Primary infection with simian immunodeficiency virus: Plasmacytoid dendritic cell homing to lymph nodes, type I IFN and immune suppression

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**List of non standard abbreviations:**

SIV : simian immunodeficiency virus
IDO : indoleamine 2,3-dioxygenase
PDC : plasmacytoid dendritic cells
Kyn : kynurenine
Trp : tryptophan

**Running title:**

PDC in SIV infection and immune suppression

**Key words:**

Simian immunodeficiency virus, indoleamine-2,3-dioxygenase, plasmacytoid dendritic cells, interferon, HIV, primary infection
Abstract

Plasmacytoid dendritic cells (pDC) are antigen-presenting cells which develop into type-I interferon (IFN-I)-producing cells in response to pathogens. Their role in HIV pathogenesis needs to be understood. We analyzed their dynamics in relation with innate and adaptive immunity very early during the acute phase of simian immunodeficiency virus (SIV) infection in 18 macaques. PDC counts decreased in blood and increased in peripheral lymph nodes, consistent with early recruitment in secondary lymphoid tissues. These changes correlated with the kinetic and intensity of viremia and were associated with a peak of plasma IFN-I. IFN-I and viremia were positively correlated with functional activity of the immune suppression associated enzyme IDO and FoxP3+CD8+ T cells, which both negatively correlated with SIV-specific T-cell proliferation and CD4+ T-cell activation. These data suggest that pDC and IFN-I play a key role in shaping innate and adaptive immunity towards suppressive pathways during the acute phase of SIV/HIV primary infection.
Introduction

Plasmacytoid dendritic cells (pDCs) are major type I interferon (IFN-I) producing cells (IPCs) in response to viral infections \( ^1 \) due to selective expression of Toll-like receptors (TLR) 7 and 9 and constitutive expression of interferon response factor 7 (IRF-7). They migrate to inflamed lymph nodes (LN) through high endothelium venules during viral and bacterial infections \( ^2,^3 \) and provide an important link between innate and adaptive immunity, enhancing natural killer (NK) cell activity and adaptive immune responses.

Blood pDC counts and pDC-dependent IFN-I production levels \textit{in vitro} decrease in patients infected with human immunodeficiency virus (HIV) \( ^4,^8 \). These decreases are generally correlated with a fall in CD4+ T-cell counts, inversely correlated with plasma viral load, and are associated with opportunistic infections \( ^5,^6,^9,^12 \). The reduction in circulating pDC numbers during HIV infection may be related to their direct infection \( ^6,^9,^13 \), or to redistribution to lymphoid organs, as suggested in the chronic asymptomatic stage of HIV infection \( ^14 \).

In nonhuman primate models of HIV infection, the IFN-I innate response is an early immunological event \( ^15,^17 \). Rhesus macaque pDCs are activated and produce IFN-I \textit{in vitro} in response to pathogenic simian immunodeficiency virus (SIV) \( ^18,^19 \) like their human counterparts in response to HIV \( ^20,^21 \). Strong depletion of pDC from blood and lymphoid tissues are reported in the end stage of SIV infection \( ^19,^22 \), and attributed to infection and to higher levels of apoptosis. However, although the HIV/SIV interplay with the host immune response during primary infection is a key event, probably determining the later progression to disease, little is known about the role of pDC in immune regulation at this early stage.

We predicted that HIV/SIV infection may have an impact on the dynamics of circulating and LN DC, and on their functions in the first few days of infection \( ^{23,^24} \) and that it may induce immune suppression through IFN-I production and indoleamine-2,3-dioxygenase (IDO)
activity. IDO is the rate limiting enzyme responsible for the extrahepatic catabolism of the essential amino acid tryptophane (Trp) and is triggered by type I and II interferons \(^\text{25-27}\). Abnormal production/activation of IDO is associated with inefficient immunological responses to infections, including HIV/SIV and cancer \(^\text{26,28-30}\) and is induced by HIV in human pDC \textit{in vitro} \(^\text{20,21}\). There is growing evidence to suggest that pDC are involved in the induction of tolerance, through IDO-dependent mechanisms \(^\text{31}\). This suggests that pDC may target immune suppression during the acute phase of HIV/SIV infection.

We focused on the dynamics of pDC during primary infection by carrying out fine time-resolution sampling of blood, and a longitudinal analysis of peripheral lymph nodes, using absolute quantification, to investigate the possible homing of these cells into lymphoid tissues. In parallel, the kinetics of virus replication, IFN-I and II responses, and IDO activity were analyzed, to improve our understanding of the contributions of virus and IFNs to IDO activation \textit{in vivo}. We also analyzed pDC and IDO dynamics as a function of the dynamics of FoxP3+CD25+CD8+ T cells and SIV-specific T-cell proliferative responses during primary infection (Karlsson et al., 2007b). Experiments were performed in 18 cynomolgus macaques, and the role of virus infection was assessed by using two different infectious doses or by using post-exposure antiretroviral treatment.
Material and methods

Animals

Young adult cynomolgus macaques (Macaca fascicularis) weighing 3 to 5 kg were imported from Mauritius and kept according to European guidelines for animal care (“Journal Officiel des Communautés Européennes”, L358, December 18, 1986). Blood sampling, biopsies and treatment were carried out in macaques anesthetized by intramuscular injection of 10 mg/kg ketamine (Rhone-Mérieux, Lyon, France). Six macaques received 5,000 animal infectious dose 50% (AID50) of SIVmac251 intravenously (iv; 5,000 AID50 group), six received 50 AID50 iv (50 AID50 group), and six were infected iv with 50 AID50 and treated from 4 hours to 28 days post-exposure (pi). This ART group was given a combination of 4.5 mg/kg AZT, 2.5 mg/kg 3TC and 20 mg/kg Indinavir twice daily, via the oral route, as previously described. Virus stock was kindly provided by Dr A.M. Aubertin (Université Louis Pasteur, Strasbourg, France).

Phenotypic characterization and intracellular staining

Blood samples were collected into CPT tubes for the isolation of PBMCs, according to manufacturer’s recommendations. Cells were incubated with FcR blocking reagent (Miltenyi Biotech) for 10 min at room temperature and labeled with monoclonal antibodies (Supplemental Table 1) specific for CD123, HLA-DR, and either CD4, or CD7, or CD11b, or CD36, or CD40, or CD45RA, or CD80, or CD86, or CD184 (CXCR4), or CD185 (CCR5), or CD206, or CD1c (BDCA-1), or with isotypic control antibodies for 20 min at +4°C. Cells were then washed in PBS, and fixed in Cell Fix (BD Biosciences). PDC were gated on CD123bright, HLA-DR+ cells as described.

For IFN-α intracellular labeling, PBMC were stimulated by incubation for 6 hours with
HSV-I at a multiplicity of infection of 0.5 or with 20 µg/ml total protein from aldriethiol-2 (AT-2)-inactivated SIVmac239 (SIV-AT2). Brefeldin A (Sigma Aldrich) was added (10 µg/ml) for the last 4 hours. Mock stimulation with AT-2-treated SupT1 microvesicles was used as a negative control. AT-2-inactivated SIVmac239 (ARP1018.1) and its negative control (ARP1018.2) were obtained from Dr. Jeff Lifson (NCI, Frederick, MD, USA), through the EU Program EVA Centralized Facility for AIDS Reagents (NIBSC, Potters Bar, UK). Intracellular staining was performed using anti-human IFN-α2a monoclonal antibody (kindly provided by Dr Jacques Banchereau, Baylor Institute for Immunology Research, Dallas, Texas, USA), as previously described 24.

**Plasma viral load, T-cell counts, and T-cell proliferation assay**

Plasma viral load was determined by real-time PCR, and T-cell counts, including FoxP3+ T cells, were determined by flow cytometry as described previously 33. T-cell proliferation assay in response to inactivated SIV was performed as reported 33.

**pDC quantification**

Absolute numbers of lineage^HLA-DR^CD123^CD1c^ pDC were quantified in whole blood using TruCount tubes 24.

Lymph node biopsies were weighed, dilacerated and passed through cell strainers (BD Biosciences). The percentage of lineage^HLA-DR^CD123^CD1c^ pDC was determined on freshly isolated cells 24. Absolute counts of pDC were obtained by multiplying the total number of lymph node cells isolated per gram of tissue by the percentage of pDC divided by 100.

**In situ immunofluorescence**
Small blocks of lymph node tissue were snap-frozen in liquid nitrogen and stored at -80°C. Ten µm sections were cut with a CM1500 cryotome (Shandon, Thermo Scientific, Courtaboeuf, France), dried for 30 mn, fixed for 10 mn in acetone, and used directly for labeling or stored at -20°C. Sections were washed in PBS and non specific sites were blocked by incubation in 0.3% BSA, 0.5% SAB, 2% NGS (normal goat serum) in PBS for 30 mn. Endogenous biotins were blocked using an avidin biotin blocking kit (Vector Laboratories, Peterborough, UK). Sections were then incubated for 90 minutes with the following primary antibodies: 25 µg/ml anti-CD123 (clone 7G3, BD Biosciences), 0.2 µg/ml anti-CD20 (clone L26, Dako), 12 µg/ml anti-CD3 (rabbit polyclonal, Dako) and appropriate isotypic controls. Sections were washed twice in 0.5% Tween 20 (Sigma Aldrich) in PBS. They were then incubated for 1 hour with secondary antibodies (0.5 µg/ml biotinylated goat-antimouse IgG1 or IgG2a (Caltag) and either 5 µg/ml Alexa 546-conjugated goat-antimouse IgG1 or IgG2a (Molecular Probes) or 10 µg/ml TRITC-conjugated goat-antirabbit IgG (Caltag). Sections were washed twice and biotinylated antibodies were detected by incubation for 15 mn incubation with 1 µg/ml streptavidin-Alexa-fluor-488 (Invitrogen). The sections were washed again, stained for nuclei for 10 mn in 1/20,000 4’, 6-diamidino-2-phenylindole (DAPI, Sigma Aldrich), then incubated with 0.5 M glycine in PBS for 30 mn. Slides were mounted with the Fluoromount Kit (Dako). All sections were analyzed under an Axiovert 100M microscope (Zeiss, Le Pecq, France) equipped with an Orca-ER CCD camera 1344x1024 pixels (Hamamatsu photonics, Hamamatsu City, Japan), and images were colorized using Image J 1.38 software.

**Determination of IDO activity**

Kyn and Trp concentrations were measured in macaque plasma from EDTA-treated blood, using high-performance liquid chromatography (HPLC) with coulometric detection 34. The
Kyn/Trp ratio was considered as an index of IDO activity, independent of baseline Trp concentrations\textsuperscript{35}.

**Quantification of Type I IFN, IFN-γ and IL-18**

IFN-I levels were measured by assessing reduction of the cytopathic effect of vesicular stomatitis virus (VSV)-infected Madin-Darby bovine kidney (MDBK) cells\textsuperscript{36}. A laboratory reference sample of human IFN-α, standardized against the NIH Ga 023-902-530 reference, was included for each titration. In uninfected cynomolgus macaques, plasma IFN-I concentration was < 2 IU/ml. ELISA kits for monkey IFN-γ (BioSource International) and human IL-18 (MBL, Nagoya, Japan), known to cross-react with macaque cytokine, were used. IFN-γ was not detectable in most animals before infection, but in some IFN-γ concentration exceeded 100 pg/ml (two in the 50 AID\textsubscript{50} group and one in the 5,000 AID\textsubscript{50} group). These macaques with high background levels were excluded from subsequent analysis.

**In vitro stimulation of pDC**

Aldrithiol-2 (AT-2)-inactivated SIVmac239 (2 μg/ml total protein, equivalent to 560 ng/ml p27) or herpes simplex virus (HSV) 1 (6 x 10\textsuperscript{5} PFU) was added to 1 ml of whole blood for 18 hours at 37°C. Plasma samples were collected and stored at –80°C until use for IFN-I titration. Negative controls of stimulation included mock-stimulation and stimulation with AT-2-treated SupT1 microvesicles.

**Statistical analysis**

The nonparametric Spearman's rank correlation test was used to investigate the relationships between parameters. The nonparametric Mann-Whitney test was used to compare different
groups of macaques, and the nonparametric Wilcoxon rank test was used to compare data from the same macaques at different time points before and after SIV infection. In some cases, we calculated the AUC for analysis of the amplitude of variation of a given parameter during primary infection. Statistical analyses were carried out with Statview software (SAS Institute, Inc., Cary, N.C.). In two-tailed tests, \( p \) values of 0.05 or lower were considered significant.
Results

Changes in pDC numbers during primary SIV infection

Twelve macaques were exposed iv to 50 AID$_{50}$ (n=6) or to 5,000 AID$_{50}$ (n=6) of SIVmac251. Profound changes in pDC numbers were observed in the blood after infection (Figure 1A). The earliest change was a significant transient increase during the first week of infection (mean 2.28 fold increase; range 1.23 – 4.22). This increase was rapidly followed by an acute decrease between days 9 and 14 (mean 16% of baseline; range 2 to 31% of baseline). Plasmacytoid DC counts decreased earlier in the 5,000 AID$_{50}$ group than in the 50 AID$_{50}$ group, but the nadir was not significantly different, mirroring kinetics and amplitudes of the viral load peak (Figure 1B). PDC numbers then gradually increased to subnormal values at 3 months p.i., staying below baseline from plasma viral load setpoint (month 3) to month 9 p.i. (data not shown, p < 0.0001).

The decrease in pDC counts paralleled the decrease in CD4$^+$ T-cell counts (Figure 1C) and pDC counts were correlated with CD4$^+$ T-lymphocyte counts at setpoint (p=0.05) and 9 months post-infection (p=0.03). PDC counts were not correlated with plasma viral loads, although dynamics of pDC in the blood were strongly related to the kinetics of viremia, higher infectious doses resulting in earlier peak of viremia and earlier changes in pDC blood counts than lower doses.

As blood pDC counts were not correlated with viral load and the acute decline of pDC levels in the blood was partly corrected during chronic infection in cynomolgus macaques, we investigated potential homing of pDC to peripheral LN. pDC were counted sequentially by flow cytometry in peripheral LN cells (Figure 2A, 2B). Before infection, pDC represented 0.6 x 10$^6$ to 11 x 10$^6$ pDC/ gram of tissue (Figure 2B) which corresponds to 0.039 ± 0.024%
of total LN cells. On day 15, they had increased significantly (Wilcoxon, p=0.002), and a further increase was observed in 11 of the 12 macaques between days 15 and 38, reaching 10 \times 10^6 to 156 \times 10^6 pDC/g (increase from baseline levels by a factor of 2 to 49 on day 38).

Nine months after infection, the number of pDC in lymph nodes was lower than during primary infection, but remained greater than preinfection levels (p=0.002). The percentages of pDC in total lymph node cells paralleled that of absolute numbers (data not shown).

The ratios of pDC to other cell populations counted by flow cytometry (CD20+ B cells, CD4+ and CD8+ T cells, CD3-CD8+/NK cells) were all increased after infection, the highest increases being observed during primary infection (data not shown) suggesting that the enrichment in pDC is selective.

The increase of pDC in lymph nodes was confirmed by in situ immunofluorescence labeling of CD123 on peripheral lymph node sections (Figure 2C, Supplemental Figure 1). In uninfected animals, CD123+ cells were mostly in the T-cell area, as shown by CD3/CD123 double staining, and were sometimes close to the B-cell area. Both the T-cell area and the B-cell follicles were infiltrated with large numbers of CD123+ cells after 38 days of infection.

To further highlight the impact of virus replication on pDC dynamics, a third group of six macaques was exposed to 50 AID50 of SIVmac251 and then treated with ART (AZT, 3TC and indinavir) four hours to 28 days after infection. As shown in figure 3A, macaques in this group showed a delay of the viral load peak of 2 days on average (mean 17 days ± 3 days vs 15 ± 3 days for the 50 AID50 untreated group, Mann Whitney test p=0.005). Viral load peaks were also lower in the treated group (median peak viral load 3.06 x 10^5 copy/ml vs. 1.39 x 10^6 copy/ml, p=0.025). In treated macaques, the transient decrease in CD4+ T-cell counts was delayed and attenuated (mean nadir 45% ± 9% of baseline CD4+ T-cell count vs 21% ± 7%, p=0.004) (Figure 3B). Similarly, the transient decrease in blood pDC absolute counts
was smaller in the treated group than in the untreated group (Figure 3C and Figure 1C). Indeed, in ART treated macaques pDC decreased to a mean nadir of 41% ± 16% of baseline counts whereas in untreated macaques the nadir of pDC was lower 17% ± 8% of baseline counts (p=0.02). The absolute numbers of pDC increased in peripheral lymph nodes following infection (Figure 3D), and this increase did not differ significantly from those in untreated macaques.

**Acute peaks of plasma IFN-I correlate with IDO activity and IL-18 production, all being closely synchronized to the increase of viremia during the acute phase**

As previously reported for rhesus macaques 15,17, IFN-I levels peaked during the increase in viremia (Figure 4A), two to three days earlier in the 5,000 AID₅₀ group than in the 50 AID₅₀ group (between day 7 and 9, mean 8.8 ± 0.4 days, vs between days 9 and 11, mean 10.2 ± 1.0, p=0.02). IFN-I concentration in plasma rapidly returned to undetectable levels in all animals on day 14. A small rebound was observed between day 14 and 28 then IFN-I was no more detectable between day 28 and 91. The increases in plasma IFN-I concentration and plasma viral load occurred at the same time. The area under the curve (day 0 to day 21) of plasma IFN-I concentration was positively correlated with that of plasma viral load (Rho=0.67, p=0.03).

We also determined plasma concentrations of IL-18, because this pro-inflammatory cytokine is induced by IFN-I 37 and induces IFN-γ in NK cells 17,38. IL-18 is also a potent chemoattractant for human pDC 39. A peak of IL-18 was evidenced in the plasma (Figure 4B). The peak of IL-18 occurred 2 to 3 days after the peak of IFN-I.

A peak of plasma IFN-γ concentrations was observed in a limited number of macaques, one of the five animals of the 50 AID₅₀ group and three of four animals of the 5,000 AID₅₀ group studied (Figure 4C). When detectable, the IFN-γ peak was temporally associated with the
peak of IL-18 and the macaques with a detectable IFN-\(\gamma\) peak were among those with the largest increase in IL-18 levels.

As IFN-I and HIV are both inducers of the immune suppression-associated enzyme indoleamine-2,3-dioxygenase (IDO) in human pDC in vitro \(^{21}\), we analyzed functional IDO activity in vivo. IDO catalyzes extrahepatic tryptophan (Trp) catabolism in the kynurenine (Kyn) pathway and measurement of their respective concentrations in serum is used as a marker of IDO activity \(^{40}\). A peak was observed for Kyn (mean peak value 2.24 ± 0.56 \(\mu\)M compared to 0.53 ± 0.08 \(\mu\)M at baseline), coinciding with the increase in plasma viremia (Figure 4D). This transient acute Kyn peak was associated with a transient decrease in Trp concentration, which returned to normal values by 3 months post-infection (Figure 4E). As a consequence, the Kyn/Trp ratio increased as viral load increased towards its peak (Figure 4F), with significantly more rapid kinetics (p=0.03) in the 5,000 AID\(_{50}\) group (peaking from day 9 to 11, mean 9.3 ± 0.8) than in the 50 AID\(_{50}\) group (day 9 to 14, mean 11.2 ± 1.6). IDO activity decreased after the peak, but remained higher at set point than at baseline (p=0.002), by a factor of 1.23 to 2.29 (mean 1.83 ± 0.27). Thus, the dynamics of IFN-I, IDO and IL-18 were highly coordinated with the exponential increase in viral load during primary infection. IFN-I and Kyn were synchronous with the exponential increase of viremia whereas IL-18 and IFN-\(\gamma\) increased later. Virus and IFN-I appeared to be the earliest inducers of IDO detected in the acute phase.

Innate immunity was strongly reduced in ART treated macaques, which showed reduced and delayed peaks of plasma IFN-I (Figure 4G). No increase in IL-18 concentration was observed (Figure 4H), and only one of four macaques analyzed in this group showed a transient, atypical increase in plasma IFN-\(\gamma\) concentration (Figure 4I). In these animals, IDO activity did not increase significantly during treatment, a slight increase being observed after treatment interruption (Figure 4J), suggesting that post-exposure treatment may attenuate
IDO-dependent immune suppression. Thus, the intensity of innate immunity is closely associated with the viral load during primary infection \textit{in vivo}, strongly suggesting that viremia needs to reach a certain threshold for an acute burst of innate immunity to occur. AUCs were calculated for each parameter, between days 0 and 21, taking into account the entire acute phase of infection in the 18 macaques (treated or untreated, Supplemental Figure 2). IDO activity (Kyn/Trp ratio) and Kyn concentration were positively correlated with IFN-I concentration (Rho=0.79, \( p=0.001 \) and Rho=0.85, \( p=0.0004 \), respectively), but not with IFN-\( \gamma \) concentration. Kyn and IFN-I concentrations were also positively correlated with plasma viral loads (Rho=0.75, \( p=0.002 \) and Rho=0.86, \( p=0.0004 \), respectively). Therefore, acute IDO activity was strongly correlated with both IFN-I concentration and viremia, but not with IFN-gamma concentration, during primary infection.

As viral load peaked after Kyn concentration, whereas IFN-I concentration peaked earlier, these data suggest that IFN-I plays a key role in the early phase of acute IDO activation although a direct virus-induced IDO activation in this study \textit{in vivo} is not excluded as reported in human pDC \textit{in vitro} \textsuperscript{21}.

**Primary infection is associated with a transient acute reduction in IFN-I production by pDC in response to viral stimulation \textit{in vitro}**

Human pDC rapidly become refractory to secondary stimulation \textit{in vitro} \textsuperscript{41}, a phenomenon proposed to account for decreased \textit{in vitro} production of IFN-I in HIV infected patients \textsuperscript{42}. As IFN-I became undetectable soon after its concentration peaked in the plasma, despite the persistence of high viral loads in our study, we compared the capacity of pDC to respond to SIV and HSV-1 stimulation before and after this peak, to evaluate a possible refractory phase \textit{in vivo}.  


In vitro, cynomolgus macaque pDC underwent rapid IFN-α synthesis in response to SIV stimulation (Figure 5A), as previously shown in response to HSV stimulation\textsuperscript{24}. These cells were also characterized extensively by flow cytometry (Supplemental Figure 3). Cynomolgus pDC share common characteristics with human and rhesus macaque pDC in response to HIV and SIV, respectively\textsuperscript{43,44}. In PBMC stimulation assays, most IFN positive cells clustered in the pDC gate and no other IFN-positive cell population was evidenced outside the pDC gate (Figure 5A). In addition, in whole blood stimulation assays, IFN-I production was strongly correlated with the amount of pDC in the blood sample (Figure 5B). Then, we tested blood samples after infection, to follow IFN-I production by pDC in response to \textit{in vitro} stimulation.

Before infection, IFN-I production per pDC was close to one IU per pDC (Figure 5C), as reported for human pDC\textsuperscript{1,45}. On day 35 after infection, IFN-I production per pDC in response to HSV was significantly lower, by a factor of about two on average (Wilcoxon, p=0.002), and then it gradually increased, with pre-infection values recovered nine months after infection. IFN-I production per pDC in response to SIV was also strongly decreased (by a log on average) on day 35 after infection (Figure 5D). Then it was partly restored but by contrast to what was observed for HSV stimulation, responses to SIV remained low 9 months post-infection (p=0.05 compared to uninfected macaques).

These data demonstrate that in addition to profound dynamic changes, pDC rapidly become refractory to IFN-I production in response to \textit{de novo} stimulation during acute infection. This decrease probably results from intense stimulation \textit{in vivo} during the acute phase of SIV infection, accounting for the rapid, but transient, acute plasma IFN-I peak.
IDO activity correlates positively with virus load and FoxP3⁺CD25⁺CD8⁺ T cells, and negatively with both antigen-specific T-cell proliferation and CD4⁺ T-cell activation

We then analyzed IFN-I and IDO dynamics in relation with the adaptive immune response. We recently described the dynamics of adaptive immunity in these macaques and showed a transient expansion of CD25brightCD8⁺ T cells, which express FoxP-3 and CTLA-4 — two molecules expressed by regulatory T cells (Tregs) — 9 to 35 days after infection. As IDO was recently shown to be induced by HIV in pDC in vitro and to correlate with viral load in HIV infection, and as pDC expressing IDO activate Tregs in a mouse tumor model, we searched for the correlation between IDO activity and FoxP3⁺CD8⁺ T-cell subset expansion in vivo. IDO activity correlated not only with plasma viral load (Spearman's rank correlation p=0.0005, Rho=0.84) but also with transient expansion of the FoxP3⁺CD25⁺CD8⁺ T-cell subset (p=0.0005, Rho=0.85) (Figure 6A and 6B). Transient expansion of the FoxP3⁺CD25⁺CD8⁺ T-cell subset also correlated with IFN-I concentrations (p=0.0015, Rho=0.77, data not shown).

In contrast, acute IDO activity negatively correlated with both SIV-specific T-cell proliferation and CD4⁺ T-cell activation (Figure 6C and 6D).

These data, confronted to the dynamic of pDC, are consistent with acute pDC dependant IFN-I production during acute infection creating a favorable environment for immune suppression by inducing IDO functional activity and FoxP3⁺CD25⁺CD8⁺ T cells which may participate in dampening the CD4⁺ T-cell SIV-specific response.
Discussion

Our data, based on longitudinal analysis of a large number of macaques with close time-course measurement in the blood and the lymph nodes, describe for the first time a rise in circulating pDC within a week after SIVmac251 infection in cynomolgus macaques suggesting early involvement of these cells in virus/host interaction. This was followed by a striking coincidence between the accumulation of pDC in the lymph nodes, and pDC decrease in the blood during the acute phase of primary SIV infection – nadir attained two weeks after infection –. The dynamics of pDC were closely correlated with the dynamics of CD4⁺ T-cell counts and plasma viral load. The transient increase of circulating pDC during the first week may be due to mobilization from lymphoid organs to the blood or to differentiation from blood precursors. Later during acute HIV or SIV infection, previous reports have shown that DC accumulate in the lymph node paracortex, but without specific characterization of pDC. The increase in pDC counts in the lymph nodes of SIV-infected macaques, demonstrated here for the first time during the acute phase of primary infection, is consistent with the increase in CD123+ cell numbers reported in the lymph nodes of HIV-infected patients later during chronic asymptomatic infection. These data contrast with other reports both in a group of HIV-1 infected patients with low CD4⁺ T-cell counts, and in SIV infected macaques with simian AIDS. One possible interpretation which would reconcile these divergent results is that pDC increase in lymphoid tissues in early stage of infection and are depleted in later stages as a consequence of infection and/or increased cell death. Several factors may account directly or indirectly to early relocation of pDC: the high levels of viral replication observed in lymphoid organs, the acute IL-18 burst we observed, as this cytokine was recently shown to act as a chemoattractant for human pDC in vitro, or chemokines and their receptors, such as CCL19/MIP3-ß/CCR7 and CCL-20/MIP-3α/CCR6,
as their mRNA levels increase in the spleen and lymph nodes during acute SIV infection 55, and as HIV induces CCR7 expression on human pDC in vitro 56.

In ART treated macaques, although transient decrease of pDC in the blood was lower than in untreated macaques, increase of pDC in peripheral lymph nodes was similar. Possible interpretations could be that pDC relocation to other lymphoid organs such as spleen or mucosal tissues could be affected by ART or that cell death and/or infection of pDC are reduced in these macaques.

Our data strongly suggest that pDC contribute to the rapid and acute increase in plasma IFN-I concentration in vivo, even though other cells, such as macrophages, might also contribute to the peak in plasma IFN-I concentration in vivo.

First, we observed that the relocation of pDC to lymph nodes and the decrease in blood pDC counts were concomitant with the increase in viral load and plasma IFN-I concentration, suggesting production at the site of virus replication in the lymphoid organs. Second, we showed that macaque pDC underwent activation, maturation and rapid IFN-I production in response to SIV stimulation in vitro, as previously described in human pDC 18,19,20. Third, pDC became unresponsive to secondary stimulation once the acute IFN-I response was over, suggesting that they were desensitized for IFN-I production in vivo as observed previously for human pDC in vitro after primary stimulation with TLR-9 ligand 41 and consistent with the reduced ability of pDC to produce IFN-I in response to in vitro stimulation in HIV infected patients 42. Fourth, we did not evidence IFN-I-positive cell clusters outside the pDC gate in our in vitro stimulation studies.

For the first time, our study monitored the acute transient burst of IDO activity in parallel with acute changes in pDC dynamics and IFN-I concentration in the early phase of viremia. The kinetic of IDO activity in cynomolgus macaques in our study and the positive correlation
observed between IDO activity and viral load are consistent with observations made in Indian rhesus macaques. The positive correlation observed between IDO activity and viral load is also consistent with published data suggesting that the virus itself induces IDO directly, through its own proteins, and indirectly, by inducing pDC dependent IFN-I production.

Although IFN-γ is a major inducer of IDO in vitro, and correlates positively with IDO activity during chronic HIV infection, in our study, during acute infection, transient increase in plasma IFN-γ concentration only occurred in a small number of macaques, over a limited time period, consistent with findings in HIV-1 acute infection. IFN-γ concentrations were correlated with plasma viral loads, but increased later and were not correlated with Kyn concentrations. Therefore, in contrast to IFN-I and the virus, IFN-γ does not appear as a major inducer in the initial acute induction of IDO activity. This does not exclude the possible later involvement of IFN-γ in the amplification of IDO activation.

We also investigated the relationship between innate and adaptive immune responses. We recently reported the dynamics of FoxP3+ CD4+ and CD8+ T cells during infection in the same animal cohort. In the present work, we analyzed the relationship between these and innate immunity. IFN-I and IDO were shown to provide important signals for the induction of CD4+ Tregs in vitro. We found no correlation of FoxP3+ CD4+ Tregs, which declined in the blood and were not increased in peripheral lymph nodes, with IFN-I or IDO activity (data not shown), although we can not exclude a different picture from other lymphoid organs which were not available in this study.

In contrast, we observed a transient expansion of CD25bright CD8+ T cells in the blood, around day 21 post-infection. These cells bear regulatory T-cell markers such as FoxP3 and CTLA-
4, were not correlated to the activation of CD8+ T cells[^33], were positively correlated with plasma viral load and negatively correlated with antigen-specific proliferative responses to SIV. We suggested they might participate in the control of helper T-cell responses during primary infection[^33]. In the present study, we show that this transient expansion strongly correlates with IDO activity and IFN-I concentration. These factors may therefore be important to create favorable environmental conditions \textit{in vivo} for the expansion of CD25\textsuperscript{bright}FoxP3\textsuperscript{+}CD8\textsuperscript{+} T cells, as previously reported \textit{in vitro} for CD4\textsuperscript{+} Tregs[^62,63], likely through the activation of pDC[^64]. Although direct evidence of regulatory function and increase of these cells in lymphoid tissues are yet lacking, as acute IDO activity and FoxP3\textsuperscript{+}CD8\textsuperscript{+} T-cell expansion both negatively correlated with antigen-specific T-cell proliferation and CD4\textsuperscript{+} T-cell activation, we speculate that FoxP3+CD8+ T cells may therefore synergize to impair SIV-specific CD4\textsuperscript{+} T-cell responses during primary infection.

Indeed, these cells share phenotype features with some of the human CD8\textsuperscript{+} regulatory T cells described in the literature[^65-68]. Alternatively we can not exclude that FoxP3 expression on CD8\textsuperscript{+} T cells during primary infection could result from activation with no regulatory function as reported in vitro for human CD8\textsuperscript{+} T cells[^69] although remarkably the dynamic of CD25+CD8\textsuperscript{+} T cells (total CD25+ CD8\textsuperscript{+} T cells, CD25 being used as an activation marker), did not correlate with FoxP3+ CD8\textsuperscript{+} T cells[^33].

Similarly, FoxP3+CD4\textsuperscript{+} Tregs were suggested to contribute to the suppression of SIV specific CD8\textsuperscript{+} T-cell responses[^30]. Indeed, cycling CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes are barely detectable before the end of the IFN-I peak at day 14 after SIV infection in cynomolgus macaques[^32] (data not shown). This observation supports the hypothesis that the anti-proliferative activity of IFN-I[^70] and/or IDO-dependent suppression[^71] may contribute to delay the expansion of antigen-specific T cells important for the control of viremia. This
environment, during increasing viremia, may provide favourable conditions for the establishment of viral reservoirs and chronic infection by dampening cellular immunity.

Post-exposure antiretroviral treatment has major repercussions on both viremia and innate immunity, weakening the IFN-I response, abolishing the IL-18 peak, limiting pDC dynamic changes during acute infection, and suppressing acute increase in IDO activity. This may favor the early development of more efficient adaptive immune responses, and better long-term viremia control as shown in this and our previous study. Low levels of IFN-I production were also observed in macaques infected with attenuated Δnef SIVmac, which also display lower viral load and develop an efficient adaptive immune response. Therefore, low viremia during primary infection is associated with lower levels of IFN-I production, weaker immune suppression, better adaptive immunity and improved control of viremia in the long term. This suggests that acute production of IFN-I early during primary HIV/SIV infections may limit the capacity of the host to fight infection as recently shown in the mouse/MCMV infection model.

In conclusion, our in vivo longitudinal study strongly suggests that greater viral expansion during primary infection leads to a stronger interferon response and transient induction of immunosuppressive agents, dampening antigen-specific CD4+ T-cell responses. IFN-I plays a major role in antiviral immunity, but our results show a strong correlation between IFN-I concentration, virus replication levels and markers of immune suppression during primary infection, suggesting opposite effects on cellular immunity. As plasmacytoid DC home to lymph nodes during the acute phase of infection and are most likely involved in the acute production of IFN-I, they appear as key elements in this duality.
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Authorship and Conflict of Interest Statements

B. Malleret contributed in writing, performed research, and analysed data.
B. Manéglier, IK, PL, MN, and LP performed research and analysed data. PB, BD, JC, TA performed research. OS-V analyzed data. AH, RLG analyzed data and contributed in writing. BV designed experiment, performed research, analyzed data, contributed in writing.

The authors declare no competing financial interests.
References


Figure legends

Figure 1: Changes in blood pDC counts, plasma viral load and blood CD4+ T-cell counts over time after inoculation with SIVmac251.

(A) Absolute counts of pDC in the blood, (B) Plasma viral loads, (C) Proportion of CD4+ T cells with respect to baseline. Solid gray lines in A, B and C: macaques infected with 50 AID50; solid black lines in A, B and C: macaques infected with 5,000 AID50; dotted gray lines in A and C: median plasma viral load of the 50 AID50 group; dotted black lines in A and C: median plasma viral load of the 5,000 AID50 group.

Figure 2: pDC dynamics in the peripheral lymph nodes of cynomolgus macaques after infection with SIV.

(A) Representative dot plot showing CD123pos CD1cneg pDC in a lineage (CD3, CD8, CD14 and CD20)neg HLA-DRpos gate, in lymph node cells. (B) Changes in the numbers of pDC in sequential peripheral lymph node biopsies over time. Solid gray lines: macaques infected with 50 AID50; solid black lines: macaques infected with 5,000 AID50. p values are given for the Wilcoxon rank test. (C) Distribution of CD123pos cells in lymph nodes of SIV-uninfected (left panel) and SIV-infected macaques (day 38 pi, right panel). Photographs are representative of 2 SIV-negative and 3 SIV-positive macaques and of the entire section observed. Serial lymph node sections (10 µm) were either double-labeled for CD123 and CD20 (upper panel), or CD123 and CD3 (lower panel), or stained with isotypic control antibodies or DAPI (Supplemental Figure 1). Magnification: x100. Inset magnification: x200 (upper left, upper right, lower left) and x400 (lower right).
Figure 3: Impact of early antiretroviral treatment on plasma viral load, CD4+ T-cell counts and pDC dynamics in blood and lymph nodes.
Changes of each parameter in 6 individual macaques infected with 50 AID$_{50}$ of SIV (solid black lines) that received antiretroviral treatment (ART) from 4 h to 28 days after virus inoculation (50 AID$_{50}$+ART group). (A) Plasma viral loads, (B) Blood CD4+ T-cell counts, (C) Absolute pDC counts in the blood, (D) Absolute numbers of pDC in peripheral lymph nodes. Dotted lines in A: median plasma viral load of the 50 AID$_{50}$ group (gray) and of the 5,000 AID$_{50}$ group (black).

Figure 4: Changes in plasma concentration of different soluble factors over time after inoculation with SIVmac251 in untreated and ART treated macaques.
Untreated macaques: (A) IFN-I, (B) IL-18, (C) IFN-γ, (D) Kynurenine, (E) Tryptophan, (F) Kynurenine:Tryptophan ratio. Solid gray lines: macaques infected with 50 AID$_{50}$; solid black lines: macaques infected with 5,000 AID$_{50}$. Dotted lines in panel A to F: median plasma viral load of the 50 AID$_{50}$ group (gray) and 5,000 AID$_{50}$ group (black).
Macaques which received post-exposure ART: (G) IFN-I, (H) IL-18, (I) IFN-γ, (J) Tryptophan and kynurenine ratio. Dotted lines in panel G to J: median plasma viral load of the 50 AID$_{50}$+ART group. Duration of ART treatment is indicated by a grey horizontal bar.

Figure 5: pDC dependant IFN-I production in response to de novo stimulation with HSV and SIV in vitro.
(A) Dot plot set showing intracellular staining of IFN-α2a. Left: SSC/IFN-alpha dot plot. Right: CD123/HLA-DR dot plot of gated IFN-pha positive cells. PBMC were either stimulated 6 h with HSV-1, or with inactivated SIV (AT-2), or mock stimulated. Representative data for 2 uninfected macaques are shown. (B) Spearman's rank correlation
analysis of IFN-I production (whole blood assay) in response to HSV and pDC concentration in blood samples for 18 uninfected macaques. (C) Longitudinal changes in IFN-I production in response to HSV. (D) Longitudinal changes in IFN-I production in response to SIV. IFN-I production per pDC is expressed as the ratio between plasma IFN-I concentration after 18 hours of stimulation and the absolute number of pDC in whole blood samples.

Figure 6: Acute IFN-I and IDO responses correlate with the transient induction of FoxP3⁺CD25⁺CD8⁺ T cells.

Spearman's rank correlations between IDO activity and plasma viral load (A), IDO activity and CD8⁺CD25⁺FoxP3⁺ T cells (B), IDO activity and SIV-specific T-cell proliferation (C) and IDO activity and CD4⁺ T-cell activation (D). AUCs were used for plasma viral loads, CD8⁺CD25⁺FoxP3⁺ T-cell numbers (day 9 to 42), and SIV-specific T-cell proliferation (day 1 to 100), to take into account the optimal periods of time covering acute dynamic changes in each of these parameters during primary infection. ART treated macaques are in open symbols whereas untreated macaques are in grey (50AID50 group) or black (5,000 AID50 group).
Figure 1
Figure 2

Absolute number (million pDC/g of tissue)

Before infection
Uninfected

Day 15
Day 38
Month 9
After infection
SIV+ day 38
Figure 4
Figure 6

A

Plasma viral load (AUC x10^7)

B

CD8^+CD25^+FoxP3^+ T cells in blood (AUC)

C

SIV-specific T-cell proliferation (AUC mean cpm x10^5)

D

Peak percentage CD25^+ in CD4 T cells

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p=0.0005
Rho=0.84

p=0.0005
Rho=0.85

p=0.04
Rho=-0.5

p=0.04
Rho=-0.5
Primary infection with simian immunodeficiency virus: plasmacytoid dendritic cell homing to lymph nodes, type I IFN, and immune suppression

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