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Myeloid dendritic cells isolated from tissues of SIV-infected Rhesus macaques promote the induction of regulatory T-cells

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Abstract

Objective—To determine whether the ability of primary myeloid dendritic cells (mDC) to induce regulatory T cells (Treg) is affected by chronic SIV infection.

Design—Modulation of DC activity with the aim of influencing Treg frequency may lead to new treatment options for HIV and strategies for vaccine development.

Methods—Eleven chronically-infected SIV⁺ Rhesus macaques were compared with four uninfected animals. Immature and mature mDC were isolated from mesenteric lymph nodes and spleen by cell sorting and cultured with purified autologous nonTreg (CD4⁺CD25⁻ T cells). CD25 and FOXP3 upregulation was used to assess Treg induction.

Results—The frequency of splenic mDC and plasmacytoid DC was lower in infected animals than in uninfected animals; their frequency in the mesenteric lymph nodes was not significantly altered, but the percentage of mature mDC was increased in the mesenteric lymph nodes of infected animals. Mature splenic or mesenteric mDC from infected animals were significantly more efficient at inducing Treg than mDC from uninfected animals. Mature mDC from infected macaques induced more conversion than immature mDC. Splenic mDC were as efficient as mesenteric mDC in this context and CD103 expression by mDC did not appear to influence the level of conversion.

Conclusions—Tissue mDC from SIV-infected animals exhibit an enhanced capability to induce Treg and may contribute to the accumulation of Treg in lymphoid tissues during progressive infection. The activation status of DC impacts this process but the capacity to induce Treg was not restricted to mucosal DC in infected animals.

Keywords

Simian Immunodeficiency Virus; Dendritic Cells; T-lymphocytes, Regulatory; Lymphoid Tissue; Pathogenesis; FACS

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Conflict of interest disclosure

The authors have no conflict of interest.

Introduction

Natural regulatory T-cells develop in the thymus as a distinct lineage expressing CD4, CD25, and the transcription factor FOXP3. These cells have a critical role in the establishment and maintenance of physiological tolerance through the suppression of auto-immune responses [1]. In addition to thymically-derived 'classical' Treg, a growing number of reports have described the peripheral conversion of CD4⁺CD25⁻ T-cells into CD25⁺FOXP3⁺ Treg [2-8]. These induced or adaptive Treg are generated during induction of oral tolerance and in response to many inflammatory processes, including persistent infections [9].

Simian immunodeficiency virus (SIV) infection of Rhesus macaques (RM) results in early and persistent accumulation of Treg in the lymph nodes [10, 11]. Similar increases in Treg frequency and number have been observed in macaque and human lymphoid and mucosal tissues during chronic-stage SIV/HIV infection [12-17], yet the causes and consequences of this increase remain unclear. Indeed, several studies have proposed that Treg protect the host by mitigating the adverse affects of immune activation [18, 19] and HIV-infection of CD4⁺ non-Treg [20]; however, other findings have suggested that Treg actively suppress virus-specific immune responses, inadvertently promoting viral persistence [21-24]. Treg accumulation in tissues during HIV/SIV may be mediated by multiple mechanisms, including increased survival [12, 14, 25], trafficking [16, 25], proliferation [13, 14, 16], and extra-thymic/peripheral conversion [26, 27]. Understanding the contributions of these various mechanisms will provide a more accurate picture of Treg dynamics in the context of HIV/SIV infection and may aid in the development of targeted immuno-therapies and vaccines.

Antigen-presenting cells (APC) have been shown to mediate peripheral Treg conversion in mice and humans. In the gut, where immunity to intestinal pathogens must be tightly coordinated with tolerance to food and commensal antigens, Treg induction by murine CD103⁺ dendritic cells (DC) appears particularly efficient [28, 29]. Several authors have reported that murine CD103⁺ gut DC are required for *de novo* Treg conversion through mechanisms involving transforming factor- β (TGF- β), retinoic acid (RA) and indoleamine 2,3-dioxygenase (IDO) [7, 30, 31]. Moreover, CD103⁺ DC in human mesenteric lymph nodes (MLN) are potent inducers of allogeneic Treg [32].

We hypothesized that the increased frequency of Treg in lymphoid tissue of SIV-infected macaques could be due to enhanced DC-mediated conversion. To test this hypothesis, we isolated immature and mature myeloid DC (mDC) from mesenteric lymph nodes (MLN) and spleen (SPL) of SIV-infected and uninfected RM, cultured them with autologous CD4⁺CD25⁻ nonTreg for 4 days, and examined levels of CD25 and FOXP3 in T-cells. Our results suggest that SIV-infection promotes DC-mediated Treg conversion in both the SPL and MLN. Interestingly, a higher level of conversion was observed for the mature DC population; however, Treg induction did not correlate with CD103 expression by DC.

Methods

Animals and viral infection

Colony-bred Rhesus macaques (*Macaca mulatta*) were obtained from the California National Primate Research Center (Davis, CA). Animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International, and the study was reviewed and approved by the animal care and use committees at UC Davis. Care and use of animals were in compliance

with institutional (National Institutes of Health) guidelines. Prior to the study, all animals were determined to be seronegative for SIV, simian T-cell lymphotropic virus type 1, and simian retrovirus. Samples were obtained from 4 mature multi-parous cycling female macaques infected via vaginal inoculation with SIV_{mac251} (5000 TCID₅₀) and 7 mature males infected via penile inoculation with SIV_{mac251} (10⁴ or 10⁵ TCID₅₀). The virology of 3 of these animals has been published [33], the others are part of a control group for an unpublished vaccine study. None of the animals received antiretroviral therapy or immune modulators before euthanasia. Real-Time PCR (sensitivity: 125 copies/ml) was used to determine plasma SIV RNA levels. Four uninfected animals were included in the study as negative controls. Clinical data at the time of euthanasia are shown in Table 1. Initial experiments were focused on conversion in spleen only due to the paucity of available tissues and did not include DC phenotyping, resulting in n=7 SPL and n=6 MLN samples for phenotypic analysis, n=9 SPL and n=6 MLN samples for conversion experiments. The number of animals for which both DC phenotyping data and conversion data were available was n=5 SPL and n=6 MLN sample-pairs.

Cell isolation and culture

Single-cell suspensions from mesenteric lymph nodes (MLN) and spleen (SPL) were generated the day of euthanasia. Each lymph node was dissected and cells were detached from the surrounding membrane using a scalpel. SPL tissue was diced and dissociated into a homogenous cell suspension using a pestle. MLN and SPL cell suspensions were passed through 70 μ m cell strainers, washed in RPMI 1640 containing 15% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2mM glutamine, and red blood cell lysis performed as needed using ammonium chloride/potassium carbonate/EDTA (ACK). Cells were rested overnight at 37°C and 5% CO₂. After overnight culture, viability was consistently > 95% (trypan blue exclusion test).

SPL CD4⁺ T-cells were purified from cell suspensions (300×10⁶ cells on average) by negative selection (CD4⁺ T Cell Isolation Kit non-human primate, Miltenyi Biotec; Auburn, CA). CD25⁻ nonTreg (<1.0% FOXP3⁺ cells post-isolation) were purified from isolated CD4⁺ T-cells using CD25 MicroBeads for non-human primates (Miltenyi Biotec).

DC were separated from an average of 300×10⁶ MLN or SPL cells on a Cytomation MoFlo Cell Sorter (Beckman Coulter, Brea, CA). Cells were stained in PBS containing 2% FBS using fluorochrome-conjugated antibodies. DAPI (1 μ g/ml, final) was added to cells prior to sorting in order to exclude dead cells. Myeloid DC (mDC) were defined as Lineage (CD3⁻CD14⁻CD20⁻NKG2D⁻EpCam⁻) but HLA-DR⁺ and CD11c⁺. Mature (CD83⁺) and immature (CD83⁻) mDC were sorted into separate tubes.

DC/nonTreg co-cultures were established in 48-well plates at a ratio of 1:10 (5×10⁴ DC: 5×10⁵ nonTreg), previously determined to be optimal for FOXP3 induction. Kinetic experiments indicated that FOXP3 induction peaked after 4 days of culture. These experimental conditions were used throughout the study.

Cell Sorting and flow cytometry

The following antibodies (Abs) were used for isolation and characterization of DC: anti-Lineage [CD14 (TuK4, Invitrogen; Carlsbad, CA), anti-CD20 (2H7, eBioscience; San Diego, CA), anti-CD3e (SP34, BD Pharmingen; San Diego, CA), anti-EpCam (9C4, BioLegend; San Diego, CA), anti-NKG2D-FITC (1D11, eBioscience)] and anti-CD83-PerCPCy5.5 (HB15e, BioLegend). Anti-HLA-DR-PE-Cy7 (L243, BD Pharmingen), anti-CD11c-AF700 (3.9, eBioscience), and anti-CD123-PE (7G3, BD Pharmingen) were included in the phenotyping panel in addition to anti-CD103 (2G5.1, AbDSerotec;

Kidlington, UK) which was labeled using the Pacific Blue mouse IgG2a Zenon Labeling Kit (Invitrogen). APC-conjugated anti-HLA-DR (L243) and PE-conjugated anti-CD11c (3.9) were included in the DC-sorting panel. All antibodies were titrated prior to use.

The following Abs were used for phenotypic characterization of Treg and nonTreg: anti-CD3-Pacific Blue (SK7), anti-CD4-PerCP-Cy5.5 (L200), and anti-CD25-PE-Cy7 (MA251) (all from BD Pharmingen), and anti-FOXP3-AF647 (PCH101) (eBioscience). In order to analyze T-cell proliferation, nonTreg were labeled before culture with 0.312 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR).

Cells were treated with 20 μ g/ml of human IgG to block Fc receptors, stained for surface markers 30 minutes at 4°C in PBS, washed, and fixed in 1% paraformaldehyde. For intracellular staining, cells were fixed and permeabilized using the FOXP3 staining buffer set (eBioscience) as per the manufacturer's protocol. Samples were analyzed on a BD LSR-II Flow Cytometer. At least 250,000 events were recorded for each sample. Doublets were excluded on the basis of scatter properties and dead cells excluded using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen). Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Statistical analysis

GraphPad Prism (GraphPad Software, La Jolla, CA) was used to graph and analyze data for statistical significance. Intra-group comparison was analyzed using the Wilcoxon test. Inter-group comparison was analyzed using the Mann-Whitney test. Linear regression analysis was used to test correlations. P values between 0.1 and 0.05 were considered trends; p values of 0.05 or less were considered significant.

Results

SIV infection reduces the frequency of SPL DC while the frequency of MLN DC remains unchanged

A loss of circulating mDC and plasmacytoid DC (pDC) has been reported in HIV/SIV infected subjects [34-40]. It has been hypothesized that infection is associated with recruitment of DC to inflamed lymph nodes, partially explaining their disappearance from blood as infection progresses [39-42]. However, results concerning the frequency of these cells in lymphoid tissues are often conflicting. Such discrepancies arise from differences in the stage of the infection and type of tissue analyzed, as well as the use of different animal models and SIV-strains [43-52]. Thus, we first investigated the frequency of DC in the MLN and SPL of SIV-uninfected and chronically infected RM. SIV-infected macaques trended toward a decreased frequency of mDC (Lin⁻HLA-DR⁺CD123⁻CD11c⁺) in the SPL compared to uninfected animals (p=0.1). In contrast, mDC frequency in the MLN was comparable in infected and uninfected macaques (Fig. 1a). Similar results were seen for pDC (Lin⁻HLA-DR⁺CD11c⁻CD123⁺) where SIV⁺ RM exhibited significantly higher levels of SPL pDC than uninfected animals (p=0.04) and differences in MLN pDC between the two groups were negligible (Fig. 1b). Additionally, within control animals a higher frequency of DC was observed in SPL compared to MLN. This was true for both mDC, which trended toward an increased frequency (p=0.06) (Fig. 1a) and pDC, where the difference was significant (p=0.04) (Fig. 1b). In contrast, no significant difference in DC frequencies was observed between tissues from the infected group. Notably, SIV infection did not change the median mDC/pDC ratios in MLN or SPL (p>0.30, Fig. 1c).

Due to limitations in the amount of tissue available we were unable to study Treg induction by both mDC and pDC. For the current study we chose to focus on the more abundant mDC population.

Maturation of mDC is enhanced during SIV-infection

Because DC maturation state can influence Treg induction [53, 54], we examined mDC expression of CD83, a molecule strongly upregulated on the surface of mature DC [55, 56]. SIV infection enhanced the frequency of CD83⁺ DC in MLN ($p=0.05$). A similar trend was observed in SPL, although this did not reach statistical significance ($p=0.15$) (Fig. 1d). The frequency of mature mDC correlated with plasma viral load ($r=0.44$, $p=0.01$) (Fig. 1e) and duration of infection ($r=0.39$, $p=0.02$) (Fig. 1f) but did not correlate with CD4 count or age (data not shown).

mDC isolated from SIV-infected macaques induce autologous Treg

Several studies have reported an accumulation of Treg in lymphoid tissues during HIV/SIV infection [12-17, 57]. However, the origin of these cells remains unclear. Recently, Banerjee et al. showed that among APC, mature mDC are the most potent inducers of Treg [2]. We therefore hypothesized that enhanced mDC-mediated conversion might play a role in Treg accumulation in lymphoid tissues during HIV/SIV infection.

To test this hypothesis, we sorted mature CD83⁺ mDC and immature CD83⁻ mDC from the MLN and SPL of uninfected or SIV-infected animals (see figure, Supplemental Digital Content 1, sorting strategy). In parallel, autologous CD4⁺CD25⁻FOXP3⁻ nonTreg were isolated from spleen. Depletion of FOXP3⁺ cells was evaluated post-purification and found to be equal in the two groups of animals (data not shown). Immature or mature mDC from the SPL or MLN were then cultivated with autologous CD4⁺CD25⁻FOXP3⁻ nonTreg for 4 days.

NonTreg cultivated alone expressed neither CD25 nor FOXP3 (Fig. 2a); however, when cultivated with MLN mDC, FOXP3 and CD25 were both upregulated on a distinct subset of cells. This induction was significantly higher in co-cultures containing mDC from infected animals than those from uninfected macaques and was observed for both mature ($p<0.01$) and immature ($p=0.05$) DC (Fig. 2b). Similarly, increased conversion was found in cultures with SPL mDC from infected macaques, mature or not ($p<0.01$ for both) (Fig. 2c). When purified CD4⁺CD25⁺ Treg (FOXP3 expression > 80%) were cultured with MLN or SPL mDC, they did not undergo proliferation (not shown). It therefore appears likely that the CD25⁺FOXP3⁺ cells present after 4 days of co-culture originated from conversion of CD4⁺CD25⁻ nonTreg rather than from expansion of contaminating CD4⁺CD25⁺ Treg. Intriguingly, in SIV-infected animals mature mDC induced more Treg than immature DC. This effect was a strong trend for MLN mDC (0.07) and significant for SPL mDC ($p<0.01$) (Fig. 2d), suggesting that the activation level of mDC strongly influences regulation of the immune response.

Numerous studies have highlighted the extent of Treg conversion in the gut [58-60]. We therefore compared the ability of mature MLN and SPL DC to induce Treg. Surprisingly, a comparable level of Treg induction was observed by mDC from MLN and SPL ($p=0.31$) (Fig. 2e). Thus, DC from both tissues may actively promote Treg accumulation in the context of SIV.

The percentage of CD25⁺FOXP3⁺ T-cells induced by mDC from SIV-infected macaques did not correlate with plasma viral load or CD4 count. In contrast, there was a trend toward an inverse correlation between the duration of infection and the percentage of induced CD25⁺FOXP3⁺ T-cells ($r=0.24$, $p=0.06$) (see figure, Supplemental Digital Content 2,

correlation analyses). This result suggests that exposure to SIV over time may either impair the capacity of DC to induce Treg or alternately, the ability of nonTreg to properly respond to DC-generated signals.

In order to further characterize the induced Treg, we labeled the isolated CD4⁺CD25⁻ nonTreg with CFSE prior to culture with mDC. Because the efficiency of Treg induction by MLN and SPL mDC was similar, we combined these data for statistical purposes. In contrast to control animals, the majority of induced FOXP3⁺ cells in cultures from SIV-infected animals proliferated ($p < 0.001$) (Fig. 3a and b), offering a potential explanation for the higher percentage of CD25⁺FOXP3⁺ cells found in these cultures.

In addition to proliferation, cell death may impact Treg frequency in our cultures. NonTreg cultivated alone showed the highest level of mortality, suggesting that culture with DC improved their viability (not shown); nonetheless, no obvious differences in cell death were observed *in vitro* for uninfected and infected animals (Fig. 3c). These results were upheld when MLN and SPL were considered separately (not shown).

CD103 expression in mDC is not correlated with induction of Treg

Iliev et al. recently described CD103⁺ DC isolated from human MLN as consistent inducers of allogeneic Treg [32]. CD103 is an integrin involved in the retention of T-cells and DC in the gastrointestinal tract [61, 62]. CD103⁺ DC produce RA, which induces Treg in association with TGF- β [7, 30, 31]. Although mDC were not sorted based on CD103 expression, we examined this molecule by flow cytometry in mature and immature mDC from MLN and SPL. The frequency of CD103⁺ DC was highest in mature DC in both animal groups, whereas immature DC expressed only very low levels of CD103 (<6%) (data not shown). We therefore asked whether an increased frequency of CD103⁺ mDC in MLN and SPL of SIV⁺ macaques could explain their higher level of Treg conversion. Unexpectedly, CD103 trended toward higher expression in mature MLN mDC from uninfected compared to infected animals ($p = 0.08$), and the percentage of SPL CD103⁺ mDC was equivalent between the 2 groups. Moreover, while uninfected RM showed a strong trend toward increased CD103 expression in MLN mDC compared to SPL ($p = 0.06$), infected macaques maintained similarly low levels in both tissues (Fig. 4a). Thus, expression of CD103 by mDC did not appear to be predictive of their capacity to induce Treg. We directly tested this hypothesis by analyzing the association between the percentage of CD103⁺ mDC in each cellular preparation from the infected macaques and their ability to induce Treg. Although there did appear to be a positive correlation, we did not find a statistically significant relationship for either MLN (Fig. 4b) or SPL (Fig. 4c).

Discussion

Treg have been shown to accumulate in lymphoid tissues during chronic SIV infection, potentially contributing to the suppression of anti-viral T-cell responses. DC play a well-established role in initiating adaptive immune responses to viral pathogens, including SIV; however DC are also known to mediate peripheral tolerance, in part through the induction of Treg [28, 63]. Because DC actively migrate to lymph nodes during SIV/HIV infection, we hypothesized that trafficking of DC during SIV/HIV infection may favor the induction of Treg within lymphoid tissues. While we saw a decreased percentage of DC in the SPL of infected RM, levels in the MLN were comparable to those in uninfected controls, and mDC from both the MLN and SPL of SIV-infected animals exhibited a more mature phenotype based on CD83 expression. These results are in agreement with previous studies showing altered maturation and activation of DC during HIV/SIV infection both in blood and lymphoid tissues, including the presence of semi-mature DC [26, 47, 48, 64-67]. Semi-mature DC express high levels of costimulatory molecules, but do not secrete cytokines

necessary for the development of effector T-cells; rather, they are often associated with the induction of tolerance [53]. Additionally, high circulating levels of lipopolysaccharide typically found in chronic HIV and SIV infection [68, 69] may result in dysfunctional DC, as prolonged *in vitro* exposure to LPS produced “exhausted” DC that could not prime T-helper 1 differentiation but instead secreted immunosuppressive IL-10 [70]. Therefore, although the mDC we studied were phenotypically mature, they could have been functionally impaired or otherwise skewed toward Treg induction.

The results of our co-culture experiments indicated that mDC from SIV-infected RM were significantly more efficient at inducing Treg than mDC from uninfected animals and it was the mature rather than the immature mDC that were most efficient at driving Treg induction. Immature DC are classically associated with the induction of tolerance; however, several reports have identified activated or semi-activated DC as efficient at inducing or expanding Treg [2, 71-73], a function that may help mitigate host tissue damage due to an over-exuberant immune response. In lymph nodes of untreated HIV-infected individuals Krathwohl et al. found a high percentage of CD83⁺IL-12⁻ semi-mature DC which induced FOXP3 expression in allogeneic T-cells *ex vivo* [26]. Mature monocyte-derived DC exposed to high doses of HIV *in vitro* also induced an immunosuppressive phenotype in allogeneic T-cells, characterized by increased expression of *FoxP3* and *Blimp-1* [74]. Furthermore, expression of costimulatory molecules on mature DC is necessary for the maintenance of self-tolerance [75]. Thus, the activation state of DC in lymphoid tissues during HIV/SIV infection may support an environment favorable to Treg induction rather than the activation of effector T-cells.

Interestingly, our results show that induction of CD25 and FOXP3 was not limited to the GALT of infected animals, as splenic DC were as efficient as MLN in this context. Neither do our results support a role for CD103 in DC-mediated Treg induction, as we did not find a significant correlation between CD103 expression by mature DC and their capacity to induce CD25 and FOXP3 in autologous T-cells; however this may be due to the low number of animals available for these analyses. It is possible a larger study would reveal a statistically significant relationship. Altogether, our data suggest that SIV infection promotes DC-mediated Treg induction in all lymphoid compartments, a finding consistent with previous reports showing increased Treg frequency in multiple lymphoid tissues of chronically infected RM, including the spleen, peripheral LN and colon [12, 15].

Although we did not directly examine the molecular mechanism(s) involved in DC-mediated Treg induction, previous studies indicate possible roles for TGF- β and IDO. TGF- β , a cytokine known to be involved in Treg conversion, is increased in lymphatic tissues during SIV- and HIV-infection and appears to be generated by Treg present there [11, 76]. TGF- β -producing Treg may then educate DC to become tolerogenic through a phenomenon termed “infectious tolerance” [77, 78], thereby perpetuating a cycle of immune-suppression. The tryptophan-catabolizing enzyme, IDO, has been implicated in SIV/HIV-mediated immune dysfunction [15, 17, 79] and several groups have described a role for IDO in driving Treg induction [5, 71, 72]. Manches et al. found that pDC exposed to HIV *in vitro* induced allogeneic Treg through an IDO-dependent mechanism [27]. However, Kwa et al. recently reported higher IDO levels in mDC from MLN of SIV⁺ RM compared to pDC. It is possible TGF- β and IDO contributed to mDC-mediated conversion in our cultures; however further study will be needed to fully elucidate the mechanisms involved.

This study did not assess the suppressive capacity of the CD25⁺FOXP3⁺ cells generated *in vitro* due to difficulties in purifying a sufficient number of viable CD25⁺ Treg post co-culture; an additional group of animals would have been required to address this question. Results of previous studies indicate that similarly-induced Treg are functionally suppressive

[22, 27, 74]; nonetheless, this important question will need to be addressed in future experiments.

To our knowledge, our study is the first to show that SIV infection increases the capacity of DC to induce FOXP3 expression in autologous T-cells *ex vivo*. These data thus expand our knowledge of how DC may influence Treg frequencies within lymphoid tissues, and provide a potential mechanism underlying increased Treg frequencies found in tissues during progressive SIV/HIV infection. Regardless of the role played by Treg in HIV/SIV pathogenesis, a better understanding of the mechanisms regulating Treg dynamics could provide new opportunities for the development of targeted immuno-therapies designed to either boost anti-HIV responses or limit hyper-immune activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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P.P. and J.M.S. performed experiments, analyzed data, and wrote the manuscript. C.J.M., B.L.S., and C.A.C. designed the experiments and wrote the manuscript.

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Figure 1. The frequency of SPL mDC and pDC is lower in infected animals than uninfected RM, but similar in MLN

Cell suspensions obtained from MLN and SPL of uninfected and SIV-infected animals were stained with a cocktail of mAbs. DC were identified as Lineage-HLA-DR⁺CD123⁺CD11c⁺ (mDC) and Lineage-HLA-DR⁺CD11c⁺CD123⁺ (pDC) by flow cytometry. The percentage of (a) mDC and (b) pDC in MLN and SPL is shown. The ratio of mDC/pDC is depicted in (c). (d) Graph represents the percentage of mature mDC in MLN and SPL before sorting. Horizontal bars correspond to the medians. P values are the result of Mann Whitney tests (*Italic*, inter-group comparison) and Wilcoxon tests (intra-group comparison). P values >0.10 are not shown. Linear regression analysis depicting the relationship between the

percentage of combined MLN and SPL CD83⁺ mDC in SIV⁺ macaques and (e) viral load or (f) duration of infection.

Figure 2. Culture of nonTreg with mDC from infected animals induces CD25 and FOXP3 expression

CD4⁺CD25⁺FOXP3⁺ nonTreg purified from the spleen of uninfected or SIV-infected macaques were cultured alone or with the indicated autologous mDC at a 10:1 ratio. After 4 days, the percentage of CD4⁺CD25⁺FOXP3⁺ Treg was assessed by flow cytometry and is shown in one representative SIV-infected animal (a). Gates were set in reference to nonTreg cultivated alone. Percentages of CD25⁺FOXP3⁺ Treg after 4 days of culture with or without (b) MLN mDC or (c) SPL mDC. Horizontal bars correspond to the medians. (d) Percentage of induced CD4⁺CD25⁺FOXP3⁺ Treg by mature CD83⁺ or immature CD83⁻ mDC in MLN and SPL, respectively. (e) Percentage of induced Treg by matched mature MLN and SPL

DC from matched infected animals. P values are the result of Mann Whitney tests (*Italic*, inter-group comparison) and Wilcoxon tests (intra-group comparison). P values >0.10 are not shown.

Figure 3. Treg induced by mDC from SIV-infected RM proliferate more than Treg induced by mDC from uninfected animals

CD4⁺CD25⁻ T-cells purified from the spleen of uninfected or SIV-infected macaques were cultured and analyzed as described in Figure 2. (a) Proliferation of induced FOXP3⁺ T-cells by mature mDC was monitored by CFSE-dilution and is shown in two representative animals. (b) Graph represents combined MLN and SPL data on the the percentage of FOXP3⁺CFSE^{low} cells after 4 days of culture with mature mDC. (c) Graph represents the percentage of T-cell death, determined by LIVE/DEAD staining after 4 days of culture with mature MLN mDC and SPL mDC. Horizontal bars correspond to the medians. P values are the result of Mann Whitney tests (*Italic*, inter-group comparison) and Wilcoxon tests (intra-group comparison). P values >0.10 are not shown.

Figure 4. Expression of CD103 is not correlated with induction of CD25⁺FOXP3⁺Treg
 (a) Percentage of CD103⁺ cells in mature CD83⁺ and immature CD83⁻ mDC is shown in MLN and SPL before sorting. Horizontal bars correspond to the medians. Correlation between the percentage of CD103⁺ mature mDC from MLN (b) or SPL (c) of infected macaques and the percentage of induced CD25⁺FOXP3⁺ Treg after 4 days of co-culture was analyzed using linear regression analysis.

Table 1

Description of animals included in the study.

	Age ^a	Gender ^b	VL ^c	CD4 ^d	Route of infection ^e	Duration of infection ^f
SIV-infected (n=11)	6 (4-14)	60% M	5.67 ± 0.3	473 ± 76	60% p; 40% v	17 (7-24)
Control (n=4)	7 (7-8)	100% M				

^aMedian age, expressed in years (range)

^bM, Male

^cmean viral load (±SE) expressed in log vRNA copies/ml of plasma

^dmean (±SE) expressed in number/μl

^ep, penile; v, vaginal

^fMedian length of infection in weeks (range).