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Abstract

Dendritic cells (DCs) are known to be essential for the induction and regulation of immune responses. Non-human primates are essential in biomedical research and contribute to our understanding of the involvement of dendritic cells in human infectious diseases. However, no direct single-platform method for quantifying dendritic cell precursors has yet been optimized in macaques to give accurate absolute blood counts of these rare-event cell populations in the blood. We adapted a rapid whole-blood assay for the absolute quantification of dendritic cells in cynomolgous macaques by four-color flow cytometry, using a single platform assay compatible with human blood. Cynomolgus macaque plasmacytoid DC (PDC) and $CD1c^+$ myeloid DCs (CD1c+ MDC) were quantified in the blood of 34 healthy macaques and the results obtained were compared with those for blood samples from 11 healthy humans. In addition, circulating PDC absolute numbers were quantified in cynomolgus macaques chronically infected with SIVmac. During infection, PDC counts decreased whereas circulating CD1c+ MDC counts increased. Information regarding absolute PDC and MDC cell counts in non-human primates may improve our understanding of the role of these cells in SIV/HIV infection and in other infectious diseases.

Introduction

Dendritic cells (DC) play a key role in the innate and adaptive immune responses [1]. They are specialized in antigen uptake , presentation and priming of naive T cells [2].

DCs are affected by infection with viruses, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and dengue [3-5]. Two populations of circulating DCs — myeloid DCs (MDC) and plasmacytoid DCs (PDC) — have been characterized in human blood, based on their phenotype and ability to acquire different mature antigen-presenting cell (APC) functions upon further stimulation. MDC preferentially express Toll-like Receptors (TLRs) 1, 2, 4, 5 and 6, which recognize various bacterial components and produce IL-12 [6]. PDC selectively express TLR7 and TLR9, which recognize bacterial CpG motifs and viral DNA and ssRNA. PDC are major producers of type I IFN upon viral activation [7].

During chronic HIV infection, PDC and MDC numbers decrease in the blood. This decline is inversely correlated with virus load and/or associated with a decrease in $CD4^+$ T-cell counts [8-16].

Non-human primates (NHPs) are essential in biomedical research [17], as they provide experimental models for pathogenesis, preclinical vaccine and therapeutic studies, particularly for HIV/AIDS. The DCs of NHPs are very similar to human DCs [18-22]. Conflicting results were initially published concerning the effect of simian immunodeficiency virus (SIV) infection on circulating DCs in rhesus macaques [19, 23]. However, recent studies have shown an early and persistent decrease in PDC counts in rhesus macaques chronically infected with SIV [21] and decreases in both PDC and MDC counts in macaques with AIDS [24].

However, no direct single-platform method for quantifying circulating DCs has been optimized for NHP models, making it difficult to obtain accurate absolute counts of these rare-event cell populations in the blood, potentially accounting for the observed discrepancies. Macaque DC have been characterized *ex vivo* in some NHPs, including rhesus macaques

(*Macaca mulatta*) [25], and dual-platform quantification has been reported for a limited number of samples [18, 21, 23, 24]. In some studies, blood DC counts were obtained by multiplying the percentage of phenotypically defined DC in the leukocyte gate on multicolor flow cytometry by the leukocyte count obtained by automated blood cell counting [21, 23, 24]. DC numbers have also been determined as a percentage in the leukocyte or PBMC (peripheral blood mononuclear cell) gate [5, 21, 26], or as a percentage in a lineage$^-$ HLA-DR$^+$ gate [18, 19], providing an indication of the proportion of these cells with respect to other blood cells, but not an absolute count.

In human blood, PDC and MDC are usually defined as CD11c$^-$CD123$^+$ and CD11c$^+$CD123$^-$ cells, respectively, within a lineage negative gate. The antibodies used to exclude lineage positive non-DC events are mostly directed against CD3 (T cells), CD19 or CD20 (B cells), CD16 or CD56 (NK cells), or CD14 (monocytes) [7]. Like their human counterparts, MDC and PDC from rhesus macaques are identified on flow cytometry as CD11c^{high} CD123^{low} and CD11c$^-$ CD123^{high} cells, respectively, in HLA-DR$^+$ lineage$^-$ gate [18, 25], but NHP lineages display several specific features. Main lineages are defined in macaques, as in humans, using antibodies against CD3 (T cells), CD14 (monocytes), and CD20 (B cells). CD56- and CD16-specific antibodies are commonly used to gate out human NK cells in DC counting strategies [27]. In macaques, NK cells cannot be excluded for DC counting by the use of CD56-specific antibodies, as these antibodies mostly stain monocytes and do not stain NK cells in rhesus macaques [28, 29]. It is also not possible to use CD16 staining to exclude NK cells, because this molecule is expressed not only on NK cells, but also on some MDC in macaques [24], as described for human DC subsets in the blood [30, 31].

The CD11c$^+$ MDC population has been shown to be heterogeneous in human blood, whereas CD1c$^+$ MDC express uniformly high levels of cell-surface HLA-DR [31], as reported for a subpopulation of CD11c$^+$ MDC in Indian rhesus macaques [24]. CD1c can therefore be

used as an alternative marker for quantifying a subpopulation of MDC, as previously reported in both humans [32] and rhesus macaques [18]. A fraction of CD1c+ MDC were recently shown to be selectively infected by HIV-1 [3]. Studies focusing on this DC population are therefore of particular interest in relation to HIV/SIV infection and disease.

Cynomolgus macaques (*Macaca fascicularis*) are one of the main NHP species used in studies aiming to increase our understanding of human infectious diseases in which dendritic cells play a key role [33-35]. They are also used as a model of SIV infection and AIDS [36-38]. However, the circulating DCs of cynomolgus macaques have never been characterized. In this study, we adapted a method based on TruCount® technology for the absolute quantification of human DCs in blood [39] to cynomolgus macaque samples. We compared DC counts in cynomolgus macaque and human blood, using the same protocol, and investigated the impact of SIVmac infection on blood DC counts in cynomolgus macaques. Using this method, we show that PDC numbers decrease, whereas $CD1c^+$ MDC numbers increase in the blood of cynomolgus macaques during SIVmac251 infection.

Materials and methods

Biological samples

Young adult male cynomolgus macaques (*Macaca fascicularis*), weighing 3 to 5 kg, were imported from Mauritius and housed according to European guidelines for animal care ("Journal Officiel des Communautés Européennes", L358, December 18, 1986). Blood samples were obtained from naive animals or animals infected with SIVmac251, after sedation by intramuscular injection of 10 mg/kg ketamine (Imalgene, Rhone-Mérieux, France). Biopsies were carried out on superficial peripheral lymph nodes in animals under ketamine sedation. SIV-infected animals received 50 or 5,000 AID_{50} of SIVmac251 primary isolate intravenously. The cell-free stock of pathogenic SIV_{mac251} was kindly provided by Dr A.M. Aubertin (Université Louis Pasteur, Strasbourg, France). Nine months after infection, these animals displayed a wide range of progression stages, defined on the basis of biological parameters. Plasma viral load varied from undetectable to 1.59×10^5 viral SIV RNA copies/mL, and CD4 T-cell counts ranged between 1,500 and 10^7 cells per μL.

Blood was also obtained from healthy human adult donors (men under the age of 40 years) who gave informed consent. Blood samples were collected into EDTA tubes (Dominique Dutscher, Brumath, France) or CPT tubes (BD Biosciences, Grenoble, France) for the isolation of PBMCs, and immunostaining was carried out within four hours of the sample being taken.

Absolute quantification of PDC and CD1c$^+$ MDC

Undiluted blood (100 μL) was stained, in TruCount® tubes (BD Biosciences, Grenoble, France), with macaque-specific or anti-human differentiation cluster antibodies selected for efficient cross-reaction with their cynomolgus macaque counterparts. These antibodies are

described in Table I and include a cocktail of lineage-specific antibodies (CD3-FITC, CD8-FITC, CD14-FITC, CD20-FITC) and antibodies used for DC characterization (HLA DR-PerCP, CD123-PE and anti-CD1c-APC (BDCA-1)). The FN18 clone specific for macaque CD3 was replaced by the UCHT1 clone for the counting of human DC, as FN18 does not cross-react with human CD3.

After 15 minutes of incubation at room temperature, 1 mL FACS lysing solution (BD Biosciences) was added to each tube to lyse red blood cells and fix samples. Samples were stored at +4°C in the dark and flow cytometry was carried out within 24 hours on an LSR I flow cytometer (BD Biosciences, Grenoble, France). For each tube, a sample volume containing 7,000 TruCount® beads was acquired, corresponding, on average, to 100,000 PBMCs. FlowJo software (Tree Star, Ashland, USA) was used for the analysis. PDC and MDC events were counted by combining gating on the following populations: PBMCs (gate G1, Fig. 1b); HLA-DR$^+$ and lineage cocktail$^-$ (gate G2, Fig. 1b); CD1c$^+$ and CD123^{low} for MDC (gate G3, Fig. 1b); CD123^{bright} and CD1c$^-$ for PDC (gate G4, Fig. 1b). The G2 gate (Lin$^-$HLA-DR$^+$) was used to gate out any non-DC cells. Indeed, CD123$^+$HLA-DR$^-$ polynuclear cells were detected in cynomolgus macaque blood (data not shown), as reported for basophil cells in human blood [40].

TruCount® beads were counted, using an appropriate gate in the FSC/SSC scattergram (gate G5, Fig. 1b). The blood volume equivalent to the acquisition file was calculated from the number of beads gated on the scattergram, the known blood sample volume (100 μl), and the known total number of beads in the sample tube, as provided on the TruCount® tube data sheet. Absolute MDC and PDC counts were calculated as follows:

$$\textbf{Blood concentration of PDC} = \frac{(\text{events in PDC gate}) \times (\text{total number of beads in True Count tube})}{(\text{events in beads gate}) \times (\text{sample volume})}$$

$$\textbf{Blood concentration of MDC} = \frac{(\text{events in MDC gate}) \times (\text{total number of beads in True Count tube})}{(\text{events in beads gate}) \times (\text{sample volume})}$$

Quantification of PDC and CD1c$^+$ MDC in lymph nodes

Lymph nodes were dilacerated and passed through a cell strainer (BD Biosciences, Grenoble, France). Freshly isolated cells were incubated with FcR blocking reagent (Miltenyi Biotech, Paris, France) for 10 min at room temperature and surface stained by incubation for 20 min at 4°C with the same cocktail of antibodies used for blood. Cells were then washed and fixed using Cell Fix (BD Biosciences, Grenoble, France). One million cells were used for quantification. The percentage of PDC and MDC in lymph node mononuclear cells defined on the basis of the FSC/SSC scattergram was determined using a gating strategy similar to that used for blood.

Phenotypic characterization of circulating PDC and CD1c$^+$ MDC

Macaque blood samples was drawn into CPT tubes for the isolation of PBMCs according to the manufacturer's recommendations. For surface staining, cells were incubated with FcR blocking reagent (Miltenyi Biotech, Paris, France) for 10 min at room temperature and stained with fluorochrome-conjugated antibodies for 20 min at +4°C. They were then washed in PBS, and fixed in Cell Fix. The monoclonal antibodies used for the phenotypic characterization of cynomolgus macaque DCs are described in Table 1. PDC phenotype was characterized by gating on CD123^{bright}, HLA-DR$^+$ cells without gating on lineage$^-$ cells, as previously described for rhesus PDC in PBMCs [26]. Control experiments indicated that this gating strategy was reliable for cynomolgus PDC in blood and lymph nodes, as the contamination of this gate with lineage$^+$ cells was negligible (data not shown). For CD1c$^+$ MDC, phenotypic characterization was carried out by gating on lineage$^-$, HLA-DR$^+$ and CD1c (BDCA-1)$^+$ cells. For PDC, CD123 PE, HLA-DR-PerCP and other APC-conjugated antibodies were used (CD4, CD80, CD86, CD40), whereas, for CD1c$^+$ MDC, Lin-FITC,

CD1c-APC, HLA-DR-PerCP and the other clusters (CD4, CD80, CD86, CD40) conjugated with PE were considered. This choice was constrained by color availability for the CD123- and CD1c-specific antibodies, preventing direct comparison of the levels of CD80, CD86 and CD40 expression between the two DC subsets.

Functional characterization of circulating PDC and CD1c$^+$ MDC

For IFN-alpha intracellular staining, PBMC were stimulated with HSV for 6 hours in presence of 10μg/ml Brefeldin A (Sigma Aldrich) for the last 4 hours. Two mM EDTA were added to avoid clumps, and cells were washed in PBS, and then incubated with anti CD123, anti HLA-DR for 15 minutes at room temperature. Cells were washed in PBS and fixed and permeabilized using Facs lysing and Facs permeabilizing solutions (BD Biosciences) as recommended by manufacturer's instructions. Anti human IFN-alpha2a (kindly provided by Dr J. Banchereau, Baylor Institute for Immunology Research, Dallas, Texas, USA) was coupled with Quantum dot 800 using Qdot antibody conjugation kit as recommended by the manufacturer (Molecular Probe Europe, Eugene, The Netherlands). Cells were incubated with QDot800 labeled anti IFN-alpha2a for 15 min at room temperature, washed and resuspended in Cellfix (BD Biosciences).

For IL-12p70 intracellular staining, PBMC were stimulated with LPS for 7 hours in presence of 10μg/ml Brefeldin A (Sigma Aldrich) for the last 3 hours. Two mM EDTA were added to avoid clumps, and cells were washed in PBS, and then incubated with anti CD3, CD8, CD14, CD20 (Lineage Ab), anti HLA-DR and anti CD1c for 15 minutes at room temperature. Cells were washed in PBS and fixed and permeabilized using Facs lysing and Facs permeabilizing solutions (BD Biosciences) as recommended by manufacturer's instructions. Cells were incubated with 1:5 dilution of biotinylated anti monkey IL-12p70 (Bender Medsystems, Vienna, Austria) for 15 min at room temperature, washed in PBS,

resuspended with Streptavidin-PE (Coulter-Immunotech, Marseilles, France) and incubated for 15 min at room temperature. Finally, the cells were washed and resuspended in Cellfix (BD Biosciences).

Statistical analysis

The nonparametric Mann and Whitney test was used to determine the statistical significance of differences in PDC or MDC numbers between species (human versus cynomolgus macaque)**,** and the nonparametric Wilcoxon rank test was used to compare DC counts before and after SIV infection, using Statview® software (SAS Institute. Inc., Cary, NE, USA). In two-sided tests, *p* values of 0.05 or lower were considered significant.

Results

Gating strategies

We used CD8 staining to gate out NK cells in our study. Nkp46 (CD345) has been described as a receptor expressed by all human NK cells involved in natural cytotoxicity [41]. An Nkp46-specific antibody was recently reported to cross-react with cynomolgus macaque NK cells [42]. We confirmed that all Nkp46^{+} lymphocytes were also stained with anti-CD8 antibodies. All Nkp46+ cells were found to be CD8^{+} in this species (Fig. 1a), making it possible to use an anti-CD8 antibody in the lineage cocktail to gate out cynomolgus NK cells. In addition, the staining of CD3, CD20 and CD14 made it possible to gate out T cells, B cells and monocytes, respectively.

In humans and rhesus macaques, MDC and PDC are defined as CD11c^{high} CD123^{low} and CD11c^{-} CD123^{high}, respectively, within the Lin^{-} HLA-DR^{+} gate on flow cytometry. However, antibodies against CD11c weakly stained lineage^{-} HLA-DR^{+} CD123^{-} cells in cynomolgus blood, as previously reported in some Chinese rhesus macaques [25]. This weak signal may reflect low levels of CD11c expression on circulating MDC in cynomolgus macaques rather than poor cross-reactivity of the antibody, as a stronger signal was obtained with cynomolgus macaque monocyte-derived DCs (data not shown). The CD11c-specific antibody therefore could not reasonably be used for MDC quantification in cynomolgus macaques, and was replaced by a CD1c (BDCA-1)-specific antibody, as in several other reported human and rhesus macaque studies [18, 32].

Quantifying PDC and CD1c^{+} MDC in whole blood

We quantified PDC and MDC, as described in the material and methods. A CD123/CD1c dot plot representation (Figure 1b) was used to count CD1c^{+}CD123^{-} (MDC)

and CD123^{+}CD1c^{-} (PDC) events in a lineage^{-} HLA-DR^{+} gate (Gate G2) after the pre-gating of mononuclear cells in an FSC/SSC scattergram (Gate 1). As labeling was carried out in TruCount® tubes, which contain a defined number of beads, these beads were also counted, using a devoted gate in the FSC/SSC scattergram (Gate 5) to determine DC concentrations, as described in the materials and methods.

We used this method to quantify circulating DCs in the blood of 18 healthy macaques. Five or six consecutive blood samples were obtained from the 18 animals over a period of two months. PDC (Figure 2a) and MDC counts (Figure 2b) showed some inter-individual variability and high intra-individual reproducibility. On this basis, PDC and CD1c^{+} MDC counts were determined for 34 blood samples from healthy cynomolgus macaques and 11 samples from healthy humans. PDC counts in cynomolgus macaque blood samples (mean 8.2 ± 5.1 PDC/μL) did not differ significantly from those in human samples (mean 10.6 ± 5.2 PDC/μL; Figure 2c). The absolute CD1c^{+} MDC count in the blood of cynomolgus macaques was 33.6 ± 12.6 CD1c^{+} MDC/μL (Figure 2d). In contrast, the mean number of CD1c^{+} MDC in human peripheral blood was much lower than that in macaques (8.4 ± 2.9 CD1c^{+} MDC/μL, $p<0.0001$), consistent with recent data reporting a mean of 11.0 CD1c^{+} MDC/μL (range 6.0-20.9), measured with a dual-platform method, in PBMCs from 99 human donors [43].

Phenotypic and functional characterization of PDC and CD1c^{+} MDC in cynomolgus macaques

We further characterized the phenotype of cynomolgus CD1c^{+} MDC and PDC. Cynomolgus PDC were HLA-DR^{dim}, CD4^{+}, CD80^{-}, CD86^{-} and CD40^{+} (Figure 3a), giving them an immature profile, as previously reported for rhesus macaque PDC in blood [18, 21, 24].

CD1c^{+} cynomolgus MDC in the blood were CD4^{+}, CD80^{low}, CD86^{+} and CD40^{+} (Figure3b) as reported for rhesus CD11c^{+} MDC-s [18, 24, 44]. Remarkably CD1c^{+} MDC in

cynomolgus macaques were all HLA-DR^{bright} (Fig.1a), as generally observed for total CD11c^{bright} myeloid DCs in rhesus macaques [24].

Circulating DCs in cynomolgus macaques therefore display several phenotypic similarities to human circulating DCs [3, 45].

Cynomolgus macaque PBMC were stimulated either with HSV to adress the function of PDC by assessing type I interferon response, or with LPS to address the function of MDC, by assessing IL-12p70 expression. Increased expression of IFN-alpha2a after HSV stimulation was evidenced in CD123+ HLA-DR+ gated PDC (Figure 4a) which confirms pDC as interferon producing cells in cynomolgus macaques. Stimulation of PBMC by LPS gave rise to increased IL-12p70 in Lin- HLA-DR+ CD1c+ gated MDC confirming their function.

PDC and CD1c$^+$ MDC in cynomolgus macaque peripheral lymph nodes

The percentages of PDC and CD1c$^+$ MDC among peripheral lymph node mononuclear cells were also determined for 18 healthy cynomolgus macaques. The gating strategy used is shown in figure 5a. PDC accounted for 0.039% ± 0.024% (n=18 macaques) of total mononuclear cells (Figure 4b), consistent with some reports for the non-human primate subspecies Indian rhesus and pigtailed macaques [46], but not with all such reports [21].

CD1c$^+$ MDC accounted for 0.027% ± 0.014% (n=18) of peripheral lymph node mononuclear cells, a proportion lower than that reported for Indian rhesus macaques [46] (Figure 5b). In contrast to what was observed in the blood, PDC were more frequent than CD1c$^+$ MDC in lymph nodes, as previously reported in rhesus macaques [46].

Changes in absolute PDC and CD1c$^+$MDC counts in blood during chronic SIV infection

Using this single-platform assay, we quantified PDC and CD1c$^+$ MDC in SIV-infected cynomolgus macaques and compared these counts with the baseline values obtained for the same animals. Nine months after infection, PDC counts were significantly lower (Wilcoxon, $p = 0.0038$) and CD1c$^+$ MDC counts were significantly higher (Wilcoxon, $p = 0.0012$) than before infection (Figure 6).

Discussion

In this study, we adapted a single-platform assay for the absolute quantification of PDC and CD1c$^+$ MDC in macaques; this assay was first described for use in the clinical follow-up of human patients [39]. This assay has the advantage of allowing direct quantification in whole blood, through a process including red blood cell lysis, but with no washing, avoiding potential bias due to cell loss and DC-T cell conjugate formation [39]. It is based on flow cytometry quantification and uses TruCount® tubes, which contain defined numbers of beads, making it possible to determine directly the volume of blood corresponding to acquisition files. We aimed to adapt this DC quantification method to macaque cells, to facilitate more reliable comparisons between and within species — an essential requirement for elucidation of the role of DCs in various human diseases by studies in non-human primate models. Using this single-platform method for the quantification of DC subpopulations, we showed that chronic SIVmac251 infection increased CD1c$^+$ MDC counts and lowered PDC counts in the blood of cynomolgus macaques.

No data have been published for cynomolgus macaques, but very different mean PDC counts in blood have been reported for healthy rhesus macaques by different groups [18, 21, 23, 24]. All these data were obtained by dual-platform quantification (multiplying percentages of DCs among white blood cells (WBC) by WBC numbers obtained by automated counting), whole-blood or PBMC staining with washing steps, and were performed on limited numbers of animals (3 to 6), potentially accounting for these differences. These discrepancies led us to adapt a whole-blood single-platform method for DC quantification in macaques. Our data for a large population of cynomolgus macaques show higher PDC counts (mean 8.2±5.1 PDC per µL) than reported for rhesus macaques. These differences may reflect true differences between subspecies (cynomolgus versus rhesus macaques), differences in the size of the populations studied or differences in quantification methods. We obtained mean counts of

10.6 ± 5.2 PDC/μL for human samples. Our results are therefore consistent with those for the Trucount® quantification of human DCs published by Vuckovic *et al*. [39], confirming that the combination of antibodies used in our study for adaptation to macaques is also appropriate for human studies. Our results are also consistent with the observation by Vuckovic *et al*. that dual-platform methods give lower DC counts than single-platform methods [39], as lower counts were obtained in previous studies using the dual-platform method on human PBMCs or whole blood [16, 47]. In healthy cynomolgus macaques, PDC counts in blood did not differ significantly from that in healthy humans, whereas lower counts have been reported in most rhesus macaque studies [18, 21, 23]. In contrast, these counts were similar to those reported for pigtailed macaques [21] and in one recent study of rhesus macaques [24]. None of these studies compared human and macaque samples with the same method — a useful way of standardizing interspecies comparisons. The discrepancies reported in rhesus macaque studies demonstrate the need for the standardization of blood DC quantification in NHPs, for which methods for absolute counting in whole blood would be helpful.

The concentration of the $CD1c^+$ subpopulation of MDC in blood was higher in cynomolgus macaques than in human samples. The concentration of $CD1c^+$ MDC in human peripheral blood (8.4 ± 2.9 cells/μL) obtained here was consistent with recent data reporting a mean of 11.0 $CD1^+$MDC/μL (range 6.0-20.9), measured with dual-platform method, using PBMCs from 99 healthy human donors [43].

The frequency of PDC in cynomolgus macaque peripheral lymph node cells (mean 0.039% ± 0.02%, range 0.013% - 0.089%) was lower than previously reported for Indian rhesus macaques (0.48%) [46] and pigtailed macaques [21]. The proportion of BDCA-1^+ MDC in cynomolgus macaques was 0.027% ± 0.014% (n=18), but we are aware of no published data for this MDC subpopulation in the lymph nodes of other NHP models for comparison.

Our data provide evidence for a longitudinal decline in PDC counts in cynomolgus macaques after SIV infection. A similar longitudinal decline in PDC numbers was recently reported in rhesus macaques and in pigtailed macaque [21]. Our data, obtained with fresh whole blood, a larger group of macaques and single-platform counting, support the notion of a decline in PDC numbers in macaque blood after SIVmac infection, as previously reported for HIV-infected patients [14 15]. Higher frequencies of PDC were initially described in SHIV-vaccinated rhesus macaques chronically infected with SIVmac but with no illness than in naive or SIVmacΔnef-vaccinated animals [19]. This paper provided no information about possible changes in absolute numbers (data were presented as percentages of DC subpopulations within the lineage$^-$ HLA-DR$^+$ gate), but larger numbers of PDC are associated with a better prognosis in HIV-infected human patients [16, 48, 49]. In our cynomolgus macaque study, PDC counts were not found to be related to plasma viral loads, in contrast to what has been reported for rhesus and pigtailed macaques [21], but consistent with some studies of HIV-1 infection in humans [9]. Indeed, this correlation has not been found in all human studies [15]. A decrease in PDC numbers was also recently reported in rhesus macaques with end-stage disease progressing to AIDS [24]. Infection and an increase in the frequency of spontaneous death have both been proposed to account for this decrease in PDC numbers in macaque with end-stage disease [21, 24]. However, this decrease may also result from relocalization to lymphoid tissues in earlier stages consistent whith observations made in HIV acute infection [50].

In contrast, we describe here an increase in CD1c$^+$ MDC counts during chronic SIV infection. Conflicting results have been published concerning the effects of SIV infection on circulating CD11c$^+$ MDC in rhesus macaques. Higher CD11c$^+$ MDC counts in SIVmac-infected rhesus macaques than in non infected animals were first reported in a group of four macaques 26 weeks after infection [23], whereas a major loss of these cells was described in

SIV-infected macaques with AIDS [24]. The discordance between these studies may be due to differences in the disease stage of the animals. Indeed, high $CD11c^+$ MDC counts have been reported in HIV-1 patients with more than 500 $CD4^+$ T cell/μL and low plasma viral load [51], whereas lower MDC counts have been reported in patients with high viral loads [9, 10], or with low $CD4^+$ T-cell counts [51]. These cross-sectional studies suggest a two-step change in MDC levels in HIV/SIV infection: an initial increase, followed by a decrease when individuals progress to AIDs. Our data for chronically infected cynomolgus macaques — a model in which viral loads and progression rates are generally lower than in Indian rhesus macaques [52], and in which we observed an increase in MDC counts — are consistent with this model.

In conclusion, this study describes the first rapid single-platform method for quantifying dendritic cells in macaque blood using TruCount beads for flow cytometry. This new assay could be used for interspecies comparisons. Using this method, we describe for the first time, a longitudinal increase in circulating MDC counts and a decrease in PDC counts during chronic SIVmac infection in cynomolgus macaques. Further investigation in lymphoid tissues is required to determine the reasons for these changes. As cynomolgus macaque PDC express CD4 and the main coreceptor for SIV, CCR5 (data not shown), the decrease in PDC counts may result from infection with SIV, cell death due to necrosis or apoptosis, as previously suggested for SIV-infected rhesus macaques with AIDS [21, 24] or a relocalization to lymphoid tissues.

The ability to quantify both PDC and MDC in cynomolgus macaques, a relevant preclinical model for various human diseases involving DCs, should make it easier to elucidate the role of these cells in these diseases.

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Legends to figures

Figure 1: Gating strategies for DC counting in blood. (a) CD8/Nkp46 dot plot gated on PBMCs shows strong staining of all Nkp46^{+} cynomolgus NK cells by CD8-specific antibody (b). Gating strategy for DC counting in the blood. Dot plots represent : (left) FSC/SSC scattergram of representative whole-blood sample and the gate used for mononuclear cell gating (gate G1); (middle) HLA-DR/lineage dot plot gated on G1, used to gate lineage^{-} HLA-DR^{+} cells (whole DCs); (right) CD1c/CD123 dot plot gated on G1 and G2 used to count CD123^{+} CD1c^{-} cells (PDC, gate G3) and CD1c^{+} CD123^{-} cells (MDC, gate G4). In the middle dot plot backgating show CD1c+ mmDC in red and CD123+ PDC in blue in the HLA-DR positive lineage negative gate. TruCount beads were gated on forward and side scatter properties, gate G5 in the left dot plot in (b), for calculation of the volume of blood corresponding to the acquisition file.

Figure 2: Absolute quantification of PDC and CD1c^{+} MDC in cynomolgus macaque and human whole blood samples. (a) Mean ± standard deviation of 6 consecutive counts of PDC in 18 healthy cynomolgus macaques over a two-month period. (b) Mean ± standard deviation of 6 consecutive counts of CD1c^{+} MDC in 18 healthy cynomolgus macaques over a two-month period. (c) PDC counts in the blood of 34 healthy cynomolgus macaques and 11 healthy human individuals. Each point represents one individual and the line represents the median for each group. (d) CD1c^{+} MDC counts in the blood of 34 healthy cynomolgus macaques and 11 healthy human individuals. Each point represents one individual and the line represents the median for each group. The P value given is for a Mann and Whitney test comparing human and macaque counts.

Figure 3: Phenotypic characterization of cynomolgus macaque PDC and CD1c$^+$ MDC. The thin line corresponds to the isotype control and the bold line shows staining with the specific monoclonal antibody.

Figure 4 : Functional characterization of cynomolgus macaque PDC and CD1c$^+$ MDC **(a)** Intracellular staining of IFN-alpha2a in PDC after 6 hours stimulation of PBMC with HSV; CD123/IFNalpha2 dot plot gated on HLA-DR+ cells are shown for two macaques. Unstimulated cells are shown as negative controls. **(b)** Intracellular staining of IL-12p70 in CD1c+ MDC after 7 hours stimulation of PBMC with LPS; CD1c+/IL-12p70 dot plot gated on lineage negative HLA-DR+ cells are shown for two macaques. Unstimulated cells are shown as negative controls.

Figure 5: DCs in cynomolgus macaque peripheral lymph nodes. **(a)** Four-color flow cytometry gating strategies for identifying DCs in macaque lymph nodes, as described for blood DCs in Figure 1b; Lineage negative HLA-DR positive CD1c positive cells (CD1c+ MDC) in red; Lineage negative HLA-DR positive CD123 positive (PDC) in blue **(b)** Frequencies of PDC and CD1c$^+$ MDC in peripheral lymph nodes from 18 non-infected cynomolgus macaques. Each point represents one individual macaque and the horizontal line represents the median for each group. n.s. = not significant in Wilcoxon rank tests.

Figure 6: Longitudinal changes in absolute PDC and CD1c$^+$ MDC counts in the blood during chronic SIVmac251 infection. Blood counts are given for a longitudinal study of a group of 18 cynomolgus macaques before and after nine month of chronic infection with SIVmac251. Each point represents one macaque and the horizontal line, the median for each date. Data obtained before and after infection were compared, using the Wilcoxon rank test.

Table I: Monoclonal antibodies used for phenotyping

Cluster	Clone	Manufacturer
CD1c (BDCA-1)	AD5-8E7	Miltenyi Biotec
CD3	FN18	Biosource
CD3	UCHT1	Beckman Coulter
CD4	L200	BD Biosciences
CD8	DK25	Dako
CD11c	S-HCL-3	BD Biosciences
CD11c	BU15	Serotec
CD14	M5E5	BD Biosciences
CD20	B9H9	Beckman Coulter
CD40	5C3	BD Biosciences
CD40	HB14	Caltag
CD80	L307.4	BD Biosciences
CD86	2331	BD Biosciences
CD123	7G3	BD Biosciences
CD335 (NKp46)	BAB281	Beckman Coulter
HLA-DR	L243	BD Biosciences

Figure 1

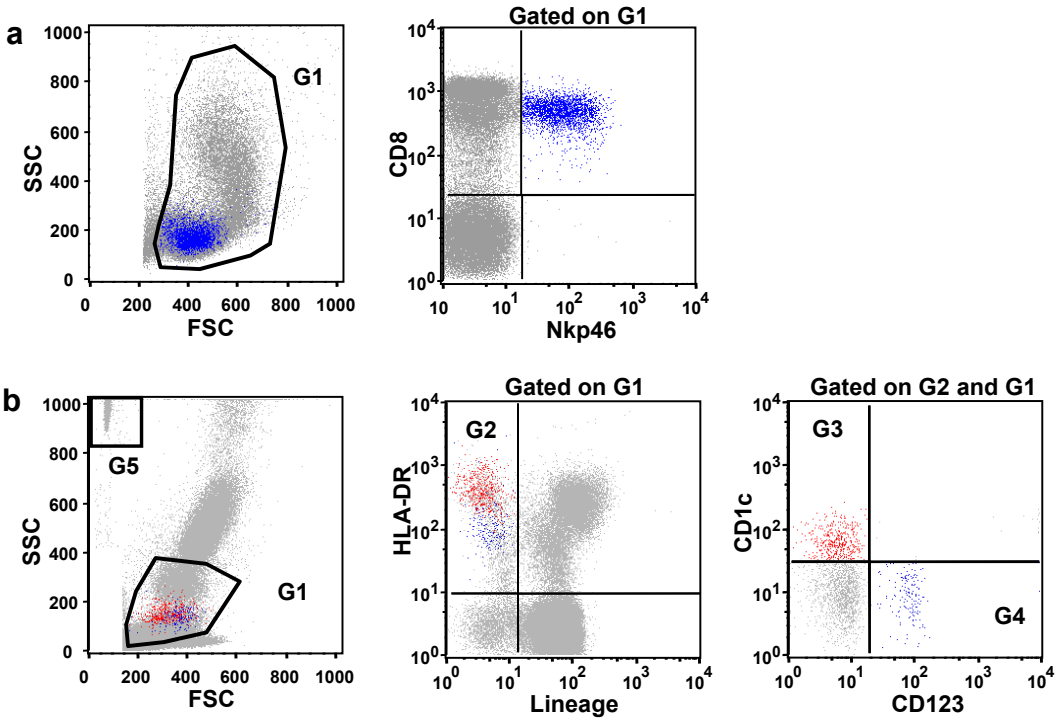

a
SSC
FSC
G1
Gated on G1
CD8
Nkp46
b
G5
G1
Gated on G1
G2
HLA-DR
Lineage
Gated on G2 and G1
G3
G4
CD1c
CD123

Figure 2

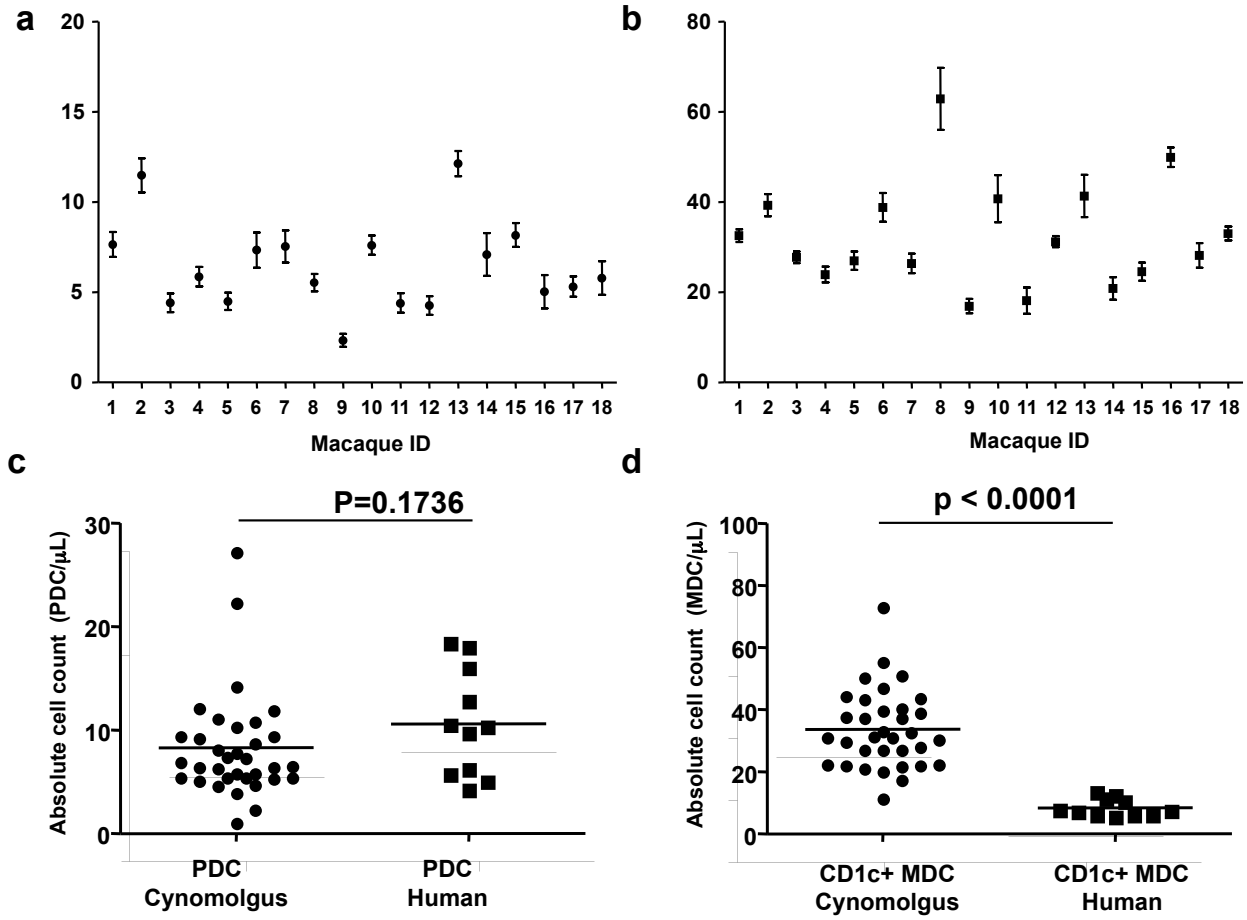

a
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Macaque ID
b
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Macaque ID
c
P=0.1736
Absolute cell count (PDC/µL)
30
20
10
0
PDC Cynomolgus
PDC Human
d
p < 0.0001
Absolute cell count (MDC/µL)
100
80
60
40
20
0
CD1c+ MDC Cynomolgus
CD1c+ MDC Human

Figure 3

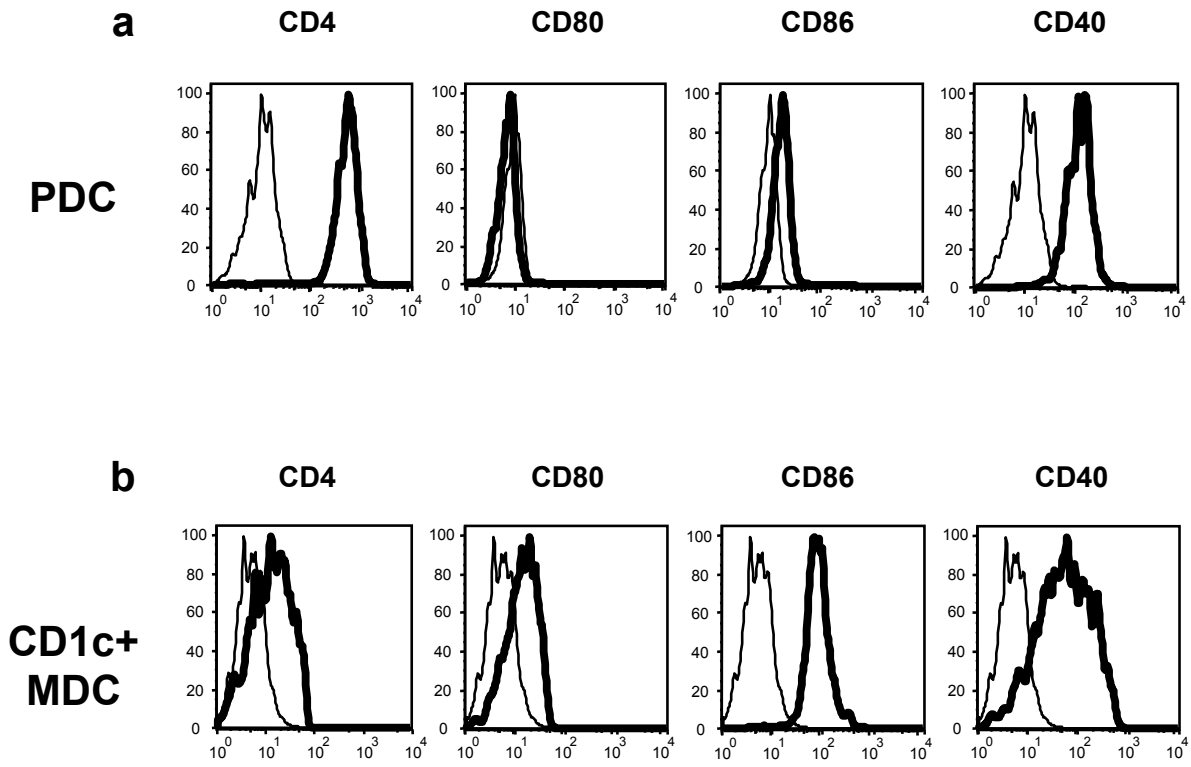


Figure 4

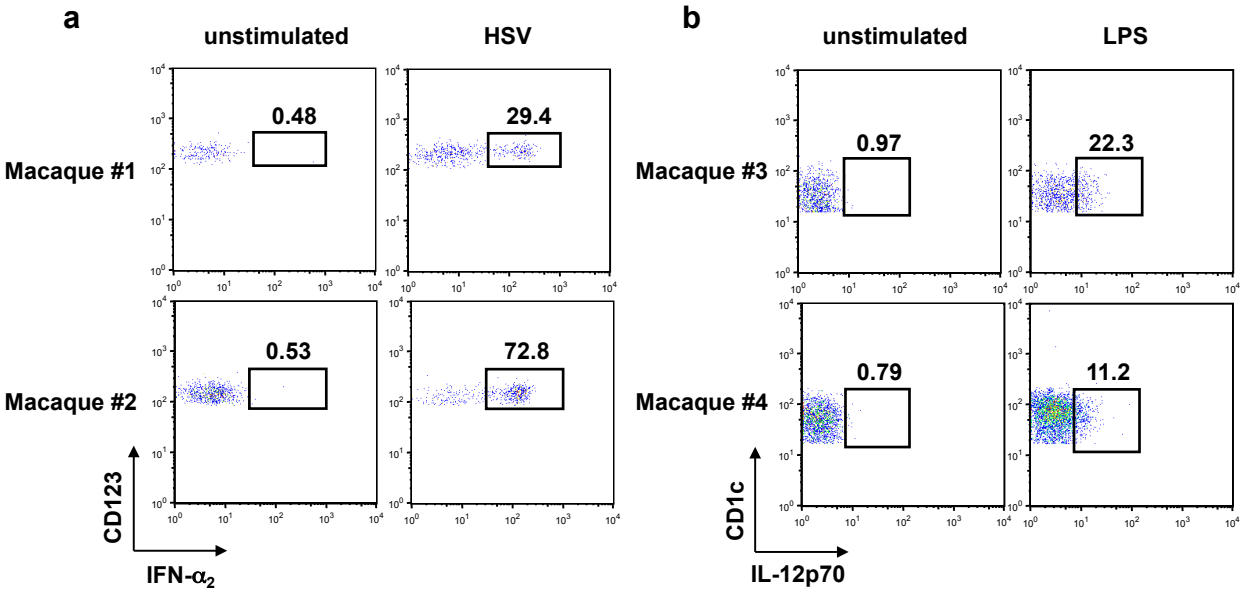
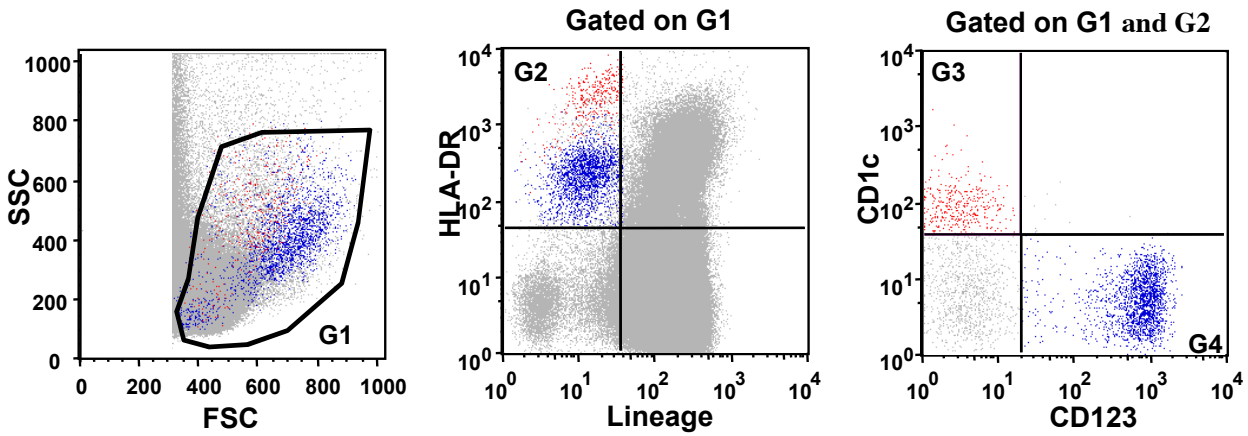

a
unstimulated
HSV
Macaque #1
0.48
29.4
Macaque #2
0.53
72.8
CD123
IFN-α2
b
unstimulated
LPS
Macaque #3
0.97
22.3
Macaque #4
0.79
11.2
CD1c
IL-12p70

Figure 5

a


SSC
FSC
G1
Gated on G1
HLA-DR
Lineage
G2
Gated on G1 and G2
CD1c
CD123
G3
G4

b

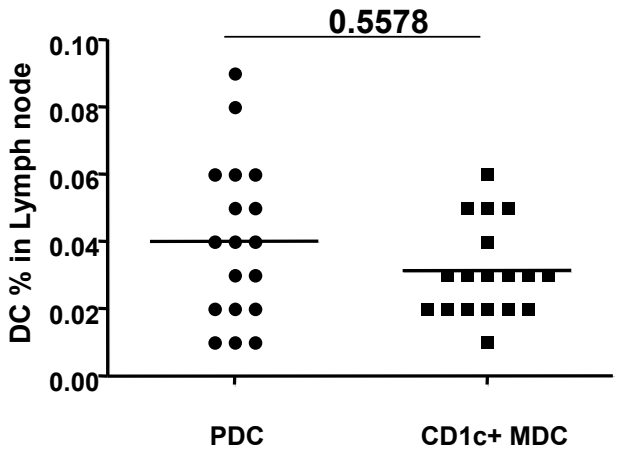
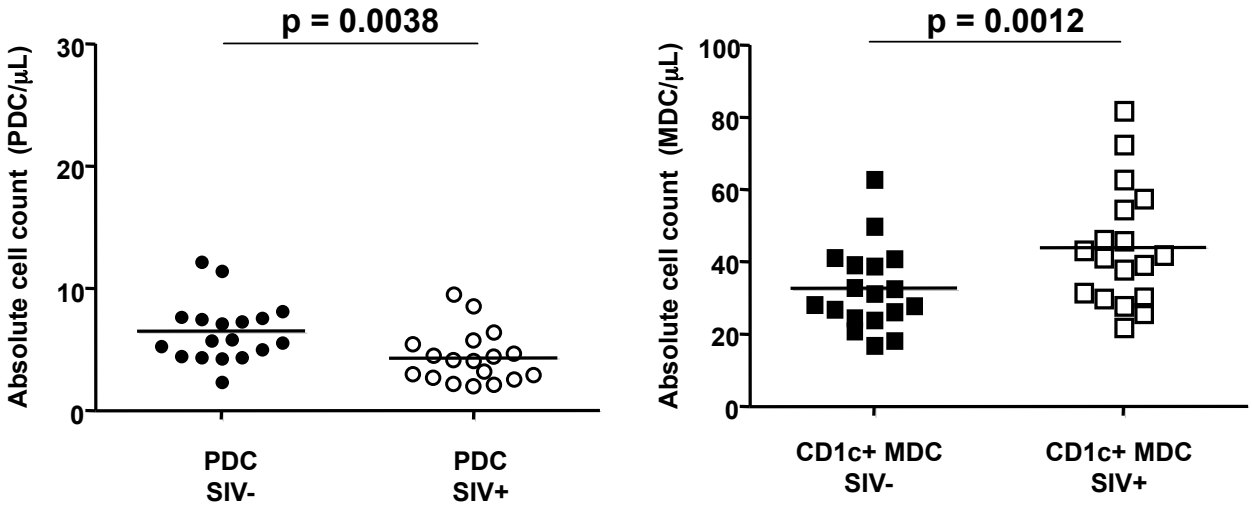

0.5578
DC % in Lymph node
PDC
CD1c+ MDC

Figure 6


p = 0.0038
Absolute cell count (PDC/μL)
30
20
10
0
PDC
SIV-
PDC
SIV+
p = 0.0012
Absolute cell count (MDC/μL)
100
80
60
40
20
0
CD1c+ MDC
SIV-
CD1c+ MDC
SIV+