Plasmacytoid Dendritic Cell Infection and Sensing Capacity during Pathogenic and Nonpathogenic Simian Immunodeficiency Virus Infection.

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Plasmacytoid dendritic cell infection and sensing capacity during pathogenic and non-pathogenic SIV infection

Running title: Low AGM pDC CD4 and CCR5 levels allow SIV infection

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Abstract

HIV in humans and SIV in macaques (MAC) lead to chronic inflammation and AIDS. Natural hosts, such as African green monkeys (AGM) and sooty mangabeys (SM), are protected against SIV-induced chronic inflammation and AIDS. Here, we report that AGM plasmacytoid dendritic cells (pDC) express extremely low levels of CD4, unlike MAC and human pDC. Despite this, AGM pDC efficiently sensed SIVagm, but not heterologous HIV/SIV isolates, indicating a virus-host adaptation. Moreover, both AGM and SM pDC were found to be, in contrast to MAC pDC, predominantly negative for CCR5. Despite such limited CD4 and CCR5 expression, lymphoid tissue pDC were infected to a similar degree as CD4+ T cells, both in MAC and AGM. Altogether, our finding of efficient pDC infection by SIV in vivo identifies pDC as a potential viral reservoir in lymphoid tissues. We discovered low expression of CD4 on AGM pDC, which did not preclude efficient sensing of host-adapted viruses. Therefore, pDC infection and efficient sensing are not prerequisites for chronic inflammation. The high level of pDC infection by SIVagm suggests that if CCR5 paucity on immune cells is important for non-pathogenesis of natural hosts, it is possibly not due to its role as a co-receptor.

Importance

The ability of certain key immune cell subsets to resist infection might contribute to the asymptomatic nature of simian immunodeficiency virus (SIV) infection in its natural hosts, such as African green monkeys (AGM) and sooty mangabeys (SM). This relative resistance to infection has been correlated with reduced expression of CD4 and/or CCR5. We show that plasmacytoid dendritic cells (pDC) of natural hosts display a reduced CD4 and/or CCR5 expression, unlike macaque pDC. Surprisingly, this did not protect AGM pDC, as infection levels
were similar to those found in MAC pDC. Furthermore, we show that AGM pDC did not consistently produce IFN-I upon heterologous SIVmac/HIV-1 encounter, while they sensed autologous SIVagm isolates. Pseudotyping SIVmac/HIV-1 overcame this deficiency, suggesting that reduced uptake of heterologous viral strains underlay this lack of sensing. The distinct IFN-I responses depending on host species and HIV/SIV isolates reveal host/virus species-specificity of pDC sensing.

**Introduction**

Chronic inflammation and immune activation in HIV-infected individuals and in SIV-infected macaques (MAC) lead to depletion of CD4$^+$ T cells and progression to AIDS. Natural hosts of SIV, such as African green monkeys (AGM) and sooty mangabeys (SM), do not display chronic inflammation or AIDS (1). This is due to resolution of inflammation before the end of acute infection, rather than to a lack of SIV recognition by the innate immune system (2). Natural hosts further differ from pathogenic HIV/SIV infections by exhibiting reduced infection rates in certain cell subsets, such as central memory CD4$^+$ T cells (Tcm) (3, 4). This relative resistance has been linked to a reduced expression of the HIV/SIV co-receptor CCR5 on natural host CD4$^+$ T cells and to a downmodulation of CD4 on activated CD4$^+$ T cells in AGM (3-5).

Plasmacytoid dendritic cells (pDC) form a rare cell population that is responsible for the vast majority of IFN-I production after HIV encounter (6). This is also true for AGM pDC as the depletion of pDC from AGM PBMC completely abrogates the IFN-I response to SIVagm stimulation (7). HIV/SIV sensing by pDC is mediated through endocytosis followed by TLR7/9 engagement. It requires CD4, but is independent of co-receptor expression (6). Data on the infection rates of pDC *in vivo* are scarce. One study reported the presence of HIV DNA in
circulating pDC of chronically HIV-infected patients (8). Another study reported high infection levels in lymph node (LN) pDC during acute SIVmac infection (9).

Here, we discovered a restricted CD4 and/or CCR5 expression on pDC in natural hosts. We evaluated the effect of low CD4 expression on the capacity of AGM pDC to efficiently sense distinct forms of SIVagm (free virus, non-infectious particles and SIVagm-infected cells). Furthermore, we examined the infection frequency of pDC during pathogenic and non-pathogenic SIV infection.

Materials and Methods

Study Approval

All animal experimental protocols were approved by either the Ethical Committee of Animal Experimentation (CETEA-DSV, IDF, France) (Notification numbers: 10-051b and 12-006) or by the Institutional Animal Care and Use Committees (IACUC) of Emory University (IACUC protocol #2000793, entitled “Comparative AIDS Program”). Animals were housed in the facilities of the CEA (“Commissariat à l’Energie Atomique”, Fontenay-aux-Roses, France, permit number: A 92-032-02), Institut Pasteur (Paris, France, permit number: A 78-100-3) or Yerkes National Primate Research Center (Atlanta, GA, USA). All experimental procedures were conducted in strict accordance with the international European guidelines 2010/63/UE on protection of animals used for experimentation and other scientific purposes (French decree 2013-118) and with the recommendations of the Weatherall report or in strict accordance with USDA regulations and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The CEA complies with Standards for Human Care and Use of Laboratory of
the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-01. The monitoring of the animals was under the supervision of the veterinarians in charge of the animal facilities.

**In vivo infections and sample collection**

Twenty-six African green monkeys (*Chlorocebus sabaeus*) of the sabaeus species with a Caribbean origin, eighteen Chinese rhesus macaques (*Macaca mulatta*), two cynomolgous macaques (*Macaca fascicularis*), sixteen Indian rhesus macaques (*Macaca mulatta*) and sixteen sooty mangabeys (*Cercocebus atys*) were used in this study. Eleven AGMs were infected via intravenous inoculation with 250 TCID$_{50}$ of SIVagm.sab92018, as described previously (10, 11). Four Chinese rhesus macaques were i.v. infected with 50 AID$_{50}$ SIVmac251 and two others were infected with 5000 AID$_{50}$ SIVmac251, as described previously (10, 12). Eight Indian rhesus macaques had been previously infected with SIVmac239 or SIVmac251 and eight sooty mangabeys were either naturally infected or infected experimentally with SIVsmE041 (13, 14).

Blood was collected by venipuncture on sodium heparin tubes and shipped to Institut Pasteur or used on site at Yerkes National Primate Research Center. Bone marrow mononuclear cells were isolated on Ficoll and tissue cells were put in suspension before staining. LNs and spleens were disrupted mechanically and rectal tissues were enzymatically degraded as described previously (12, 15).

**Viral stimulations**

Freshly isolated PBMCs were cultured at 0.5x10$^6$ cells/well in 24-well plates (Costar) at 37˚C, 5% CO$_2$ for eighteen hours with or without virus. Cell viability was measured using trypan blue and a Countess (Life Technologies) cell counter. The SIVagm strains used, have been previously
described: SIVagm.sab92018 (11), SIVagm.sabD46 and SIVagm.tanB14 (16), SIVagm.sab1c (11, 17) and SIVgri1 (18, 19). Free SIVagm was added to PBMCs at a concentration of 1500 ng/mL p27, unless indicated otherwise. HSV-1 was added at a TCID50 of 2x10^{5}. HIV-1.Bal-VSV, which is endocytosed independently of CD4 (20), was kindly provided by A. David and AT2-inactivated SIVagm as well as control microvesicles by Dr. Jeff Lifson (National Cancer Institute, Frederick, MD). SIVmac251-VSV was produced by co-transfecting 293T cells with SIVmac251Δenv and VSV-G expression vector using SuperFect (Qiagen), as described previously (21). SIVagm isolates were grown on SupT1 cells, as these cells express Bonzo and are susceptible to SIVagm while SIVmac isolates were grown on CEMx174 as these cells express Bob and are susceptible to SIVmac (22).

Production of infected cells

Cells were infected as previously described (23). Briefly, SupT1 cells were exposed for one hour at 37˚ C to 3.3x10^{4} TCID_{50} per 10^6 cells under constant agitation. Infection levels were assessed by measuring SIV Gag+ cells using flow cytometry (see below).

Functional Interferon alpha assay

Bioactive IFN-I levels were quantified as described earlier (7). In short, Mardin-Darby Bovine Kidney (MDBK) cells were incubated with UV-inactivated supernatants for 18 hours, after which the cytopathic effect of vesicular stomatitic virus was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). Alternatively, a cell line stably transfected with a luciferase gene under an IFN-I inducible promoter was used to measure IFN-I levels, as described previously (23). The limit of detection threshold was set at 2 IU/mL.

Flow cytometry
SM flow cytometric data were acquired at Yerkes National Primate Center and data for AGM were acquired at Institut Pasteur. MAC data were acquired at both Yerkes and Pasteur, with similar results. The following antibodies were used to identify and characterize pDC phenotype and function in whole blood or isolated tissue cells: CD3 (SP34-2), CD4 (L200), HLA-DR (L243), CCR5 (3A9), CD123 (7G3) (all BD Biosciences), CD20 (2H7, ebioscience), CD4 (M-T466), BDCA-2 (AC144) (all Miltenyi) and CD4 (SFC12T4D11, Beckman Coulter). FcR Blocking Reagent (Miltenyi) was used to block unspecific antibody binding and in experiments using tissues other than blood, Live/Dead cell viability (Invitrogen) was used to exclude dead cells. For intracellular staining, cells were labeled with surface-binding antibodies and fixed with 4% paraformaldehyde and permeabilized using saponin prior to incubation with anti-CD4. For intracellular SIVagm detection, anti-p24 staining (KC57, Beckman Coulter) was used after cell permeabilization with the IntraPrep Permeabilization Reagent Kit (Beckman Coulter) according to manufacturer’s instructions (23). Events ranging from one hundred to one thousand pDC were collected on a BD LSR-II flow cytometer, running BD FACS Diva 6.0 software, and analyzed with FlowJo 9.4.10 (TreeStar). Anti-mouse compensation beads (BD Biosciences) and Arc Amine Reactive Compensation Bead Kit (Life Technologies) were used to define compensation levels. An isotype control antibody was used to define CCR5⁺ cells.

**Cell sorting**

Splenocytes were frozen in 10% DMSO in liquid nitrogen until use. Cells were thawed in the presence of DNAse I (10 IU/mL, Roche) and washed in FCS. Cells were labeled with antibodies against CD3, CD20, HLA-DR, CD4, CD123 and with Live/Dead reagent in the presence of FcR Blocking Reagent. Cells were sorted using a FACS Aria II sorter (BD), running on BD FACS Diva 6.0.
Sorted pDC and CD4+ T cells were purified a second time to increase purity of the two fractions. CD4+ T cells were also isolated from LNs cells using anti-CD4 beads and magnetic stands (Miltenyi) following manufacturer’s protocol, after which purity was verified by flow cytometry.

**SIV DNA quantification**

DNA was extracted as follows: samples were lysed in NaCl (3M), EDTA (0.5M, pH 8), SDS (10%, Bio-Rad) and proteinase K (1mg/mL, Qiagen) in a 45 min incubation at 55 °C. Then, NaCl (5M) was added and incubated at 4 °C between 15 and 60 min, followed by centrifugation for 15 min at 3000 rpm at 4 °C. DNA was then precipitated from the supernatant in phenol:chloroform:isoamyl alcohol 25:24:1 (pH = 8, Sigma Aldrich). After DNA extraction, viral DNA was measured by qPCR in duplicate, using primers and probes designed specifically for SIVagm.sab and SIVmac (11, 15). SIVagm and SIVmac plasmids were used as standards to calculate SIV DNA copy numbers. CCR5 DNA quantification was used to normalize the viral levels to the number of cells (24). Sample preparation, enzyme mix preparation and PCR set-up were performed in three separate rooms to avoid PCR contamination. Positive and negative controls were used to exclude sample contamination.

**Fluorescence microscopy**

Fluorescence microscopy was done as follows with markers against NKp30 (AF29-4D12, Miltenyi), CD123 (5B11, Biolegend), DAPI and SIVagm env RNA (made from the region amplified by 5'-GAG GCT TGT GAT AAA ACT TAT TGG GAT-3' and 5'-AGA GCA GTG ACG CGG GCA TTG AGG-3' primers and labeled with fluorochrome Alexa 488 (Life Technologies)). Briefly, cryopreserved sections were permeabilized by incubating in 0.5% (v/v) Triton X-100. This was
followed by hybridization of probe and mounted antibodies on tissue. Secondary antibodies were used with to visualize the bound antibodies. Donkey anti-mouse IgG-CFL 594 (Santa Cruz) was used to detect CD123 and Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit (Life technologies) to reveal the NKP30 antibody. As negative controls for SIV RNA in situ hybridization, we used a RNAse degraded probe, taken up in hybridization buffer, as well as lymph nodes of uninfected animals. Images were acquired on a Confocal Laser Scanning Microscope Leica TCS SP8, running LAS AF 3 (Leica Application Suite Advanced Fluorescence).

**Statistics**

Statistical inference analyses were performed using Prism 5.0 (GraphPad). The non-parametric Wilcoxon signed rank test and Mann-Whitney test were used to test paired and non-paired observations, respectively. In case of multiple testing of unpaired data, a non-parametric Kruskal-Wallis test, followed by a Dunn’s multiple comparison test, was used. Multiple testing of paired data without missing values was done by a Friedman test, followed by a Dunn’s multiple comparison test.

**Results**

**Low CD4 expression on AGM pDC**

Given the importance of CD4 for HIV/SIV sensing by pDC, we measured CD4 on pDC from uninfected AGM, SM and MAC (Figure 1). PDC were defined as CD3⁻CD20⁻HLA-DR⁺CD123⁺ cells (Figure 2A) (2). MAC and SM pDC displayed similar CD4 levels as on CD4⁺ T cells (Figure 1A and B). Surprisingly, AGM pDC expressed CD4 at >1 log lower levels than CD4⁺ T cells (p < 0.001).
As (i) CD4 MFI on CD4+ T cells was similar between AGM and MAC and (ii) two additional CD4 antibody clones confirmed low CD4 expression on AGM pDC (Figure 2B), it is unlikely that low CD4 on AGM pDC was due to species-specific antibody issues. The absence of intracellular CD4 in AGM pDC indicated that recycling from the cell surface was not the underlying mechanism of this low expression (Figure 2C). AGM pDC from primary, secondary and tertiary lymphoid tissues all expressed low levels of CD4 (Figure 2D).

**Efficient SIV-sensing by AGM pDC**

The low CD4 expression on AGM pDC is paradoxical, since CD4 is essential for HIV/SIV sensing by pDC and AGM pDC have been shown to efficiently sense SIVagm (7, 12). Nonetheless, a lower production of IFN-I has been described during SIVagm infection in vivo (12, 25). We wondered whether low CD4 on pDC could have subtle effects on SIV sensing in vivo. For instance, only sensing of infectious SIVagm particles has been investigated (12, 25), while (i) most virions produced in vivo are non-infectious and (ii) sensing of virus-infected cells is more efficient in human and MAC (23). We stimulated peripheral blood mononuclear cells (PBMC) of a large number of uninfected AGM and MAC with infectious SIV, using HSV as a control for CD4-independent sensing. No quantitative differences in IFN-I production were observed between AGM and MAC (Figure 3A). Both SIV-infected cells and AT2-inactivated SIVagm also induced normal IFN-I responses (Figures 3B and 3C). Longitudinal measurement showed that CD4 levels on pDC did not further decrease during SIV infection (Figure 3D). Altogether, these data demonstrate that low CD4 on AGM pDC did not impair their capacity to sense SIVagm.

**SIV isolate-dependent sensing of AGM pDC**
We wondered how SIVagm is sensed by pDC despite low CD4 expression. CD4 is a highly polymorphic molecule among primates. We raised the hypothesis that given the long circulation of SIVagm in the AGM population, SIVagm is well-adapted to AGM CD4 (26). This could entail that SIVagm, but not heterologous viruses, can elicit robust IFN-I responses by AGM pDC. We tested this hypothesis by stimulating AGM (sabaeus) and MAC PBMC with nine SIV/HIV isolates (Figure 4A, B). The three SIVagm.sab isolates and the SIVagm.tan isolate induced robust IFN-I production by AGM pDC. SIVagm.gri did not induce an efficient response, which corresponds with the observation that the CD4 genes of the sabaeus and tantalus AGM species are more closely related to each other than to the CD4 gene of the grivet AGM species (26).

In contrast, HIV-1 and two out of three SIVmac (SIVmac.251 and SIVmac.STM) isolates did not induce robust IFN-I production by AGM pDC (p < 0.05 or p < 0.01), even at a high viral dose. Only SIVmac239 induced IFN-I production from AGM PBMC, already at a low viral dose. Low dose of SIVmac239 also induced high levels of IFN-I in by MAC PBMC. At the highest dose, SIVmac239 was more cytotoxic for AGM PBMC than SIVmac251 (p < 0.05, Figure 4C). However, at a dose of 150 ng/mL p27, SIVmac239 did not influence viability, so it is unlikely that the strong response to SIVmac239 was related to dying cells, which are known to induce IFN-I responses (Figure 4C). It should be noted that SIVmac239 has been shown to be highly virulent in vivo compared to SIVmac251 (27). In contrast to AGM pDC, MAC pDC produced IFN-I upon stimulation with all SIV/HIV isolates, including SIVagm (Figure 4B).

If the lack of SIVmac/HIV sensing by AGM pDC is indeed due to low CD4, then forcing viral endocytosis should overcome this sensing deficiency. In line with this, HIV-1, or SIVmac251, pseudotyped with VSV-G were sensed similar to SIVagm (Figure 4D). Indeed, pseudotyped HIV-1...
and SIVmac251 induced higher levels of IFN-I than their wild-type isolates (p < 0.05 and p < 0.01, respectively, Figure 4D). Altogether, these findings indicate a virus-host co-adaptation in AGM for viral sensing by pDC.

**Predominance of CCR5 negative pDC in natural hosts**

We next examined CCR5 expression on AGM pDC, since co-receptor expression, in addition to CD4, is essential for infection. MAC pDC were predominantly CCR5+ (median 92.3%), while the majority of AGM and SM pDC did not express detectable levels of CCR5 (7.7% and 40% CCR5+ pDC, respectively; Figure 1A and 1C). In SMs, the percentage of CCR5+ pDC was lower after SIV infection (18.4%, p = 0.02; Figure 1C).

**PDC are highly infected during pathogenic and non-pathogenic SIV infection**

We then addressed the question if these reduced CD4 and CCR5 expressions associate with low infection of AGM pDC. To test this, we purified splenic pDC and CD4+ T cells of chronically SIV-infected AGM and MAC and measured cell-associated viral DNA. Cells from uninfected animals were never positive for viral DNA (data not shown). Spleen pDC and CD4+ T cells from MAC harbored a median of $8.1 \times 10^4$ and $9.2 \times 10^3$ copies per million cells, respectively (p = 0.37, Figure 5A). AGM harbored $3.2 \times 10^4$ and $4.1 \times 10^3$ copies per million splenic pDC and CD4+ T cells, respectively (p = 0.44, Figure 5A). AGM and MAC pDC were thus infected to a similar extent *in vivo*. As infection levels were similar between pDC and CD4+ T cells, potentially contaminating CD4+ T cells in the pDC fraction cannot explain the levels of detected SIV DNA in pDC. As pDC have limited phagocytic capacities, the high levels of viral DNA in these cells are also unlikely to be associated with engulfed infected T cells.
To further demonstrate the infection status of AGM pDC, we immunohistochemically examined a LN of a chronically infected AGM (Figure 5B). CD123 and SIVagm RNA signals overlapped, while NKp30, which is not expressed on pDC, did not co-stain with CD123 or SIVagm RNA.

We then measured CD4 and CCR5 expression on splenic pDC of chronically infected AGM and MAC to exclude phenotypic differences compared to pDC from blood or from lymphoid tissues of uninfected animals. Splenic pDC of SIV-infected AGM, but not MAC, expressed low levels of CD4 (Figure 6). Only 4.7% of AGM splenic pDC had detectable CCR5 expression, while 92.9% of MAC splenic pDC were CCR5$^+$ ($p = 0.0079$). In conclusion, pDC of AGM and MAC were infected at high levels despite a restricted CD4 and CCR5 expression on AGM pDC.

**Discussion**

Such low CD4 expression as the one we discovered on AGM pDC is remarkable given the evolutionary conserved high expression of CD4 on mammalian pDC, including primate, murine, cattle and swine pDC (6, 28, 29). The role of CD4 in pDC biology is currently unknown, but this low expression raises questions on the physiological impact for AGM. The low CD4 levels on pDC were sufficient to allow SIVagm endocytosis and subsequent sensing and we demonstrated for the first time that natural host pDC can sense SIV-infected cells. In contrast, most SIVmac and HIV-1 isolates tested were not sensed by AGM pDC. Pseudotyping increased the efficiency of HIV-1/SIVmac251 sensing, which indicates that inefficient sensing of heterologous virus was due to restricted viral uptake. Altogether, this suggests an adaptation between SIVagm and its host-specific CD4. Similar to our findings, it has been shown that HIV-1 poorly interacts with MAC CD4 compared to human CD4 and that HIV-1 could thus infect cells expressing low levels of human CD4, but not cells expressing low levels of MAC CD4 (30). Low CD4 expression also did
not prevent AGM pDC from being infected as they were infected at high levels \textit{in vivo}. Since the pDC sensing capacity and infection levels were similar between AGM and MAC, these factors do not determine the level of chronic inflammation.

Our study reveals a high infection rate of pDC in secondary lymphoid tissue during chronic SIV infection. These data resemble those of acute SIVmac infection, where pDC were found to be infected to similar levels as CD4\(^+\) T cells in LN (9). Since only very few data on pDC infection \textit{in vivo} are available, this is important and underlines the potential role of pDC as a viral reservoir. Of note, the ratio of pDC to CD4\(^+\) T cells is low, approximately 1:300 in lymph nodes (2). Therefore, the majority of viral burden in SIV infection is still associated with CD4\(^+\) T cells. However the contribution of pDC should not be underestimated, given their presence in mucosae, their capacity to migrate to lymph nodes and their ability to efficiently transmit HIV/SIV to CD4\(^+\) T cells.

The lack of correlation between the frequency of CCR5\(^+\) pDC and SIV infection status does not support the hypothesis that absence of CCR5 expression protects against target cell infection (4). In line with this, a mutation in the SM CCR5 allele, disrupting functional CCR5 expression, does not diminish SIVsm infection prevalence (31). This can be explained by the fact that SIVs, including SIVagm, efficiently use alternative co-receptors, such as CXCR6 (Bonzo) and GPR15 (BOB) (22, 31). It is however possible that the low percentage of CCR5 expression in natural hosts is related indirectly to their resistance to disease. Indeed, while CCR5 is not a specific gut-homing receptor, it can induce migration to inflamed tissues (14). Such extremely low levels of CCR5\(^+\) pDC in natural hosts could therefore be related to the lack of pDC accumulation in the gut after infection (32). Our results therefore suggest that evaluating the function of CCR5 in
seeding of viral reservoirs outside its role as a HIV co-receptor is warranted. Teasing out such
mechanisms could be helpful for curative approaches.

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during primary SIV infection in African green monkeys are associated with protection against AIDS. J Clin Invest 115:1082-1091.


Figure 1. CD4 and CCR5 expression on blood plasmacytoid dendritic cells. (A) CD4 and CCR5 expression on pDC (blue) and Lineage^+ cells (grey) of one representative Chinese cynomolgous macaque, African green monkey, sooty mangabey and Indian rhesus macaque. (B) CD4 expression on pDC from SIV-negative Chinese rhesus macaques (n=8, open squares), Indian rhesus macaques (n=1, open upward triangles), AGM (n=13, open circles) and SM (n=11, filled
grey circles). CD4 mean fluorescent intensity (MFI) on pDC was normalized (%) to the CD4 MFI on CD4+ T cells. The horizontal, dashed line designates equal expression compared to CD4+ T cells. (C) The percentage of CCR5+ pDC was determined for SIV-negative Indian rhesus macaques (n = 11, open upward triangles), Chinese cynomolgous macaques (n = 2, open downward triangles), AGM (n = 7, open circles) and SM (n = 8, grey filled circles) and chronically SIV-infected Indian rhesus macaques (n = 8, open upward triangles), AGM (n=3, open circles) and SM (n = 8, grey filled circles). Symbols represent individual animals and lines and bars represent medians and interquartile ranges. * Kruskal-Wallis, p < 0.05, *** Kruskal-Wallis, p < 0.001.

Figure 2. Low CD4 expression on AGM pDCs. (A) Dot plots showing the gating strategy used to identify pDCs, which are FSC/SSC, singlets, Lineage (CD3/CD20)- cells and HLA-DR+ CD123hi cells, for one representative animal. (B) Low CD4 expression on AGM pDCs (n = 3) was confirmed using two additional monoclonal anti-CD4 antibodies (M-T466 and SFCI12T4D11). Histograms of representative animals are shown, depicting CD4 expression on CD4- cells (grey, solid), pDCs (blue, solid with contour) and Lineage+CD4+ cells (red, solid with contour). MFI values of CD4 for the three cell populations are shown in the table. (C) Intracellular and extracellular staining of AGM pDCs (n = 3). Histograms of a representative animal are shown, cell surface expression CD4 expression is depicted for CD4- cells (grey, solid) and for pDCs, intracellular (blue, solid with contour) and extracellular (green, solid with contour) expression is shown, indicating an absence of an intracellular CD4 pool in AGM pDCs. (D) Low CD4 expression was observed on pDCs from bone marrow (n = 1 AGM), LN (n = 1 AGM) and rectal biopsy (n = 1 AGM). Histograms depict CD4 expression on CD4- cells (grey, solid), pDCs (blue, solid with contour) and Lineage+ CD4+ cells (red, solid with contour). MFI values of CD4 are listed in the tables.
Figure 3. SIV sensing capacity of AGM pDCs. (A) SIVagm.sab92018 was used to stimulate AGM (n = 22) and SIVmac251 for Chinese rhesus MAC (n = 14) PBMCs. Alternatively, PBMCs were stimulated with HSV-1. (B) CD4 MFI was followed throughout SIVagm infection on four AGMs. Median and interquartile ranges are shown for CD4^+ Lineage^+ cells (red), pDCs (blue) and CD4^- Lineage^- cells (grey). (C) SIVagm-infected SupT1 cells and free virions were used to stimulate AGM PBMCs (n = 5). (D) SIVagm or AT2-inactivated SIVagm was used to stimulate AGM PBMCs (n = 8). Individual symbols represent distinct animals. Median and interquartile range are shown. Vertical dashes separate the different viral preparations used to test pDC sensing * Friedman, p < 0.05.

Figure 4. Viral and host determinants of SIV sensing by pDC. PBMC from (A) AGM (n = 5-23) and (B) Chinese rhesus MAC (n = 2-15) were stimulated with medium (mock), five SIVagm, three SIVmac or HIV-1 (BAL) at three concentrations: 1500 ng/mL, 150 ng/mL and 15 ng/mL p24/p27. Median values are represented by a horizontal line (blue and red for SIVagm and HIV/SIVmac, respectively). * Wilcoxon, p < 0.05, ** Wilcoxon, p < 0.01, compared to equal dose SIVagm.sab92018 stimulation. nd = not determined. Tox = cytotoxic in 18 hour culture. (C) AGM PBMC (n=10) were stimulated with medium (grey circle), 15, 150 or 1500 ng/mL p27 SIVmac251 (red square) or SIVmac239 (blue triangle) for 18 hours after which viability was measured. Median and interquartile ranges are depicted. * Wilcoxon, p < 0.05. (D) AGM PBMC were stimulated with HIV (n = 14), VSV-G pseudotyped HIV (n = 10), SIVmac251 (n = 10), VSV-G pseudotyped SIVmac251 (n=9) or SIVagm.sab92018 (n = 23) at 150 ng/mL p24 or p27. Median values and interquartile ranges are presented by a line and bars, respectively. * Kruskal-Wallis,
p < 0.05, ** Kruskal-Wallis, p < 0.01, *** Kruskal-Wallis, p < 0.001. Symbols represent individual animals, horizontal dashed lines represent the limit of detection.

**Figure 5. In vivo pDC infection.** (A) PDC and CD4+ T cells of chronically SIV-infected AGM (open circles, n = 7) and Chinese rhesus MAC (black circles, n = 5) were sorted from 2x10^8 to 4x10^8 splenocytes, yielding a median of six thousand pDC after two subsequent sorts (purity 91%). CD4+ T cells were also purified from lymph node (LN) cells (purity 97%). SIV DNA was normalized to CCR5 and represented as copies per million cells. Symbols represent individual animals. CD4+ T cells in LN were infected to a higher extent in MAC than AGM (* Mann-Whitney, p = 0.016). SIV DNA copy numbers were similar between spleen and LN CD4+ T in both species. (B) Fluorescence microscopy was performed on a LN of one chronically infected AGM. DAPI (blue) shows nuclei, CD123 (yellow) is expressed on pDC, SIV RNA (red) shows infected cells and NKp30 (green) is a marker not expressed on pDC. The merge shows an overlap from CD123 and SIV signals.

**Figure 6. CD4 and CCR5 expression on splenic AGM and MAC pDCs.** (A) CD4 expression was measured on pDCs from spleen of chronically SIV infected Chinese rhesus MACs (n=5) and AGMs (n=5). The mean fluorescent intensity (MFI) of CD4 on splenic pDCs was normalized (%) to the CD4 MFI on splenic CD4+ T cells of the same animal. The horizontal, dashed line designates equal CD4 expression to CD4+ Lineage+ cells. Symbols represent individual animals and line and bars represent the median and interquartile range, respectively. (B) The percentage of CCR5+ splenic pDCs was determined for SIV-infected Chinese rhesus MACs (n = 5) and AGMs (n = 5). Symbols represent individual animals and the line and bars represent the median and interquartile range. ** Mann-Whitney, p < 0.01.
A

B

C

D

SFCi12T4D11 clone

M-T466 clone

Bone marrow cells

Lymph node cells

Rectal biopsy cells
A

B

C

D

Mock SIV HSV

AGM MAC

CD4+ Lineage+
pDC CD4- Lineage+

Day p.i

Free SIV SIV-infected cells

Mock Uninf cells

SIV 15 ng
Inf cells 2.5%
SIV 150 ng
Inf cells 10.25%
SIV 1000 ng

IFNβ (IU/mL)

IFNβ (IU/mL)

Empty microvesicles
AT2-SIV/aggm
SIV/aggm

Mock 150 ng/mL 1500 ng/mL