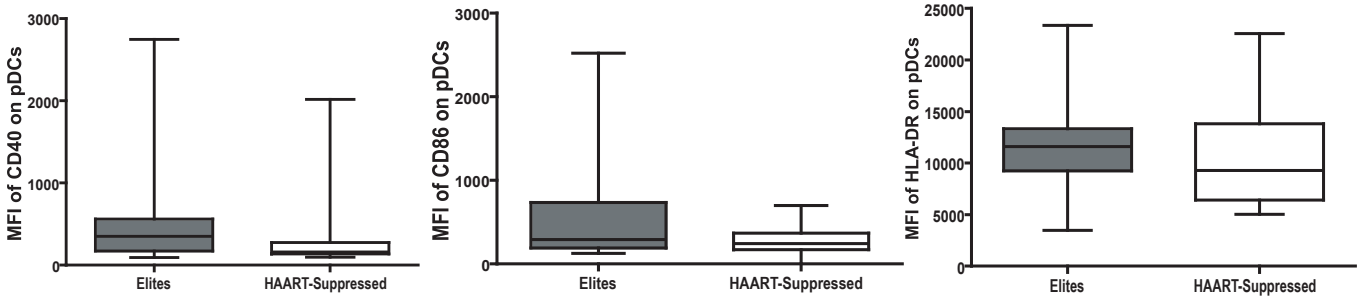

MFI of CD40 on pDCs
MFI of CD86 on pDCs
MFI of HLA-DR on pDCs
Elites
HAART-Suppressed

Figure 3.

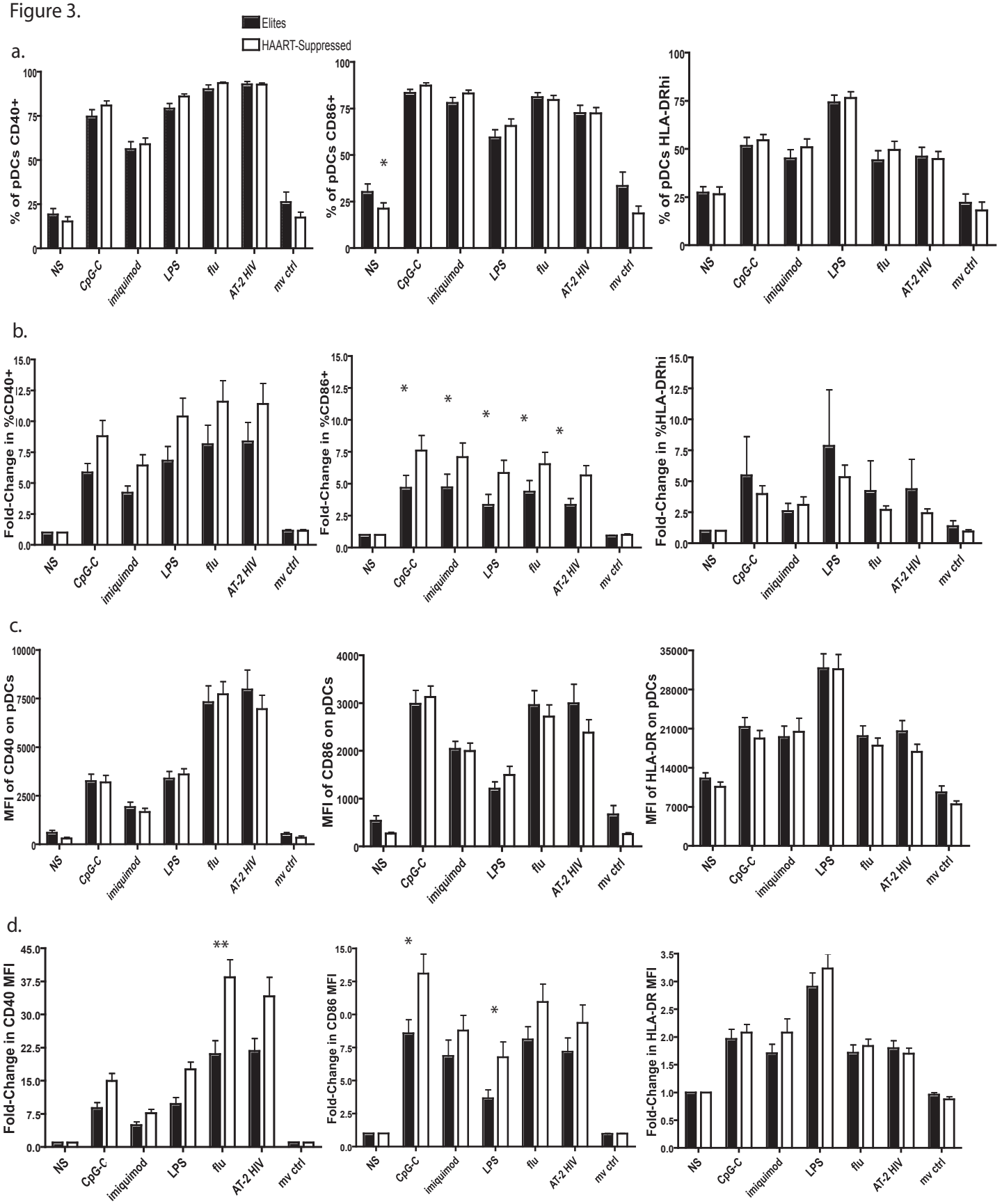
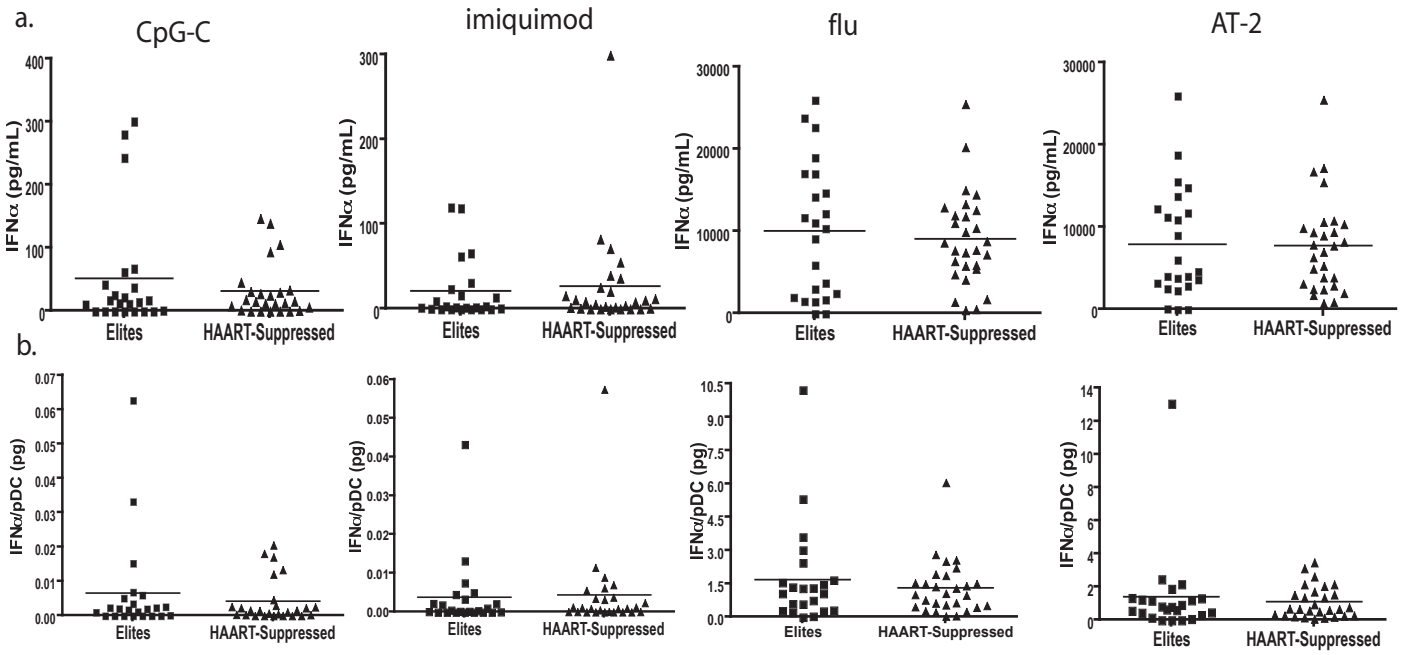

Elites
HAART-Suppressed
a.
% of pDCs CD40+
% of pDCs CD86+
% of pDCs HLA-DRhi
b.
Fold-Change in %CD40+
Fold-Change in %CD86+
Fold-Change in %HLA-DRhi
c.
MFI of CD40 on pDCs
MFI of CD86 on pDCs
MFI of HLA-DR on pDCs
d.
Fold-Change in CD40 MFI
Fold-Change in CD86 MFI
Fold-Change in HLA-DR MFI
NS
CpG-C
imiquimod
LPS
flu
AT-2 HIV
mv ctrl

Figure 4.


a.
CpG-C
imiquimod
flu
AT-2
IFNα (pg/mL)
Elites
HAART-Suppressed
b.
IFNα/pDC (pg)
Elites
HAART-Suppressed

CHAPTER 3

PROLOGUE

Similar to what has been shown in human HIV infection, simian immunodeficiency virus (SIV) infection in non-human primates can follow either a pathogenic or a non-pathogenic course. In non-human primate SIV infection, African (natural host) species generally undergo non-pathogenic infection, while natural hosts, including such species as the African Green Monkey, Chimpanzee, Sooty Mangabey and Mandrill, among others, generally undergo a non-pathogenic infection.[1] SIV infection in non-human primates is commonly used as a model for human HIV infection, particularly to try to understand the nature of non-pathogenic lentiviral infection, and what immune system factors may drive such a disease course, as opposed to the more common pathogenic outcome in human HIV infection.[2] As has been found in human HIV infection, pathogenicity is generally associated with higher levels of non-specific bystander immune activation, while non-pathogenic infections, as seen in the natural host species, are generally associated with much lower levels of non-specific immune activation.[3,4] Unlike some HIV-seropositive humans, however, (such as the elite controllers studied in Chapter 2 of this work), natural SIV host species maintain high viral loads during the course of infection, limiting pathogenicity despite their high levels of virus (ref). We and others have conjectured that this may make these animals similar to those rare individuals who fail to progress towards AIDS during long-term HIV infection, despite maintaining relatively high levels of virus (some long-term nonprogressors).[5]

Given the similarities between SIV infection in non-human primates and HIV infection in humans, there has been a great deal of interest in parsing the specifics of the immune response in non-pathogenic SIV infections in natural hosts. During the course of this thesis work, significant advances have been made towards this end. One of the most significant with regards to the work discussed here was a study that showed fundamental and important differences in the ability of TLR7 and TLR9 to mediate IFNα production in response to stimulation via their respective ligands. Specifically, this work showed that plasmacytoid dendritic cells (pDCs) from one natural host species, the sooty mangabey, fail to produce high levels of IFNα under the same conditions in which pDCs from both a non-natural host species (rhesus macaques), and from humans, do so.[6]

In our laboratory, there has a long-standing interest in understanding the immune response of the natural host the African green monkey (AGM) to SIV infection, and how it differs from that of non-natural host species, such as the pigtail (PM) and rhesus macaque (RM). For this thesis work, the role of pDCs in the course of SIV infection was examined more closely. It seemed logical to ask if, like the sooty mangabey, the AGM would also show a defect in IFNα production in response to TLR7 and TLR9 stimulation, as compared to the rhesus macaque. Additionally, we wanted to know whether other important functions of pDCs, such as their ability to upregulate activation/maturation markers important for their maturation, migration, and function, such as CD40, CD86, and HLA-DR, would be different between the natural and non-natural SIV host species. To further parse whether this was a defect specific to the IFNα pathway, or a more universal defect in TLR7 and/or TLR9 signaling in AGMs (and, presumably, other

natural SIV host species), we also examined whether the only other cell type known to express and signal through both TLR7 and TLR9, B cells,[7] also showed similar differences in activation/maturation marker expression upon stimulation between the two species.

We were also interested in the role of pDC-expressed indoleamine-2,3-deoxygenase (IDO) in these immune responses. Early data from others in the laboratory had pointed to significant differences in IDO expression and activity between natural and non-natural host species during the course of SIV infection. This thesis work also further explored such differences within the context of pDCs, by examining their expression of IDO on uninfected and chronically infected animals, to see if similar differences existed. Briefly, we did find important differences in IDO production and activity, as well as the *ex vivo* activation capacity of both pDCs and B cells when stimulated with TLR7 and TLR9 ligands between natural (AGM) vs. non-natural (PM and RM) SIV host species. AGMs showed much lower levels of IDO activity and expression, particularly during chronic infection, when compared to PMs and RMs, and they also showed the same fundamental defect in signaling across TLR7 and TLR9 as was described in the sooty mangabey. We believe that these results indicate that innate immune system function may contribute to the state of chronic, non-specific immune activation seen in non-natural SIV hosts, and that the ability of natural host species such as the AGM to limit such responses may be an important determinant of the non-pathogenic infection course seen in these animals. The results of this work are described in more detail in the enclosed manuscript entitled "The Role of Indoleamine-2,3-Deoxygenase in Pathogenic Simian Immunodeficiency Virus

Infection."

See attached manuscript draft entitled “The Role of Indoleamine-2,3-Deoxygenase in Pathogenic Simian Immunodeficiency Virus Infection”

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Working Title: "The Role of Indoleamine-2,3-Deoxygenase in Pathogenic Simian Immunodeficiency Virus Infection"

Jeffrey E. Mold, Rebecca A. Botelho, David Favre, Joseph M. McCune

ABSTRACT

Increased levels of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) are known to be associated with greater pathogenicity in simian immunodeficiency virus (SIV) infection of non-natural SIV hosts (rhesus and pigtail macaques, RMs and PMs). Here we examined whether conversely lower levels of IDO might be present in a natural host of SIV (the African green monkey or AGM). We found that this was indeed the case, as measured by levels of IDO mRNA and protein in the blood and lymph nodes of infected animals, as well as by levels of IDO enzymatic activity in the periphery during infection. All measures of IDO expression and activity remained elevated in PMs and RMs during chronic infection, but not in AGMs. We also examined whether plasmacytoid dendritic cells (pDCs), which have recently been shown to express IDO following HIV infection, showed differences in IDO expression and other markers of activation between RMs and AGMs. RM pDCs did show higher levels of activation in both uninfected, and more profoundly, in chronically animals as compared to those from AGMs. To characterize whether the observed differences in pDC function might represent, as recent literature might suggest, an overall difference in signaling across TLR7 and TLR9, we also compared B cell activation between the two species, and found similar results to those seen in the pDCs. Overall, our data suggest a picture of chronic innate immune activation in PMs and RMs compared to AGMs, and we would suggest that this difference may help to drive observed differences in disease outcome.

INTRODUCTION

Simian immunodeficiency virus (SIV) infection of non-human primate species has long been used as a model of human immunodeficiency virus (HIV) infection in humans.[1] The most commonly studied non-human primate species can be generally divided into two categories: African species, which are natural hosts for SIV infection (including the Sooty Mangabey, African Green Monkey, Chimpanzee, Mandrill, and others), and Asian species, which are non-natural hosts for SIV infection (including the Rhesus and Pigtail Macaque species, among others).[2] While natural hosts of SIV undergo a non-pathogenic and non-progressive infection upon exposure to SIV, non-natural host species undergo a progressive and pathogenic infection leading to the development of an AIDS-like syndrome, similar to what is seen in humans following HIV infection (1,2). One major driver of the development of pathogenic SIV infections in non-natural host species is higher levels of immune activation and inflammation compared to those seen in natural host species (1,2).[3,4] This trend towards greater immune activation is evident beginning early in acute infection, and persists during the chronic phase of pathogenic infections, where it is thought to be self-reinforcing and to promote the eventual development of AIDS in these animals.[5,6]

One immune modulator that has been linked with greater pathogenicity in SIV infection of non-natural host rhesus Macaques is the enzyme indoleamine-2,3-deoxygenase (IDO).[7,8,9] IDO is the first and rate-limiting enzyme in the catabolism of the essential amino acid tryptophan via the kynurenine pathway.[10] IDO has been extensively studied for its many roles in the immune system, which include functions as diverse as direct

antimicrobial activity ('tryptophan starvation') and promotion of T regulatory cell responses to antigens, including induction of maternal tolerance to the allogeneic fetus during pregnancy.[11,12,13,14] IDO gene transcription is tightly regulated, and is responsive to inflammatory signals, with the IDO gene promoter containing sequence elements that are responsive to both IFNα and, more potently, IFNγ.[15,16] IDO expression, however, is not necessarily correlated with enzymatic acticity – in dendritic cells (DCs), for instance, ligation of CD80/CD86 by CTLA-4 on T cells appears to be required for activation of IDO enzymatic activity.[17]

Plasmacytoid dendritic cells are a specialized subset of DCs that stand at the intersection of innate and adaptive antiviral immunity.[18] Recent studies have found important differences in pDC function between natural and non-natural hosts of SIV infection, with humans having pDC function that more closely resembles the latter. Specifically, pDCs from natural hosts were shown to have impaired production of IFNα in response to SIV and other TLR7 and TLR9 ligands.[19] Given both the striking differences in pDC production of IFNα in response to TLR ligands in natural vs. non-natural host species, as well as the recently-described capacity of pDCs to express IDO,[20] which appears to be essential for their unique ability to function as specialized regulatory dendritic cells, we wondered whether both IDO expression and activity especially in pDCs, might differ between natural and non-natural host species of SIV (12).

Given the differences in signaling across TLR7 and TLR9 that have been described between pDCs in natural vs. non-natural host species (19), it also made sense to examine

the only other cell type known to express both of these toll-like receptors: B cells.[21] Specifically, we wondered if the apparent defect in TLR7 and TLR9 signaling observed in pDCs of the sooty mangabey was specific to that cell type or represented a more universal difference in intracellular TLR signaling between natural and non-natural SIV host species.

In this study, we examined several cohorts of natural (African Green Monkey or AGM) and non-natural non-human primate SIV host species (Pigtail and Rhesus Macaques or PM and RM) during the course of SIV infection. We found that IDO was significantly elevated in the periphery and lymphoid tissues of SIV-infected PMs and RMs vs. AGMs, and that this elevation remained higher during chronic infection in the non-natural vs the natural host species. We also found that pDCs from uninfected RMs expressed higher levels of IDO both before and after activation, compared to pDCs from AGMs. RM pDCs also had a higher capacity for *in vitro* production of IFNα in response to TLR7 and especially TLR9 ligrands. In chronically infected aimals, these effects were magnified. As with pDC function, B cells from RMs showed much higher capacity for stimulation across TLR7 and TLR9 vs those from AGMs. Taken together, these data paint a picture of a failure to downregulate the innate immune system during chronic infection in non-natural host species and a resultant fundamental difference in intracellular TLR signaling in natural vs. non-natural SIV host species, which is likely to contribute to the development of pathogenic outcomes in these animals by promoting a chronic state of innate immune activation and inflammation.

RESULTS

Elevated expression of IDO and IFNγ transcript during acute and chronic infection in Pigtail Macaques vs. African Green Monkeys

As part of ongoing efforts in our laboratory and others to understand the differences in the immune response between natural and non-natural hosts of SIV disease, and specifically how natural hosts manage to undergo non-pathogenic courses of infection despite high levels of viral replication. During the course of a study in which we examined global gene expression during SIV infection in natural (African green monkey) and non-natural (Asian pigtailed macaque), certain interesting trends with regards to IDO and related gene expression emerged. For this study, four animals of each species were infected intravenously with 600 $TCID_{50}$ of SIVagm.Sab92018 at day 0. Samples of blood and lymph nodes were collected at baseline (day -14), at peak viremia (day 10, acute infection), and at termination of the experiment (day 45, chronic infection). Additional characterization of the results of this study have been published in separate manuscripts.[22,23]

Microarray analysis was conducted on the blood and lymph node samples collected at the uninfected, acutely infected, and chronically infected time points. To determine upregulation of mRNA from relevant genes, we measured the fold-change in mRNA expression at acute or chronic infection time points vs. baseline. As shown in Figure 1a, IDO mRNA was significantly elevated during both the acute and chronic phases of

infection in pigtail macaques (PMs) as compared to African Green Monkeys (AGMs). This higher upregulation of IDO was seen in both the peripheral blood and lymph nodes of PMs vs. AGMs.

It has previously been shown that IDO requires IFNγ in order to be enzymatically active (17). Therefore, we looked at expression of IFNγ mRNA, as well as the expression of mRNA for the IFNγ receptors IFNγR1 and IFNγR2. As shown in Figure 1b, the expression of both IFNγ and the IFNγ receptors was significantly elevated in PMs vs. AGMs during acute infection in both peripheral blood and lymph nodes. In the case of chronic infection, differences in mRNA expression of IFNγ and the IFNγ receptors between the two species were not significant, but did show a trend in the same direction, indicating that, as with IDO mRNA, their expression may remain elevated in the non-natural SIV host species during the chronic phase of infection.

Immunohistochemistry shows higher expression of IDO in lymph nodes of acutely and chronically infected Pigtail and Rhesus Macaques vs. African Green Monkeys

As part of the study described above, lymph node biopsies were also preserved in paraffin and then sectioned for immunohistochemistry analysis. We also obtained additional lymph node samples from ongoing studies involving different African green monkeys as well as rhesus macaques, another non-natural host of SIV infection. Using a custom-made rabbit polyclonal antibody, we analyzed these sections for their expression of IDO, to determine whether the results seen in the microarray studies done on lymph

node tissue could be confirmed at the protein level in these tissues. Figure 2a-f shows representative sections from the lymph nodes of acutely infected and chronically infected AGMs vs. PMs and rhesus macaques (RMs). As shown in these representative sections, the expression of IDO is much higher in the LNs of the two macaque species vs. the AGMs during both acute and chronic infection, suggesting that IDO-expressing cells are migrating to the lymph nodes at a higher rate during SIV infection in these animals and/or that resident lymph node cells are upregulating IDO to a greater degree in the non-natural hosts. This is consistent with other published reports showing high expression of IDO in the lymph nodes of macaques undergoing pathogenic SIV infection (7,8).

We also quantitated this effect by calculating the expression of IDO as a function of surface area in these tissue sections. These numbers were calculated using Aperio Imagescope software (Aperio Technologies, Inc, Vista, CA), which measured the total number of positive (brown) pixels in a given tissue section as a function of the total hematoxylin-stained section of lymph node. The results support the idea that IDO is being expressed much more strongly in the lymph nodes of animals undergoing pathogenic infection (PMs and RMs) at both the acute and chronic stages, than in the lymph nodes of animals undergoing non-pathogenic infections (AGMs) (Figure 2g). Again, these results are consistent with published reports linking higher levels of IDO expression in lymphoid tissues with more pathogenic infections in macaque species (7,8). Such studies have not previously been published in African Green Monkeys or other natural SIV host species, and our results provide further support for the role of IDO in the

inflammatory processes which lead to pathogenic infection in SIV-infected non-natural SIV host species, but not in natural host species.

Higher and more prolonged upregulation of IDO activity in the periphery during SIV infection in Pigtail and Rhesus Macaques vs. African Green Monkeys

Since it has been shown that the expression of IDO protein by DCs and other cells doesn't always mean that the expressed IDO is enzymatically active (17), we also measured IDO activity in the periphery of pathogenically vs. non-pathogenically infected animals. Since IDO is the rate-limiting enzyme in the metabolism of tryptophan via the kynurenine pathway, IDO activity can be easily measured by determining the ratio of kynurenine to tryptophan (Kyn-Tryp) in the plasma of animals or humans, or in the supernatants of cell cultures.[24]

As shown in Figure 3, we determined several interesting trends about the Kyn-Tryp ratio in the periphery of animals undergoing pathogenic vs. non-pathogenic SIV infections. First we show Kyn-Tryp ratio data from the same animals used for the transcript expression studies (Figure 1, Figure 3a-b). We see that IDO activity in the periphery is elevated during acute infection in the AGM, peaking around Day 10 post infection, and then subsequently returns to baseline levels during chronic infection (Figure 3a). By contrast, the PMs from the same study upregulate IDO activity earlier in infection (peaking around Day 3 post infection), and to a greater degree than that seen in the AGMs (Figure 3b). Additionally, IDO activity in the PMs remains elevated above

baseline (pre-infection) levels in a statistically significant manner, indicating a sustained increase in IDO activity in these animals during chronic SIV infection.

Next we examined the kinetics of IDO activity during a longer course of infection in AGMs vs. RMs (Figure 3c-d). These animals were from separate, ongoing experiments in the laboratory examining the course of SIV infection. However, the data from the AGMs in this experiment (Figure 3c) shows a similar trend to that seen in the AGMs in the other experiment – an early upregulation of IDO activity (though in this experiment the peak was seen much earlier – Day 1 vs. Day 10 post infection), and a subsequent downregulation to baseline levels during the chronic phase of infection. By contrast, the RMs shown in Figure 3d had a similar early upregulation of IDO enzymatic activity early in infection (peak at Days 7-14 post infection), but they failed to downregulate IDO activity during chronic infection. As shown in Figure 3d, this effect was statistically significant, with IDO activity remaining elevated over baseline (uninfected) levels during chronic infection. This persistent upregulation of IDO activity in non-natural SIV host species suggests that prolonged IDO enzymatic activity is correlated with, and may play a role in, the persistent immune activation and inflammation which are associated with pathogenic SIV infection as seen in pigtail and rhesus macaques.

Higher IDO expression and secretion of IFNα in pDCs from uninfected rhesus macaques vs. African green monkeys

Based on recent reports that TLR9 signaling in plasmacytoid dendritic cells (pDCs) appears to be defective, or at least lower, in Sooty Mangabeys as compared to RMs (Mandl *et al*), as well as reports that TLR9 signaling can lead to strong upregulation of IDO by pDCs (Boasso *et al*), we decided to examine TLR9 signaling and expression of IDO in pDCs from SIV-uninfected AGMs vs. those from RMs. In addition to IDO expression in pDCs, we also examined expression of the co-stimulatory molecule CD86 and its upregulation following stimulation across TLR9 – CD80-86 ligation, along with IFNγ signaling, are important for enzymatic activation of IDO (Munn *et al* 2004). We also examined additional markers of pDC activation/maturation, CD40 and HLA-DR.

As shown in Figure 4a, baseline (i.e. before stimulation) expression of the activation/maturation markers CD40, CD86, and HLA-DR was similar on pDCs from both species. However, IDO expression was significantly higher in the RMs as compared to the AGMs, both as measured by the percentage of pDCs positive for IDO, as well as by the mean fluorescence intensity (MFI) of IDO on pDCs. Following stimulation, there was very little difference in upregulation of the activation/maturation markers CD40, CD86, and HLA-DR. However, in terms of IDO expression, RM pDCs maintained their high level of expression following stimulation, while pDCs from AGMs downregulated IDO. This phenomenon supports the data from Figures 1-3 suggesting that RMs are unable to downregulate IDO expression despite stimulation by TLR7 ligands.

We also examined IFNα secretion following stimulation with TLR7 and TLR9 ligands *in vitro*. As shown in Figure 4d, IFNα secretion was significantly higher in RMs vs. AGMs

following stimulation across both TLR7 and TLR9. These results agree with those of Mandl *et al*, and provide further support for a hypothesis of higher capacity for innate immune activation in non-natural vs. natural SIV host species.

Higher activation across TLR9 in peripheral B Cells from uninfected rhesus macaques vs. African green monkeys

To attempt to parse whether the apparent difference in pDC signaling between the non-natural host species (RMs) and the natural host species (AGMs) was restricted to pDCs (and therefore likely to be tied to pDC-specific differences between the two species, and particularly to differences within the IFNα signaling pathway), or whether it might be a more universal defect in TLR9 and/or TLR7 signaling, we examined whether B cells, the only other common cell type besides pDCs that expresses both TLR9 and TLR7, might show differences in activation with stimulation across TLR7 and/or -9.

As shown in Figure 5a, baseline expression of CD40 was slightly higher in AGMs as compared to RMs, as measured by the percentage of B cells positive for CD40. Upon stimulation, however, these animals showed a trend for higher levels of activation (i.e. upregulation of activation/maturation markers over baseline) in the RMs vs. the AGMs. This trend was restricted to upregulation of CD40 and CD86 following TLR7 and/or TLR9 stimulation, and was not apparent with regards to changes in HLA-DR expression on B cells. These differences suggest that a more universal difference may exist in the signaling pathways downstream of TLR7 and TLR9 in natural vs. non-natural SIV host species.

Higher IDO expression, IFNα secretion and activation across TLR9 in peripheral pDCs from chronically infected rhesus macaques vs. African green monkeys

Given the finding, shown in Figures 1-3, that IDO expression and activity remain elevated in non-natural host species vs. natural host species during chronic SIV infection, we decided to examine whether differences in pDC activation would extend during chronic infection as well. For these experiments, we used samples from chronically infected RMs and AGMs (chosen based on the persistent activation/inflammation seen in the RMs but not in the AGMs), to try to verify that differences in pDC function were intrinsic to the different species and were present in the setting of chronic infection, during which we posit that ongoing innate immune activation may drive continued inflammation and related pathogenesis.

As shown in Figure 6a, baseline (i.e. non-stimulated) expression of IDO by pDCs was significantly higher in RMs vs. AGMs as measured both by the percentage of pDCs expressing IDO and also the mean fluorescence intensity of IDO on the pDCs. This agrees with the previous data in this paper, and also with published reports showing that IDO expression and activity are higher in the case of pathogenic SIV infections in the macaque as compared to non-pathogenic infections in the macaque (7,8,9). The other major difference seen between the two species at baseline was the percentage of peripheral pDCs expressing the costimulatory molecule CD86 – this was significantly higher in AGMs vs. RMs, however, the significance of this difference is unclear.

Next we examined the capacity of pDCs to upregulate activation/maturation markers following *in vitro* stimulation with TLR7 and TLR9 ligands. As shown in Figure 6b, RMs showed a larger increase in the percentage of pDCS positive for both CD40 and HLA-DR compared to AGMs following stimulation with the TLR9 ligand CpG-C. Figure 6c shows that this same trend was apparent when we measured the mean fluorescence intensity (MFI) of activation/maturation markers and on pDCs on both species following TLR9 stimulation. These differences suggest that peripheral pDCs isolated from non-natural hosts of SIV have a higher capacity for stimulation across TLR9 compared with those from natural host species. Similarly to what was seen in uninfected animals (Figure 4), IDO expression was downregulated in the AGM pDCs following stimulation across both TLR9 and TLR7, while it remained the same in the RMs.

Figure 6d shows that IFNα secretion following *in vitro* stimulation remained higher in RMs as compared to AGMs during chronic infection. While the differences were not quite as profound as those seen in uninfected animals, these data agree with recent reports suggesting intrinsically lower capacity for IFNα secretion by pDCs from Sooty Mangabeys, another natural host species for SIV, as compared to RMs (Mandl *et al*).

Higher activation across TLR9 in peripheral B Cells from chronically infected rhesus macaques vs. African green monkeys

As shown in Figure 7a, baseline (i.e. before stimulation) expression of the activation/maturation markers CD40 and CD86 was significantly higher on B cells from RMs vs. AGMs, as measured by the percentage of pDCs expressing these markers as well as, in the case of CD86, the mean fluorescence intensity on pDCs. In Figure 7b, we see that upon stimulation across both TLR9 and TLR7, B cells from the non-natural SIV host species (RMs) show significantly higher increases in the percentage of B cells positive for the different activation/maturation markers as compared to those from the natural SIV host species, the AGM. Figure 7c shows that, as with the pDCs, the trend is the same when we measure the MFI of the activation/maturation markers on B cells following stimulation in the two species. When taken together with the pDC data, these data suggest an intrinsic difference in the signaling pathways of TLR9 and possibly also TLR7 between the RMs and the AGMs. Furthermore, these data suggest that it is likely that this difference is not restricted to the IFNα-producing portion of the TLR9 signaling pathway, since this portion of the signaling pathway has only been shown to be active in pDCs, and we observed similar (if not stronger) signaling differences in B Cells between the species.

DISCUSSION

Many studies have shown the strong association between non-specific immune activation and inflammation and progression of lentiviral infections (1,2). Specifically, inflammatory processes have been shown to be crucial in the development of a pathogenic response to SIV infection in non-natural nonhuman primate host species of SIV vs the non-pathogenic case seen in natural (African) hosts of SIV infection (3-6). Several lines of evidence had pointed to a potential role of the major IFNα-secreting cell population, pDCs, in these inflammatory processes in non-natural hosts of SIV (25,26). A recent study showed stark differences in the abilities of pDCs from non-natural SIV hosts (rhesus macaques) vs natural SIV hosts (sooty mangabeys) to signal across TLR7 and TLR9, and specifically, showed defects in the ability of sooty mangabey pDCs to secrete IFNα in response to *in vitro* stimulation (19). Here, we have confirmed and extended these results in the context of additional nonhuman primate models of lentiviral infection.

Taken together, our data show an overall picture of higher inflammation and capacity for innate immune activation in non-natural hosts of SIV vs. natural hosts. In comparing non-natural vs. natural SIV host species, we found higher levels of IDO expression and activity in tissues and periphery of SIV-infected RMs and PMs vs. AGMs. We also found higher expression of IDO by pDCs in both uninfected and chronically infected RMs vs. AGMs. Higher levels of IDO have been strongly linked with pathogenic

infections in both HIV-infected humans and SIV-infected primates (7,8,9), although the mechanism by which IDO might drive disease progression remains controversial.

In this paper, we also determined that both pDCs and B Cells from the periphery of SIV-uninfected and chronically infected RMs vs. AGMs showed a higher capacity for activation in response to stimulation with TLR7 and TLR9 ligands, in agreement with recent data showing a higher capacity for production of IFNα by pDCs from non-natural host animals (Mandl *et al*). This last we also found to be true for AGMs – they produce significantly less IFNα following TLR7 and TLR9 stimulation *in vitro* as compared to RMs, similar to what has been seen for the sooty mangabey. However, it is important to note the possibility that *in vitro* pDC data may be misleading – it was recently shown that pDCs during human HIV infection responded differently *in vitro* vs. *in vivo* with regards to their ability to produce IFNα.[25] Despite this caveat, we feel that our data paint a compelling picture of ongoing (and in some cases, enhanced) higher capacity for innate immune activation in non-natural SIV host species as compared to natural SIV host species. This difference appears to be intimately linked with signaling through TLR7 and TLR9, and it will be interesting to see if downstream signaling molecules in these pathways can be shown to be fundamentally different between the two types of host species.

It should also be noted that while the lesion studied here in African green monkeys appeared to be confined to pDCs and B cells, it is also possible that other cells types may make IFNα and/or IDO. It is clear that AGMs are capable of producing IDO

immediately following infection, as shown in Figure 3, and previous studies have also shown that IFN-response genes are upregulated directly following infection of AGMs (22,23). This may indicate that the apparent lesion we characterized here relates primarily to persistent infection, and that very early-stage responses to infection are not, in fact, appreciably different between natural and non-natural host species with regards to IFNα and/or IDO. Regardless, our data clearly show a defect in *in vitro* IFNα production during both uninfected and ongoing/chronic phases of SIV infection in the AGM. This could plausibly have a significant impact on the course of disease progression, because type I IFNs are known to drive inflammatory responses, and pDCs specifically have been shown to drive innate immune activation and to be associated with inflammation in the context of HIV/SIV infection (9, 19, 22, 23, 25, 26).[26]

Similarly, the absence of IDO production by pDCs and possibly other cell types during chronic infection in the AGM could also have a significant impact on lentiviral disease progression. Multiple studies have suggested an important role for tryptophan and tryptophan metabolite levels in T cell activation and the balance of T effector and T regulatory cells.[27,28,29] Given what we now know about the importance of non-specific T cell activation and inflammation in driving pathogenic SIV and HIV infections, it is possible that a prolonged upregulation of IDO as seen in the pathogenic non-natural host models of non-human primate SIV infection has a profound effect on the balance of T cell activation and levels of regulatory T cell activity, and thus on disease progression.

Despite the results presented here, it is important to consider several additional factors when interpreting this story. The first is whether fold-changes in IDO and other markers of activation on specific cell types following *in vitro* stimulation are a relevant measurement in the setting where baseline levels are non-equivalent between the species, as seen here. While it is possible that AGM and other natural SIV hosts are relatively resistant to activation when compared with non-natural hosts, it is also possible that cells from these animals have simply reached their activation threshold and cannot be activated further. This would in fact imply the opposite of the conclusion we have drawn here – it would suggest that natural host pDCs and B cells actually undergo higher levels of activation *in vivo*. Another important caveat to consider is the relatively small number of animals studied. While the results did appear relatively homogenous within species cohorts, it is possible that the observed effects could simply be due to sampling bias, and that a larger study would fail to confirm these results.

Many open questions remain based on the results presented here. First, it will be interesting for future studies to examine why and how IDO expression and activity are apparently dampened following primary infection in non-pathogenic settings, as seen here in the AGM. One thing that is clear from studies of the role of tryptophan metabolism in chronic immune activation is that this dampening effect on IDO activity is likely to have beneficial effects – sustained upregulation of IDO activity has been shown to be associated with higher levels of pathogenicity in a wide variety of disease settings.[30,31,32] While the full complement of factors controlling the production and activation of IDO have not yet been elucidated, it is possible that there is some sort of

underlying genetic difference in either CD80/CD86 or in the IFNγ pathway, both of which are required for IDO activation; IFNγ also helps to control IDO mRNA production, by binding to IFNγ-responsive elements in the IDO promoter.[33,34] It is also possible that there is a more universal difference in the mechanisms that account for dampening of the immune response in natural SIV hosts as compared to non-natural hosts. An extremely diverse set of elements controls the dampening of an immune response, including mRNA stability and the production of specific lipid mediators, and it is clear that the stage is set very early in an inflammatory response for the termination of that response.[35,36,37,38] Therefore, it seems reasonable to ask whether fundamental differences in the nature of the innate immune response between pathogenic vs nonpathogenic lentiviral infections may account for the apparent differences in the downregulation of immunomodulatory factors such as IDO as well as the differences in levels of chronic inflammation during chronic infection.

Finally, and perhaps most important, it will be important to determine how these findings in the various animal models of lentiviral infection relate to the situation in human HIV infection. While the situations in so-called human "elite controllers" and natural hosts of SIV are fundamentally different – for example in that natural hosts of SIV undergo high levels of viral replication during infection whereas elite controllers suppress the majority of viral replication – it is still possible that parallels exist in the ways that these two groups respond to a given stimulus. Furthermore, if such parallels did exist, it would provide important clues as to the nature of non-pathogenic lentiviral infection in both

humans and non-human primates. For this reason, if no other, it is worth rigorously pursuing this line of inquiry.

MATERIALS AND METHODS

Microarray study of PM and AGM tissues and blood

Immunohistochemistry for IDO

HPLC for measurement of kynurenine and tryptophan in plasma

***In vitro* Stimulation and flow cytometry**

Cryopreserved PBMCs from chronically infected RMs and AGMs were thawed and then were re-suspended in RPMI-based media containing FCS, L-glutamine, and Penicillin-Streptomycin, and incubated 20-24 hours at 37°C. Stimulations were used as follows: CpG-C (ODN M362 from Invivogen, San Diego, CA) was used at 5 μM. Imiquimod (Invivogen, San Diego, CA) was used at 20μM. Supernatants were isolated following stimulation, and stored at -20°C for later analysis.

Following stimulation, cells were washed with a "PBS-plus" buffer [containing phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2.5 mM EDTA]. The same buffer was used to prepare cocktails of fluoresceinated antibodies against surface markers, including CD123 PE, CD3 Pacific Blue, CD40 APC, CD86 FITC, HLA-DR PE-Cy7 (all BD Biosciences, San Jose, CA), CD20 ECD, and CD14 QDot605 (Invitrogen, Carlsbad, CA). An amine dye (Invitrogen, Carlsbad, CA) was used to discriminate live from dead cells according to the manufacturer's instructions. Cells were stained for 30 minutes at 4° C, and then washed twice with PBS-plus.

Cells were subsequently permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) as per the manufacturer's instructions, then stained with intracellular antibodies: rabbit anti-IDO primary antibody (Axxora, San Diego, CA) and Alexa-700 conjugated goat anti-rabbit (Molecular Probes). Samples were resuspended in PBS-plus buffer following staining and run on an LSR II flow cytometer (BD Biosciences, San Jose, CA). All data were analyzed using FlowJo software (Treestar).

ELISA for IFNα

ELISAs to detect IFNα secretion were performed using the *Verikine*™ Human Interferon-Alpha Serum Sample ELISA kit (PBL Biomedical Labs, NJ), according to the manufacturer's directions. Briefly, supernatants were diluted as necessary, and incubated in antibody-coated plates for 1 hour at 26° C. Plates were washed and then a biotin-conjugated secondary detection antibody was added. Following secondary antibody incubation, the plates were washed and then incubated with a streptavidin-conjugated horse radish peroxidase (HRP) enzyme. Finally, a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate for the HRP was added, causing the development of a color which was analyzed using a plate reader to measure the optical density of each sample. The concentration of IFNα was then calculated based on known standards included with each test.

FIGURE LEGENDS

Figure 1. Microarray studies of IDO, IFNG, and IFNGR expression in blood and lymph node of SIV-infected pigtail macaques (PM) and African green monkeys (AGM). Time points were was follows: baseline was day -14 before infection, acute infection was day +10 after infection, and chronic infection was day +42/study termination. (a) Fold-change in expression of IDO gene mRNA – fold change was calculated as a function of uninfected expression in the same animals, and data was averaged across 4 animals of each species. (b) Fold-change in expression of IFNG, IFNGR1 and IFNGR2 genes, calculated same as (a).

Figure 2. Immunohistochemistry of lymph node sections from pigtail macaques (PM), rhesus macaques (RM), and African green monkeys (AGM), and quantitation of IDO staining. Representative sections are as follows: (a) PM acutely infected, (b) RM acutely infected, (c) AGM acutely infected, (d) PM chronically infected, (e) RM chronically infected, and (f) AGM chronically infected. All sections were stained with a polyclonal anti-IDO antibody, and the brown staining (3,3',5,5'-Tetramethylbenzidine (TMB) substrate reaction with HRP-conjugated secondary antibody) represents IDO. (g) Quantitation of IDO staining in lymph nodes, done by counting positive (brown) pixels as a percentage of total tissue area on the slide, using Aperio Imagescope software (Aperio Technologies, Inc, Vista, CA), showing that IDO expression is upregulated in a stronger and more sustained fashion in lymph nodes from RMs and PMs vs AGMs. *$p<0.05$, **$p<0.01$.

Figure 3. Measurement of Kynurenine-Tryptophan (Kyn-Tryp) ratio in the periphery of PMs, RMs, and AGMs as a measurement of IDO enzymatic activity. (a) and (b) PMs and AGMs who were simultaneously infected with SIV, shows that IDO enzymatic activity is upregulated higher and sooner in PMs, and remains elevated during chronic infection. (c) AGMs from a different study, shows that IDO activity returns to baseline during chronic infection and remains there over a longer time course than that shown in (a) and (b). (d) RMs also show sustained upregulation of IDO during longer-term chronic infection, similar to PMs and different than AGMs. *$p<0.05$.

Figure 4. Uninfected RMs show higher expression and upregulation of IDO and IFNα on pDCs compared to AGMs. (a) Baseline expression of CD40, CD86, HLA-DR, and IDO on pDCs, showing both the percentage of pDCs positive for a given marker (left) and the mean fluorescence intensity (MFI) of those markers on the pDCs (right). (b) Changes in the percentage of pDCs positive for CD40, CD86, HLA-DR and IDO following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). (c) Changes in the MFI of CD40, CD86, HLA-DR and IDO on pDCs following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). (d) Secretion of IFNα (pg/mL) following stimulation with CpG-C (TLR9 ligand) and imiquimod (TLR7 ligand). *$p<0.05$.

Figure 5. Uninfected RMs show higher expression and upregulation of activation markers on B cells compared to AGMs. (a) Baseline expression of CD40, CD86 and HLA-DR on B cells, showing both the percentage of B cells positive for a given marker (left) and the mean fluorescence intensity (MFI) of those markers on the B cells (right). (b) Changes in the percentage of B cells positive for CD40, CD86 and HLA-DR following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). (c) Changes in the MFI of CD40, CD86 and HLA-DR on B cells following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). *$p<0.05$.

Figure 6. Chronically infected RMs show higher expression and upregulation of IDO, activation/maturation markers and IFNα on pDCs compared to AGMs. (a) Baseline expression of CD40, CD86, HLA-DR, and IDO on pDCs, showing both the percentage of pDCs positive for a given marker (left) and the mean fluorescence intensity (MFI) of those markers on the pDCs (right). (b) Changes in the percentage of pDCs positive for CD40, CD86, HLA-DR and IDO following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). (c) Changes in the MFI of CD40, CD86, HLA-DR and IDO on pDCs following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). (d) Secretion of IFNα (pg/mL) following stimulation with CpG-C (TLR9 ligand) and imiquimod (TLR7 ligand). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 7. Chronically infected RMs show higher expression and upregulation of activation markers on B cells compared to AGMs. (a) Baseline expression of CD40, CD86 and HLA-DR on B cells, showing both the percentage of B cells positive for a given marker (left) and the mean fluorescence intensity (MFI) of those markers on the B cells (right). (b) Changes in the percentage of B cells positive for CD40, CD86 and HLA-DR following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). (c) Changes in the MFI of CD40, CD86 and HLA-DR on B cells following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand). Fold change following activation is calculated based on the baseline numbers shown in (a). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

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FIGURE LEGENDS

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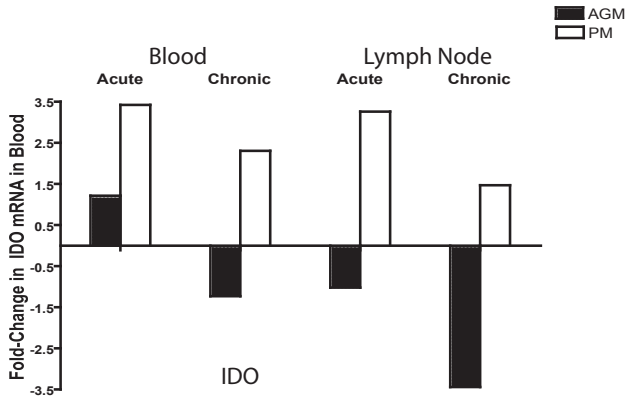
Figure 6. Secretion of IFNα and IFNγ is higher in RMs vs AGMs following *in vitro* stimulation. (a) IFNα secretion following following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand), as measured via ELISA. (b) IFNγ secretion following following *in vitro* stimulation with CpG-C (TLR9 ligand) or imiquimod (TLR7 ligand), as measured via ELISA. **$p<0.01$.

Figure 1.

a.


AGM
PM
Blood
Lymph Node
Acute
Chronic
Acute
Chronic
Fold-Change in IDO mRNA in Blood
3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
-3.5
IDO

b.

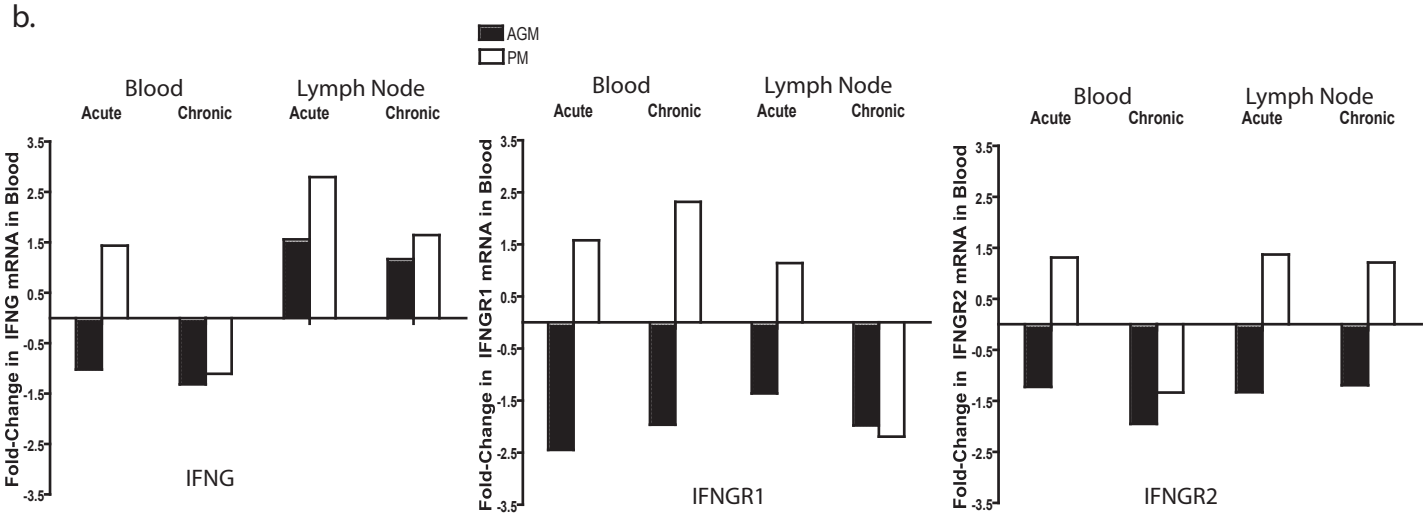
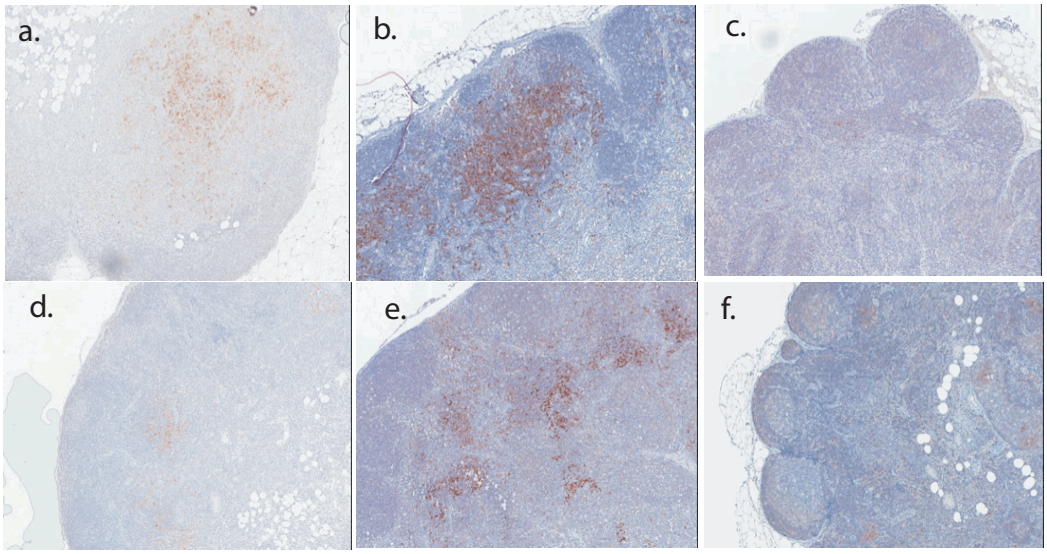

AGM
PM
Blood
Lymph Node
Acute
Chronic
Acute
Chronic
Fold-Change in IFNG mRNA in Blood
IFNG
Fold-Change in IFNGR1 mRNA in Blood
IFNGR1
Fold-Change in IFNGR2 mRNA in Blood
IFNGR2

Figure 2.


a.
b.
c.
d.
e.
f.

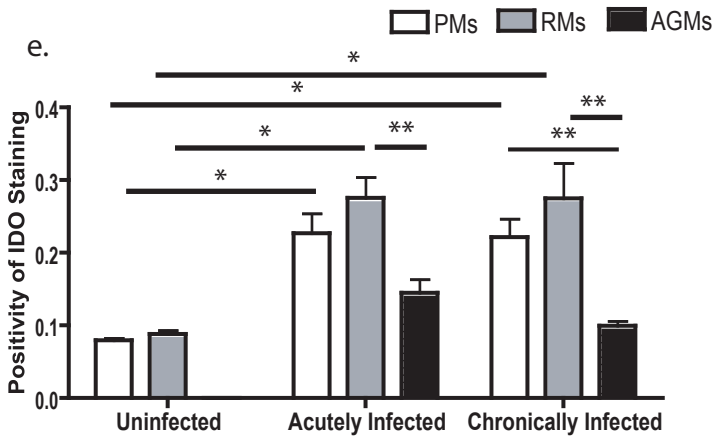

e.
PMs
RMs
AGMs
Positivity of IDO Staining
0.4
0.3
0.2
0.1
0.0
*
*
*
*
**
**
**
Uninfected
Acutely Infected
Chronically Infected

Figure 3.

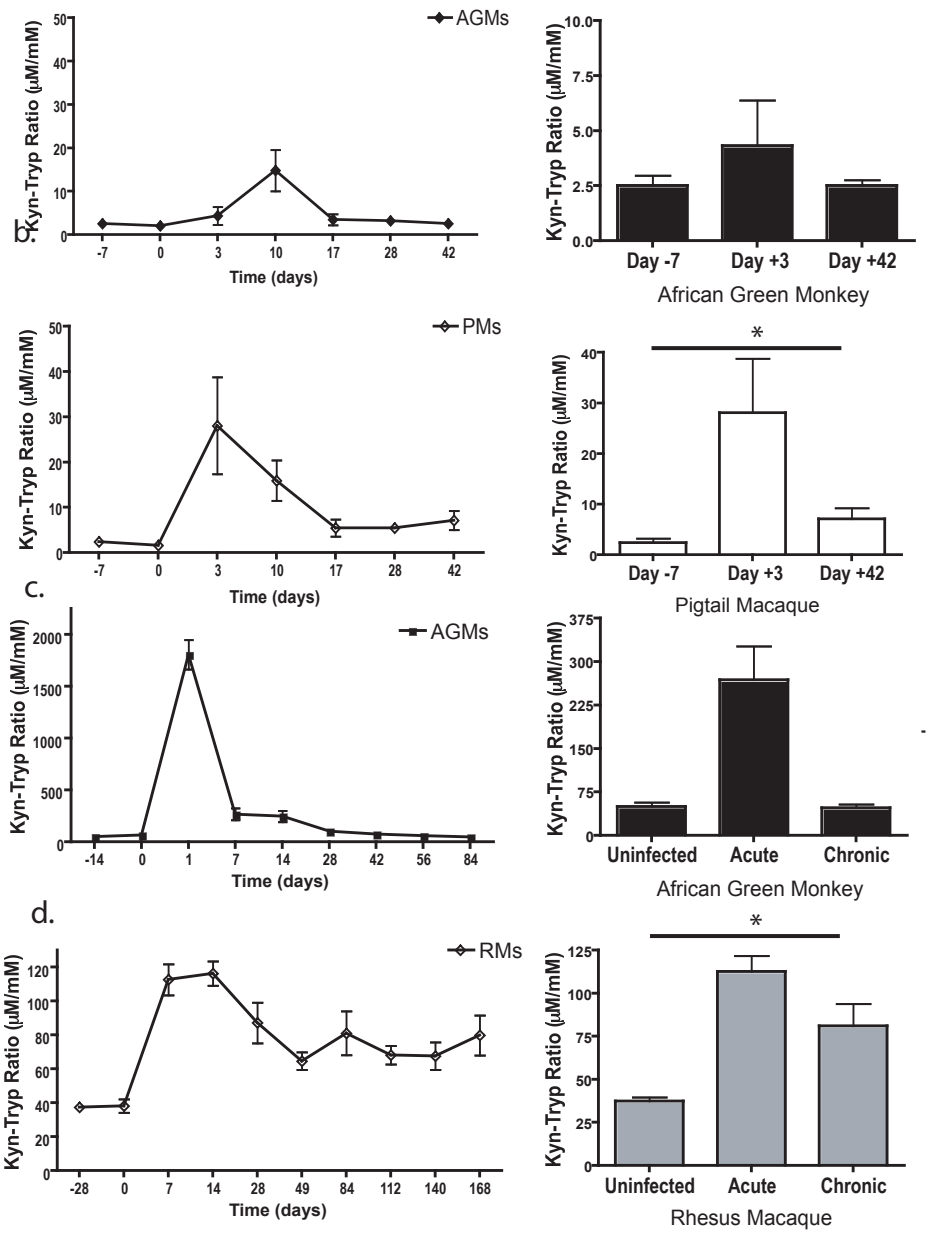
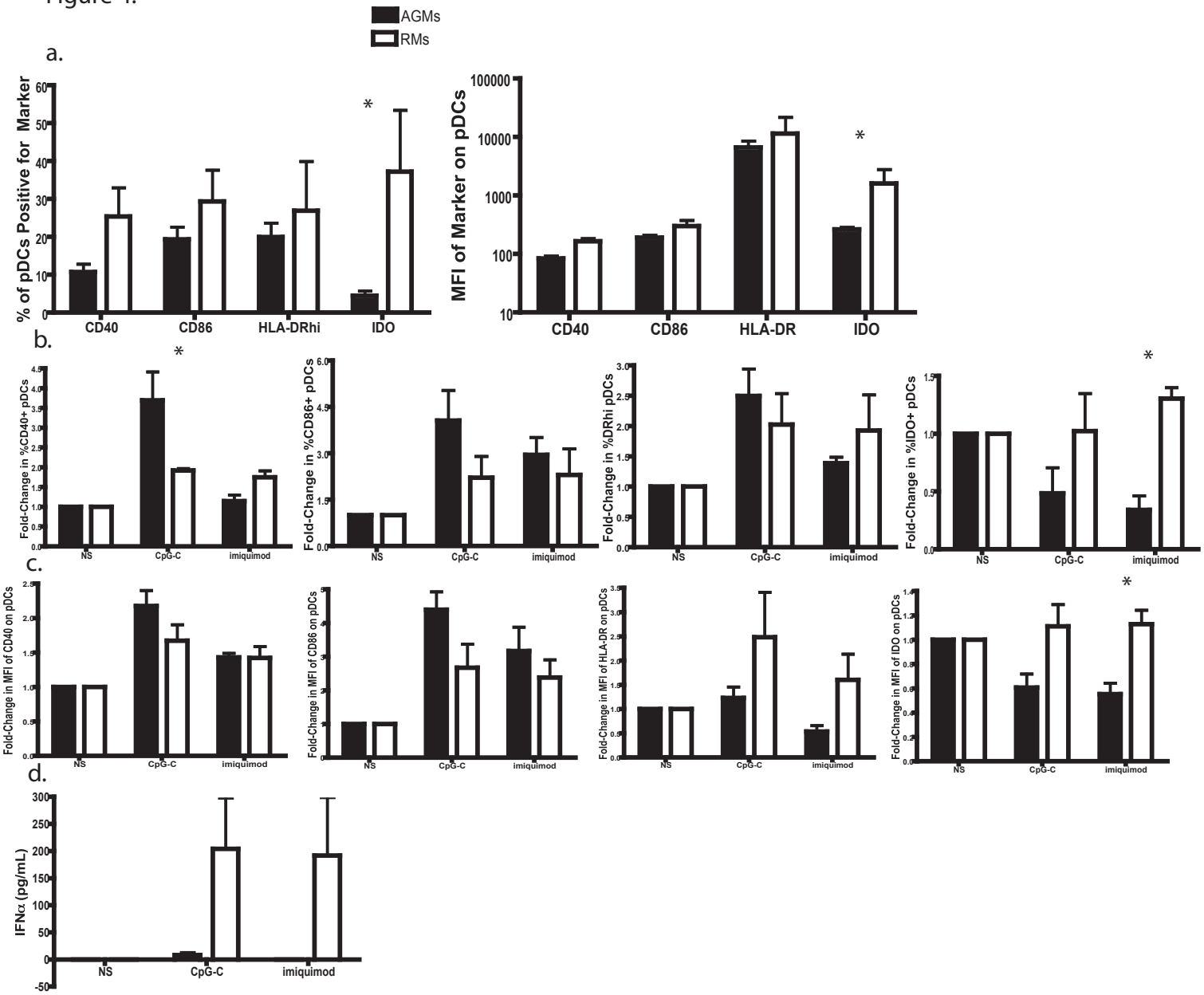
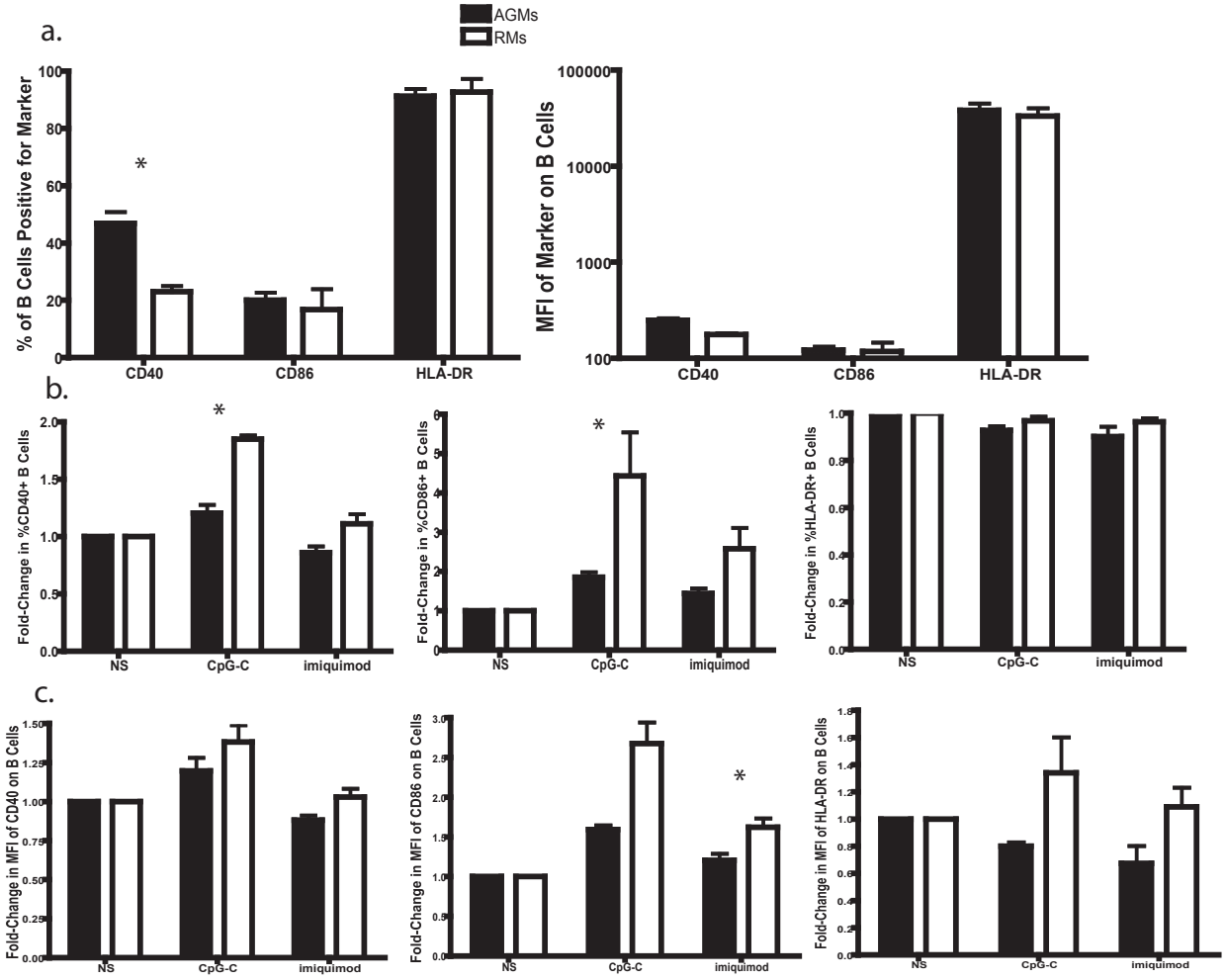

a.
AGMs
Kyn-Tryp Ratio (µM/mM)
Time (days)
-7
0
3
10
17
28
42
Day -7
Day +3
Day +42
African Green Monkey
b.
PMs
Pigtail Macaque
*
c.
AGMs
-14
1
7
14
28
42
56
84
Uninfected
Acute
Chronic
African Green Monkey
d.
RMs
-28
49
84
112
140
168
Rhesus Macaque

Figure 4.



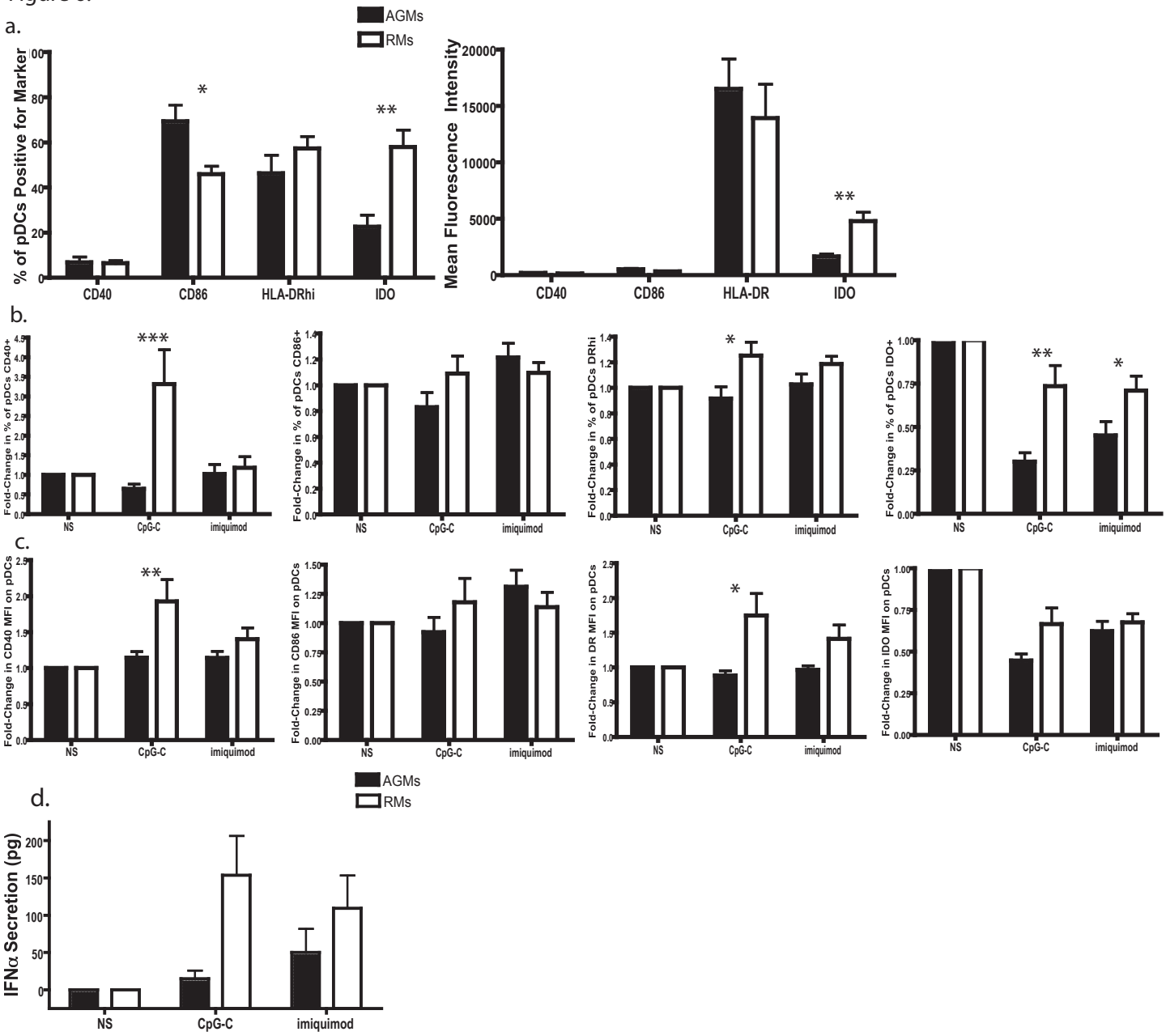
*p < 0.05

Figure 5.



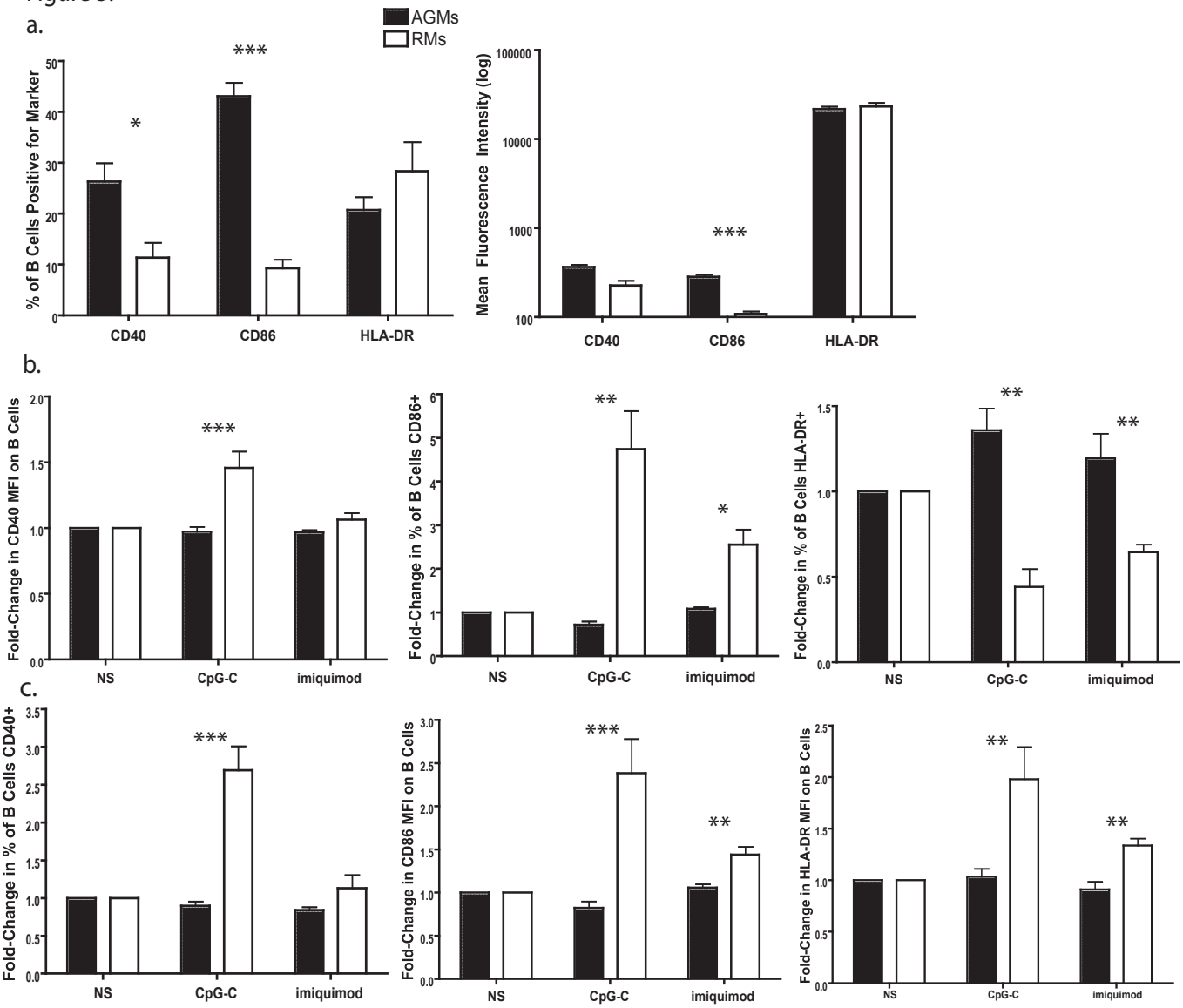
*p < 0.05

Figure 6.



*p < 0.05, **p < 0.01, ***p < 0.001

Figure 5.



*p < 0.05, **p < 0.01, ***p < 0.001

CONCLUSION

Overall, the cumulative results of this thesis work indicate that while important differences in pDC functionality appear to exist between natural and non-natural hosts of SIV infection, and while these differences in plasmacytoid dendritic (pDC) cell function may help to drive differential disease outcomes in this setting, such differences do not appear to exist between HIV-seropositive elite controllers and other HIV-seropositive patients who do not control viremia. Given the importance of understanding the mechanisms by which elite controllers manage to control viremia, and, in most cases, avoid disease progression, this work provides an important indication that pDC number and/or function, at least during chronic infection, are unlikely to drive these processes. As indicated in the discussion in Chapter 2, it will be important to assess whether pDCs might play a role at an earlier point in disease (i.e. during primary infection) in terms of determining later pathogenicity and disease progression outcomes. Also, this work, as most other work in this field, focused exclusively on pDCs in the periphery HIV-seropositive patients; it will be important for future work to try to determine if pDC and other immune cell functions in lymphoid tissue differ between elite controllers and other classes of HIV-seropositive patients. It may also be interesting to focus future studies on the connections between pDC functionality and the functionality of other immune cell types, such as T cells. Preliminary studies based on the results discussed here indicate that some connections between T cell function and pDC function may exist – larger studies may be warranted in the future to explore whether such a connection does indeed exist, either among elite controllers, or among all HIV-seropositive patients.

While the data presented here did not show a similarity in pDC function between natural hosts of SIV and HIV elite controllers, as we had originally hypothesized might exist, intriguing preliminary data from long-term nonprogressors (LTNPs) had previously indicated that in some cases, such a connection might exist. Specifically, as discussed in the attached manuscript draft entitled "HIV-Seropositive "Elite Controllers" Have Apparently Normal Plasmacytoid Dendritic Cell Function," these findings showed that a small cohort of LTNPs, some with high and some with low viral load, were refractory to TLR9 stimulation, similar to what is seen in natural hosts of SIV infection. It is possible that such findings could eventually be duplicated if a larger sample of patients were examined, and thus that there may be individuals with an apparent defect in their pDC compartment similar to the one seen in natural hosts of SIV, which is protective during chronic lentiviral infection.

Our work supported and extended a finding of an important lesion in natural hosts of SIV infection with regards to signaling across TLR7 and TLR9, and pDC function more widely, as well as IDO expression and activity, both in pDCs and universally. It will be interesting and important to further explore these differences. It would be of particular interest to evaluate the upstream and downstream signaling pathways that may lead to downregulation of both IDO and/or TLR7 and TLR9 signaling in natural but not non-natural SIV host animals during chronic infection. Such differences may be genetic, or they may be driven more by post-translational modifications and/or interactions. Either way, determining the mechanisms that drive downregulation of the harmful inflammatory pathways of the innate immune system, particularly during chronic infection in these

animals, might provide further important clues to the processes regulating ongoing pathogenicity and eventual progression to an AIDS-like syndrome in non-natural host species. Such understanding could provide important future directions in exploring and understanding the rare but extremely heterogeneous group of HIV-seropositive individuals who control HIV infection and show limited or no progression towards AIDS, even in the absence of antiretroviral treatment.

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