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# SIV Infection Induces Accumulation of Plasmacytoid Dendritic Cells in the Gut Mucosa

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## Abstract

Multiple studies suggest that plasmacytoid dendritic cells (pDCs) are depleted and dysfunctional during human immunodeficiency virus/simian immunodeficiency virus (HIV/SIV) infection, but little is known about pDCs in the gut—the primary site of virus replication. Here, we show that during SIV infection, pDCs were reduced 3-fold in the circulation and significantly upregulated the gut-homing marker  $\alpha_4\beta_7$ , but were increased 4-fold in rectal biopsies of infected compared to naive macaques. These data revise the understanding of pDC immunobiology during SIV infection, indicating that pDCs are not necessarily depleted, but instead may traffic to and accumulate in the gut mucosa.

In peripheral blood and lymphoid tissues of primates, there are 2 major types of dendritic cells (DCs), myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), identifiable by mutually exclusive expression of the cell-surface integrin CD11c, and the IL-3R $\alpha$  chain CD123, respectively [1]. pDCs, also referred to as natural-interferon-producing cells, respond to microbial pathogens by rapidly producing interferon (IFN)- $\alpha$ , as well as other proinflammatory cytokines such as interleukin-12 (IL-12) and tumor necrosis factor (TNF)- $\alpha$ , initiating a cascade of both innate and adaptive immune responses. Although pDCs have been primarily characterized in peripheral blood samples, multiple studies have revealed that pDCs develop in the bone marrow or the thymus and then emigrate to numerous lymphoid and nonlymphoid tissues under steady-state conditions [2]. However, at present, very little is known about pDCs in human tissues other than blood and lymph nodes, in part due to limited access to tissue samples.

As first reported over a decade ago, multiple groups have verified that pDC numbers are severely reduced in blood and lymph nodes during human immunodeficiency virus (HIV) infection [3]. Furthermore, loss of

pDCs begins during primary infection, is associated with increasing viral loads, and is only partially reversible by highly active antiretroviral therapy. Although pDCs do harbor infectious virus, infection rates are relatively low, suggesting the loss of pDCs from blood and lymph nodes is likely dependent on factors besides direct infection. A comparable loss of pDCs has been demonstrated in simian immunodeficiency virus (SIV)-infected macaques [4]. Studies of acute pathogenic lentivirus infection in macaques have demonstrated a transient increase of pDCs in peripheral blood due to rapid egress from the bone marrow, followed by rapid depletion of circulating pDCs and the death of pDCs in lymph nodes [4, 5]. However, in a nonpathogenic lentiviral model, acute SIVagm infection of African green monkeys resulted in a transient reduction of pDCs in blood associated with an accumulation in the lymph nodes [6]. Multiple studies have also suggested cytokine production by pDCs is impaired in HIV/SIV infection. While this is a generally accepted phenomenon, subsequent research indicates that much of the perceived pDC dysfunction *ex vivo* is likely to reflect overstimulation *in vivo* [7]. Furthermore, although pDCs

have been described in the gastrointestinal tract, and despite the dramatic effects of HIV/SIV infection on pDCs in the blood, little is known about the effects of pDCs in the gastrointestinal mucosa, the primary site of virus replication.

## METHODS

### Animals

Twenty-five Indian rhesus macaques (*Macaca mulatta*) were analyzed: 15 SIV-naive macaques and 10 macaques infected chronically with SIVmac239. All animals were free of simian retrovirus type D, simian T-lymphotrophic virus type 1, and herpes B virus, and were housed at the New England Primate Research Center and maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Guide for the Care and Use of Laboratory Animals.

### Cell Processing

Macaque peripheral blood mononuclear cells were isolated from ethylenediaminetetraacetic acid (EDTA)-treated blood by density gradient centrifugation over lymphocyte separation medium (MP Biomedicals, Solon, OH) and contaminating red blood cells were lysed using a hypotonic ammonium chloride solution. Rectal biopsies were collected using 1.9 mm fenestrated endoscopic biopsy forceps (Olympus America, Center Valley, PA). Biopsy pieces and/or colonic tissue sections were weighed in bulk (Mettler balances MS104S or PB602, Mettler-Toledo, Columbus, OH), then mononuclear cells were isolated by both enzymatic and mechanical disruption as described previously [8].

### Polychromatic Flow Cytometry and Fluorescent Bead-Based Cell Quantification

Flow cytometry staining of mucosal mononuclear cells was carried out for cell-surface and intracellular molecules using standard protocols and the monoclonal antibodies noted in Table 1 [9]. LIVE/DEAD Aqua dye (Invitrogen, Carlsbad, CA) and isotype-matched controls and/or fluorescence-minus-one controls were included in all assays. All acquisitions were made on an LSR II flow cytometer (BD Biosciences, La Jolla, CA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Absolute quantification of cell subsets in blood and mucosal biopsy samples was performed using polychromatic flow cytometry gating strategies previously described [4, 8, 9] and shown in Figure 1A in combination with fluorescent bead-based assays optimized in our laboratory [8, 10].

### Intracellular Cytokine Staining

Mucosal mononuclear cells were resuspended in Roswell Park Memorial Institute medium 1640 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum alone or with imiquimod (Sigma-Aldrich) at a final concentration of 10  $\mu$ M. GolgiPlug (brefeldin A) and Golgi-Stop (monensin) were added at final concentrations of 6  $\mu$ g/mL to all samples and then cultured for 12 hours at 37°C in 5% CO<sub>2</sub>. After culture, samples were surface-stained using markers to delineate pDCs as shown in Figure 1. Cells were permeabilized using Caltag Fix and Perm, and intracellular cytokine staining was performed for macrophage inflammatory protein (MIP)-1 $\beta$  (fluorescein isothiocyanate conjugate, clone 24006, R&D Systems), IFN- $\alpha$  (phycoerythrin conjugate, clone 225.C, Chromaprobe, Maryland Heights, MO) and TNF- $\alpha$  (Alexa 700 conjugate, Mab11).

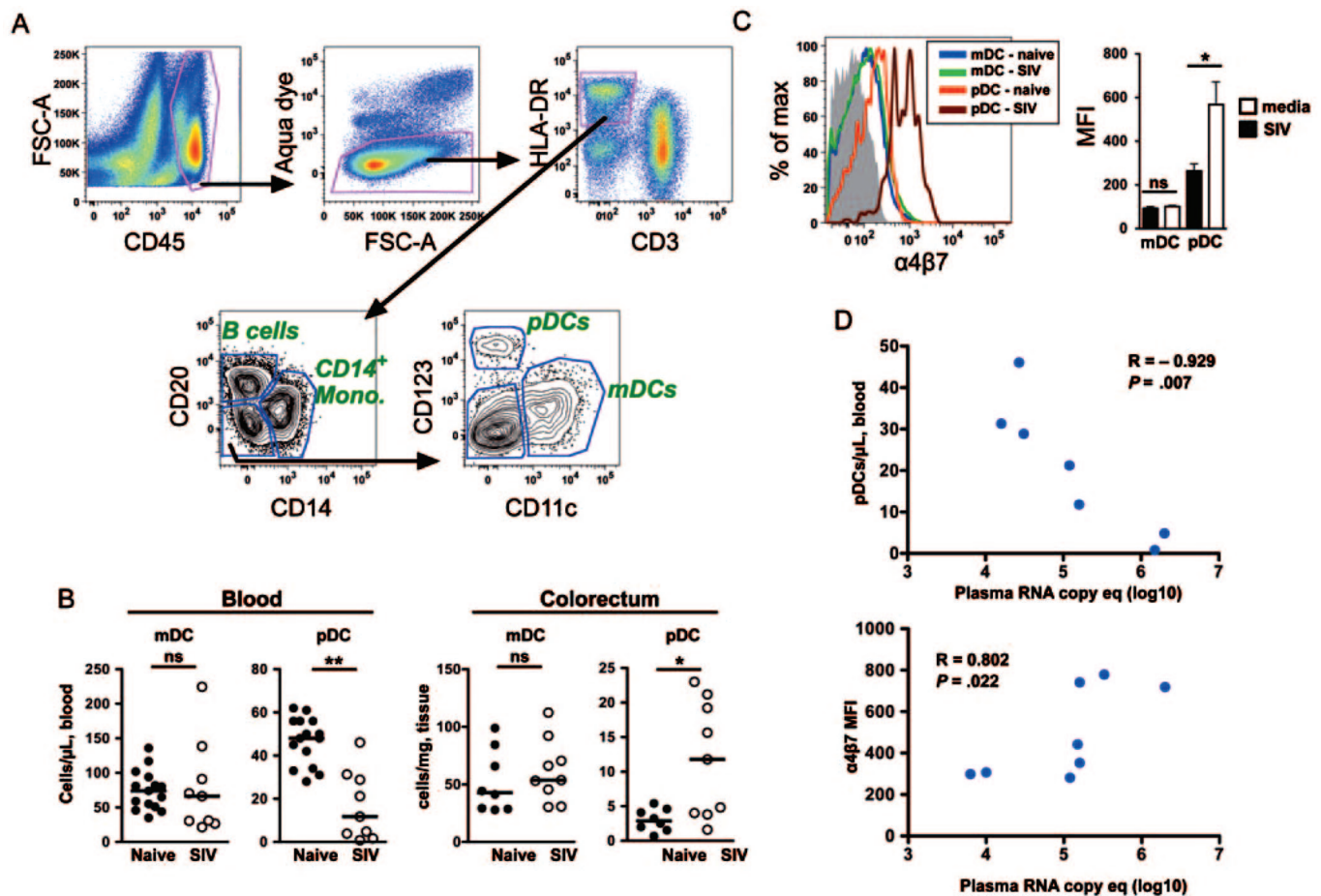
**Table 1.** Antibodies Used in Polychromatic Flow Cytometry Analyses

| Antibody                   | Clone     | Fluorochrome              | Manufacturer                       |
|----------------------------|-----------|---------------------------|------------------------------------|
| anti- $\alpha$ 4 $\beta$ 7 | A4B7      | APC                       | NIH NPRR <sup>a</sup>              |
| anti-CD3                   | SP34.2    | APC-Cy7                   | BD Biosciences (La Jolla, CA)      |
| anti-CD11c                 | S-HCL3    | PE, APC                   | BD Biosciences                     |
| anti-CD14                  | M5E2      | Alexa700, PE-Cy7          | BD Biosciences                     |
| anti-CD16                  | 3G8       | FITC                      | BD Biosciences                     |
| anti-CD20                  | L27       | PerCp-Cy5.5               | BD Biosciences                     |
| anti-CD45                  | D058-1283 | Pacific Blue <sup>b</sup> | BD Biosciences                     |
| anti-CD123                 | 7G3       | PE, PE-Cy7                | BD Biosciences                     |
| anti-HLA-DR                | Immu-357  | PE-Texas Red              | Beckman-Coulter                    |
| anti-IFN- $\alpha$ 2       | 225.C     | PE                        | Chromaprobe (Maryland Heights, MO) |
| anti-MIP-1 $\beta$         | 24006     | FITC                      | R&D Systems (Minneapolis, MN)      |
| anti-TNF- $\alpha$         | MAb11     | Alexa700                  | BD Biosciences                     |

*Abbreviations:* HLA-DR, human leukocyte antigen-DR; IFN, interferon; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

<sup>a</sup> NIH Nonhuman Primate Reagent Resource.

<sup>b</sup> In-house custom conjugate.



**Figure 1.** Dendritic cell dynamics in blood and colorectal mucosae of naive and SIV-infected macaques. *A*, Representative gating strategy for pDCs and mDCs. *B*, Absolute numbers of pDCs and mDCs in peripheral blood and colorectal biopsies were enumerated using a bead-normalized flow cytometry assay [8]. Black horizontal lines indicate medians. *C*, MFIs of cell-surface expressed  $\alpha 4\beta 7$  on peripheral blood pDCs and mDCs. Bars represent means  $\pm$  SEM of 9 animals per group. *D*, Plasma viral loads were correlated with absolute numbers of blood pDCs (upper panel) and cell-surface expression of  $\alpha 4\beta 7$  on blood pDCs (middle panel). Mann–Whitney *U* tests were used for naive-versus-SIV comparisons and Spearman correlation tests were used to assess associations with plasma viral loads. \**P* < .05; \*\**P* < .001.

*Abbreviations:* FSC-A, forward-scatter area; HLA-DR, human leukocyte antigen–DR; mDCs, myeloid dendritic cells; MFIs, median fluorescence intensities; Mono., monocyte; pDCs, plasmacytoid dendritic cells; SIV, simian immunodeficiency virus.

### Plasma Virus Load Quantification

RNA copy equivalents were determined in EDTA-treated plasma using a quantitative real-time reverse-transcription polymerase chain reaction assay based on amplification of conserved sequences in *gag* [11]. The limit of detection for this assay was 30 viral RNA copy equivalents/mL plasma.

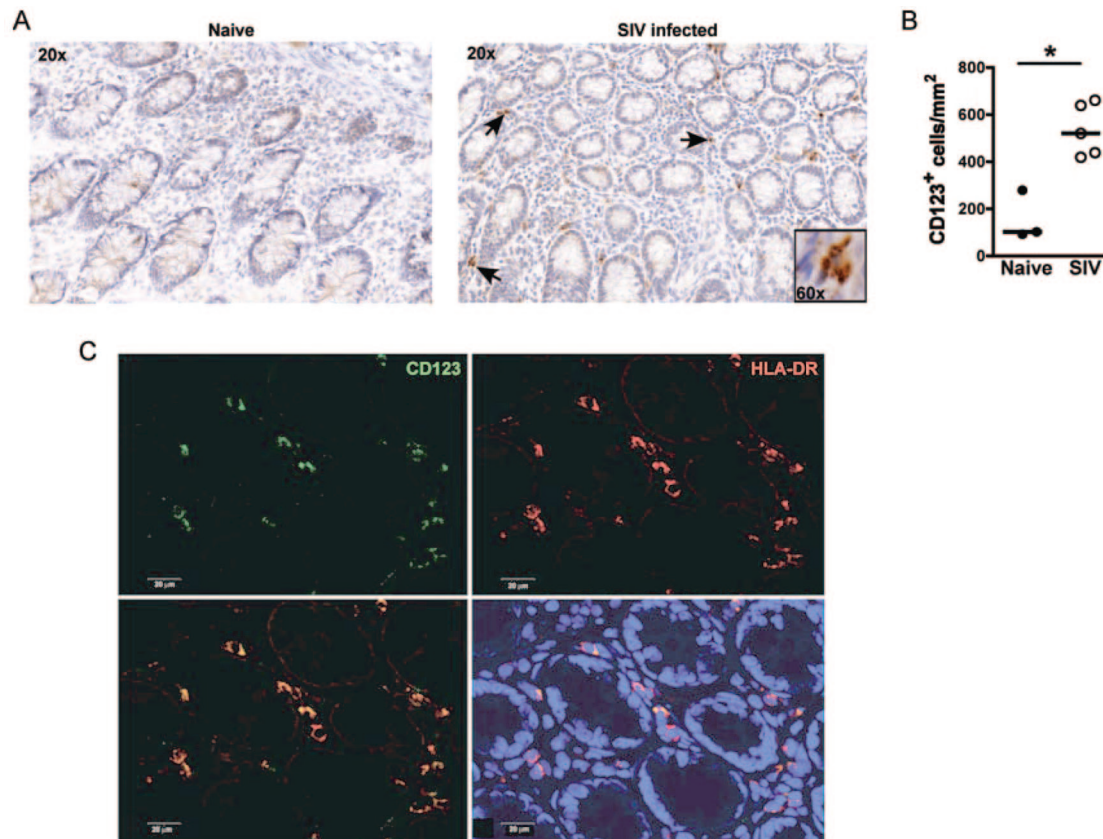
### Immunohistochemistry and Confocal Microscopy for pDCs In Situ

Identification and quantification of CD123<sup>+</sup> cells by immunohistochemistry was performed as described previously [12]. Briefly, immunochemically stained whole tissue sections were scanned using Scanscope. A digital slide of each CD123 stain was opened in ImageScope and lamina propria areas were selected with ImageScope drawing tools for analysis. CD123<sup>+</sup> cells were

quantified by using a positive pixel count algorithm in the Spectrum Plus analysis program (version 9.1). The parameters of the algorithm were manually tuned to match the CD123<sup>+</sup> markup image accurately over background DAB stain. Once the parameters were set, the algorithm was applied automatically to all digital slides to measure the number of CD123<sup>+</sup> cells.

Confocal microscopy on colorectal sections was performed as previously described [13]. Briefly, tissue sections were stained with primary antibodies to CD123 and human leukocyte antigen (HLA)–DR, and then secondary antibodies labeled with Alexa Fluor 488 (green) and Alexa Fluor 555 (red), respectively. Cell nuclei were counterstained blue with TOTO-3. Sequential images at wavelengths for each fluorophore were collected using a Bio-Rad MRC 1000 Confocal Microscope at  $\times 60$  and were processed in Adobe Photoshop 7.0.





**Figure 2.** Gut mucosal pDCs in situ. *A*, Representative immunohistochemistry slides of pDCs (stained brown) in colorectal tissue from naive (left panel) and chronically SIV-infected macaques (right panel). *B*, Quantitative analysis of gut mucosal pDC in naive ( $n = 3$ ) and SIV-infected ( $n = 5$ ) macaques. Total CD123<sup>+</sup> cells were calculated per mm<sup>2</sup> as described in the “Methods” section. The Mann–Whitney  $U$  test was used to evaluate significance;  $*P < .05$ . *C*, Immunofluorescent images of CD123 (green) colocalizing with HLA-DR (red) on the surface of pDCs in colorectal tissue. Cell nuclei are counterstained in blue.

*Abbreviations:* HLA-DR, human leukocyte antigen–DR; SIV, simian immunodeficiency virus.

### Statistical Analyses

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla, CA). Nonparametric Mann–Whitney and Spearman correlation tests and unpaired  $t$  tests were used where indicated and  $P < .05$  were assumed to be significant in all analyses.

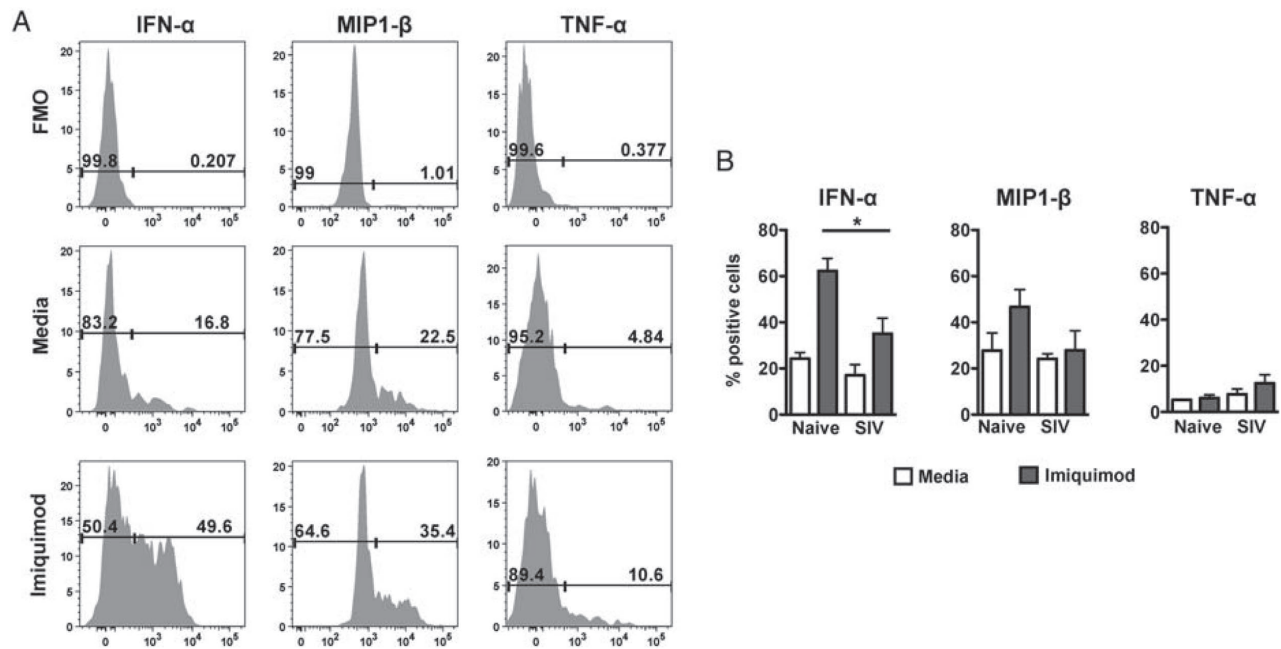
## RESULTS

### During Chronic SIV Infection, pDCs Are Reduced in Peripheral Blood but Accumulate in the Gut Mucosa

We first identified pDCs and mDCs among live CD45<sup>+</sup> mononuclear cells that were HLA-DR<sup>+</sup> and negative for common lineage markers (CD3, CD14, CD20) and found them easily distinguishable in both blood and rectal mucosa by mutually exclusive expression of CD123 and CD11c (Figure 1A). Next, we applied our 8-color flow cytometry panel to fluorescent bead quantification assays optimized in our laboratory for both blood and rectal biopsies to quantify absolute numbers of pDCs and mDCs in chronically SIV-infected and naive rhe-

sus macaques (Figure 1B). These analyses revealed no change in absolute numbers of circulating mDCs, but a significant reduction of circulating pDCs in chronically infected animals ( $P = .0005$ ), similar to previously published observations [4, 5]. However, in colorectal biopsies, pDC numbers were increased 4-fold in SIV-infected animals, while no change was observed in absolute counts of mDCs. Interestingly, the pDCs that did remain in peripheral blood of infected animals had significantly higher levels of cell-surface  $\alpha 4\beta 7$ , a MAD-CAM-1-binding integrin, which has been previously shown to mediate trafficking and retention of T and natural killer (NK) cells in the gut mucosae [9, 14] (Figure 1C). In infected animals, both the loss of pDCs in the peripheral blood and the upregulation of  $\alpha 4\beta 7$  on blood pDCs were significantly related to plasma viral load (Figure 1D). However, neither blood pDC numbers nor plasma viral loads significantly correlated with pDC numbers in colorectal biopsies (data not shown).

To further confirm our observations that pDCs accumulate in the gut mucosae during SIV infection, we next performed immunohistochemistry analysis of CD123<sup>+</sup>



**Figure 3.** Mucosal pDC function. *A*, Representative flow cytometry plots showing percentages of colonic pDCs (as gated in Figure 1) expressing intracellular cytokines with and without stimulation. *B*, Percentages of colonic pDCs from naive and SIV-infected macaques positive for intracellular IFN- $\alpha$ , MIP1- $\beta$  and TNF- $\alpha$ , without stimulation (media) and with imiquimod added to cultures. Bars represent means  $\pm$  SEM of 4 animals per group. Unpaired *t* tests were used for naive-versus-SIV comparisons; \**P* < .05. Only statistically significant differences are shown. *Abbreviations:* FMO, fluorescence minus one; IFN, interferon; mDCs, myeloid dendritic cells; MIP, macrophage inflammatory protein; pDCs, plasmacytoid dendritic cells; SIV, simian immunodeficiency virus; TNF, tumor necrosis factor.

cells. These studies verified that pDCs were significantly increased in colorectal specimens of SIV-infected macaques and showed that most CD123<sup>+</sup> cells were found in the lamina propria (Figure 2*A* and 2*B*). Using confocal microscopy, we also demonstrated that the vast majority of colorectal CD123<sup>+</sup> lymphocytes were HLA-DR<sup>+</sup>, further confirming their identity as pDCs (Figure 2*C*).

#### Gut pDCs from Naive and SIV-Infected Macaques Are Potent Cytokine-Producing Cells

Many previous studies have suggested that during chronic HIV/SIV infection pDCs are dysfunctional. However, subsequent studies indicate that this perceived dysfunction is at least partially attributable to overstimulation *in vivo* [7]. Furthermore, these studies did not address pDCs in the mucosae. Using a 3-function intracellular cytokine-staining assay, we sought to evaluate the functional capacity of colonic pDCs. Because pDCs are relatively rare in the gut mucosa, for functional assays we used mononuclear cells isolated from colons taken at necropsy from naive and SIV-infected macaques. Not surprisingly, relatively high frequencies of pDCs were positive for IFN- $\alpha$  secretion even without stimulation, and production was significantly enhanced by addition of the Toll-like receptor 7 ligand imiquimod (Figure 3). However, IFN- $\alpha$  production in response to imiquimod stimulation was significantly less in pDCs from infected animals, suggesting some refractoriness to stimulation.

Interestingly, high frequencies of colonic pDCs were also positive for MIP1- $\beta$  production, a C-C chemokine receptor type 5 (CCR5)  $\beta$ -chemokine known to block HIV/SIV infection [15], but produced only low levels of the proinflammatory cytokine TNF- $\alpha$ .

## DISCUSSION

Here, we present data indicating that the currently accepted pathogenesis of systemic pDC depletion during lentivirus infection is, at least in part, inaccurate. While many studies of both HIV-infected humans and SIV-infected macaques indicate pDCs are depleted in peripheral blood and lymph nodes, these studies have not analyzed pDCs in the gut mucosa, the primary site of virus replication. We present here, alternatively, that while pDC numbers are diminished in the blood of chronically SIV-infected macaques, they are accumulating in the gut mucosa.

Similar to what has been previously observed in both HIV and SIV infections [3, 4], we found reduced numbers of pDCs in the circulation of chronically SIV-infected macaques, which negatively correlated with viral load. However, most previously published results have reported decreased frequencies of pDCs, and we verify here for the first time a loss of absolute numbers of pDCs as determined by a more accurate bead-based

enumeration assay. Next, we observed in these same animals increased numbers of pDCs in colorectal biopsies from infected animals, also determined by a highly sensitive bead-based enumeration assay [8]. These data suggested that as pDCs are reduced in blood, they accumulate in the gastrointestinal tract—a hypothesis supported by mouse studies showing that pDCs accumulate in tissues where there is active viral replication due to the many strategies pDCs have evolved to “sense” viral products [16].

To explore potential mechanisms of trafficking, we next evaluated expression of the gut-trafficking and retention integrin  $\alpha 4\beta 7$  on blood pDCs. In infected animals,  $\alpha 4\beta 7$  was significantly upregulated, correlating with increasing viral load. While not definitive, these data suggest increased  $\alpha 4\beta 7$  expression on pDCs as a plausible mechanism for increased trafficking and retention of these cells in the gastrointestinal mucosa, a phenomenon also observed with T and NK cells during chronic lentivirus infections [9, 14]. While increased  $\alpha 4\beta 7$  expression appears to be related to increased pDCs in the gut, the exact mechanisms responsible for this altered trafficking repertoire remain unclear. It could reflect upregulation of  $\alpha 4\beta 7$  on previously  $\alpha 4\beta 7$ -negative differentiated pDCs by retinoic acid imprinting as has been observed for T cells [14], or alternatively an emigration of  $\alpha 4\beta 7^+$  pDCs from a currently undefined reservoir.

Previous studies have reported that blood pDCs are functionally impaired during HIV and SIV infection. Similarly, we found a modest, but significant, reduction in IFN- $\alpha$  production by mucosal pDCs from SIV-infected macaques. However, it is difficult to determine from these analyses whether pDCs from infected animals are truly dysfunctional in IFN- $\alpha$  production, or if this reflects the fact that pDCs are overstimulated in vivo and therefore have reduced IFN- $\alpha$ -producing capacity ex vivo. This phenomenon has been previously described to account, in part, for reduced cytokine production by blood pDCs from HIV-infected patients [7]. Regardless, the 4-fold increase in absolute numbers of pDCs in the gut mucosa suggests that overall IFN- $\alpha$  production is likely to be increased, and we have previously found that in many infected macaques, IFN- $\alpha$  is upregulated in the gut mucosa [17]. Although there may be multiple sources of IFN- $\alpha$  in the SIV-infected gut, pDCs could contribute to both IFN- $\alpha$ -induced chronic immune activation and IFN- $\alpha$ -dependent cell apoptosis that have been described in chronic lentivirus infection [18]. Furthermore, these data indicate that pDCs in both naive and infected animals are a potent source of MIP-1 $\beta$ . An overall increase of MIP-1 $\beta$  in the gut during infection could potentially have both beneficial and detrimental effects. First,  $\beta$ -chemokines such as MIP-1 $\beta$  have been previously shown to inhibit HIV/SIV infection by blocking

the CCR5 coreceptor [15]. Alternatively, however, pDC secretion of MIP-1 $\beta$  could also recruit CCR5 $^+$  memory CD4 $^+$  T cells, the primary HIV/SIV target cells, to the gut, thereby fueling virus replication. A similar mechanism of pDC-dependent recruitment of memory CD4 $^+$  T cells to the vaginal tract during transmission has recently been proposed [15].

In summary, these data advance our understanding of pDC immunobiology during lentivirus infection by revising the current dogma that they are depleted and suggesting instead that they accumulate in the gut mucosa. Given the complex network of virus-sensing mechanisms found in pDCs, in retrospect it is perhaps not surprising that these cells accumulate in the primary site of virus replication. However, the consequences of accumulating pDCs in the gut mucosae in regard to chronic immune activation, cell apoptosis, and target cell recruitment will require further study.

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While our manuscript was under review, another group published a manuscript that reports similar findings (Kwa S, Kannanganat S, Nigam P, Siddiqui M, Shetty RD, Armstrong W, Ansari A, Bosinger SE, Silvestri G, Amara RR. Plasmacytoid dendritic cells are recruited to the colorectum and contribute to immune activation during pathogenic SIV infection in rhesus macaques. *Blood* 2011; 118:2763–73).

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R. P. Johnson and R. K. Reeves have filed a patent application on bead-based quantification of mucosal lymphocytes. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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