TLR3–Responsive, XCR1⁺, CD141(BDCA-3)⁺/CD8α⁺-Equivalent Dendritic Cells Uncovered in Healthy and Simian Immunodeficiency Virus–Infected Rhesus Macaques

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TLR3–Responsive, XCR1+, CD141(BDCA-3)+/CD8α+ Equivalent Dendritic Cells Uncovered in Healthy and Simian Immunodeficiency Virus–Infected Rhesus Macaques

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In mice, CD8α+ myeloid dendritic cells (mDC) optimally cross-present Ags to CD8+ T cells and respond strongly to TLR3 ligands. Although equivalent DC have been identified by comparative genomic analysis and functional studies in humans as XCR1+CD141+ mDC and in sheep as CD26+ mDC, these cells remained elusive in nonhuman primates. To remedy this situation, we delineated precisely DC and monocyte populations by 12-color flow cytometry and transcriptional analyses in healthy rhesus macaques. We identified a new mDC population, with strong phenotypic and transcriptional homology to human CD141+ and murine CD8α+ mDC, including XCR1 membrane expression as a conserved specific marker. In contrast, high CD11c expression was not characteristic of mDC in macaques, but of CD16+ monocytes. Like their human and murine homologs, simian XCR1+ mDC had much stronger responses to TLR3 stimulation than other myeloid cells. The importance of this new mDC population was tested in SIV mac251 infection, the most relevant animal model for pathogenic HIV-1 infection and vaccination. This population increased sharply and transiently during acute infection, but was reduced in blood and spleen during advanced disease. The identification of XCR1+ mDC in rhesus macaques opens new avenues for future preclinical vaccinal studies and highlights XCR1 as a prime candidate for targeted vaccine delivery.

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Mouse CD8α+ myeloid dendritic cells (mDC) respond strongly to TLR3 ligands and excel at CD8+ T lymphocyte cross-priming, as compared with the other DC subsets, conventional mDC, and plasmacytoid DC (pDC) (1–3). CD8α+ mDC express high levels of molecules involved in cellular Ag uptake, virus recognition, or cross-talk with CD8 T cells, altogether promoting cross-presentation to CD8+ T lymphocytes. These molecules include CD205 (lectin mediating phagocytosis and CpG uptake) (4), CLEC9a (C-type lectin involved in the uptake of apoptotic/necrotic cells) (5), cell adhesion molecule 1 (CADM1), which mediates adhesion to activated CD8+ T lymphocytes and NK cells (6–10), and TLR3 (that recognizes virus-derived dsRNA and is triggered by the polyinosinic-polycytidylic acid [poly(LC)] adjuvant) (11). An equivalent DC subset has been identified by comparative genomic analysis and functional studies in human CD141+ and murine CD8α+ mDC, including XCR1 membrane expression as a conserved specific marker. In contrast, high CD11c expression was not characteristic of mDC in macaques, but of CD16+ monocytes. Like their human and murine homologs, simian XCR1+ mDC had much stronger responses to TLR3 stimulation than other myeloid cells. The importance of this new mDC population was tested in SIV mac251 infection, the most relevant animal model for pathogenic HIV-1 infection and vaccination. This population increased sharply and transiently during acute infection, but was reduced in blood and spleen during advanced disease. The identification of XCR1+ mDC in rhesus macaques opens new avenues for future preclinical vaccinal studies and highlights XCR1 as a prime candidate for targeted vaccine delivery.

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and NK cells. Vaccination strategies targeting CLEC9a (19) or CD205 have shown promising results in mice (20) and humanized mice (21) and are currently considered for translation into humans. Nonhuman primates, and particularly macaques, are privileged animal models for such preclinical vaccinal studies. Although CD205 targeting has been shown to be immunogenic in macaques (22), studies in humans have demonstrated that CD205 is quite promiscuously expressed in primates (23). Other receptors would therefore be better suited for selective targeting in primates. Further complicating the matter, equivalent cells have not yet been identified in primates. Therefore, we designed a strategy based on our previous work comparing sheep, mouse, and human DC subsets. We reasoned that macaque DC equivalent to sheep SIRPα+ CD26+, mouse CD8α+, and human CD141+ mDC should express the same conserved membrane markers, share the same transcriptomic signature, and be highly reactive to TLR3 triggering. Indeed, we were able to: 1) identify lineage (lin)+ XCR1+ mDC in rhesus macaques using polychromatic flow cytometric labeling with Abs and fluorochrome-labeled XCL1; 2) confirm by real-time PCR the expression pattern of all of the genes tested as compared with other macaque myeloid cells; and 3) demonstrate their unique high reactivity to TLR3 triggering for TNF-α production and CD40 upregulation. Hence, for the first time, to the best of our knowledge, we rigorously identified the macaque DC homologous to mouse CD8α+ and human CD141+ professional cross-presenting mDC.

Identification of the equivalent of murine CD8α+ mDC in macaques is important for vaccination. Indeed, cross-presentation is required for presentation to cytotoxic CD8+ T lymphocytes of Ag sources other than live viruses. Live attenuated viruses cannot be used for vaccination against many pathogens. For instance, in SIVmac251 infection, the best animal model for HIV-1 infection, the most protective vaccinal viruses can revert to pathogenic strains (24).

To illustrate the importance of our new myeloid subset definition, we tested the variations of dendritic and monocyteic subsets in SIVmac251 infection, the most relevant animal model for pathogenic HIV-1 infection. A prophylactic or therapeutic vaccine against HIV is needed to prevent pathogenic infection or induce immune response recovery during infection, in conjunction with antiretroviral treatments (which cannot be interrupted without viral replication resumption). HIV-1 infection induces CD4+ T lymphocyte depletion and AIDS. It induces mDC and pDC depletion, at least in patients with high viral loads (25–34). Among DC subsets, CD141+ mDC show the deepest attrition (35). Conversely, CD14++ CD16++ nonclassical monocytes (36, 37) accumulate in the blood from viremic or AIDS patients (38–40). SIVmac251 infection induces similar variations as HIV-1 infection for pDC (41–43). In animals with progressive infection, mDC are depleted (44, 45), whereas in animals remaining disease free for 60 wk, their numbers increase (41, 45). CD14+CD16+ intermediate and CD14+/−CD16++ nonclassical subsets are increased, particularly at the AIDS stage, and newly recruited and dividing monocytes/macrophages are found in SIV encephalitis lesions (46–50).

In this study, we took advantage of our novel phenotypic key to characterize unequivocally XCR1+ mDC and rigorously distinguish DC and myeloid subsets in macaques. We examined how infection with a pathogenic immunodeficiency virus affects the homeostasis of DC and other myeloid cell subsets kinetically and not only in blood but also in a secondary lymphoid organ, the spleen, and how this correlates with viral burden. We found that during SIV infection, XCR1+ mDC increased transiently in the circulation during the first days of acute infection and were depleted in blood and spleen during advanced disease.

Materials and Methods

Blood and spleen macaque samples and mononuclear cell isolation

Ten healthy and 17 SIV-infected rhesus macaques (Macaca mulatta) were studied (51) (Table I). Among healthy macaques, two (078016 and 066160) had previously received IL-7 injections >1 y prior to euthanasia. SIVmac251 infection was performed i.v. using 50 AID50 (infectious doses per monkey). Sacrifice was performed at the acute (n = 8), chronic (n = 2), or AIDS (n = 7) stages. PBMC and spleen mononuclear cells (SMC) obtained after Ficoll/Paque gradient centrifugation were frozen and conserved in liquid nitrogen, unless sorted before freezing. Research was approved by animal experimentation ethical committee Paris 1 (2008-0006, 2010-0009, and 2011-0001).

Twelve-color flow cytometric analysis using Abs and vaccibodies

The Abs used for FACS analyses and flow cytometric cell sorting were all mouse anti-human mAbs, except the chicken anti-human CADM1 IgY primary Ab and its secondary conjugate donkey anti-chicken IgY, associated with PerCP. We used the following mAbs that were all designed and validated for flow cytometry: isotype controls mouse IgG1–Pacific Blue (clone MG128; Invitrogen/Life Technologies); mouse IgG2a–FITC (clone A12689; Beckmann Coulter); and mouse IgG1–allophycocyanin and mouse IgG1–PE (clone MOPC-21), both from BD Biosciences. Specific anti-human Abs that cross react with rhesus macaque molecules were: anti-human CD14 (clone HED1, 1/100; clone CD1523–FITC, 1/100; clone G155-178, 1/100); anti-human CD1a (clone HLA-DR–PE-Cy7 (MHC class II [MHC-II]; clone L243, 1/100), anti-human CD16–APC-H7 (clone 3G8, 1/40), anti-human CD20–FITC (clone 2H7, 1/10), anti–nonhuman primate CD45–V500 (clone 2D1, 1/25), anti-human CD16–PE (clone 4D11, 1/10), anti-human CD205–PE (clone MG38, 1/10), and anti-human CD141 (clone BDCA-3–PE) (clone IA4), all from BD Biosciences; anti-human CD1c–Alexa Fluor 700 (clone 3.9, 1/10; eBioScience); anti-human CD1c (BDCA-1)–Pacific Blue (clone L161, 1/500; BioLegend); anti-human CLEC9a–APC (clone 683409, 1/40; R&D Systems); anti-human CD20-PE–Texas Red (clone HI47, 1/10), anti-human CD16–Alexa Fluor 700 (clone 3G8, 1/10, 1/100); and anti-human CD14–Qdot655 (clone TuK4, 1/100) (Invitrogen/Life Technologies); chicken IgY anti-human CADM1 (clone 3E1 used at a final dilution of 1/500; MBL International); and donkey Ig anti-chicken IgY Fab2–PerCP (1/50 final dilution; Jackson Immunoresearch Laboratories). Fluorescence-minus-one (FMO) controls were used to define the positivity of various molecules for the different cell subsets that could be defined with the other Abs used in our flow cytometric panel. In these FMO samples, the proper fluorochrome-conjugated isotype controls were added in place of the Ab directed against the molecule for which the limit of positivity was required. To define the number of FMO samples, multiple FMOs were combined within a single sample but only for Abs coupled to fluorochromes that had absolutely no spectral overlap. Therefore, a fluorescence-minus-three control sample was used in which isotype controls coupled to Pacific Blue, FITC, and allophycocyanin were combined to delineate the positivity for CD1c (Pacific Blue), CD123 (FITC), and Clec9a (allophycocyanin). We also used an FMO for PE, which was our variable channel (CD162-PE, CD205-PE, or CD141 minus secondary Ab-PE). Concerning CD16, the limit between CD16– and CD16+ monocytes was easily defined as the CD14+ CD16– classical subset always appeared as a round, well-defined population. The limit between nonclassical CD14+ CD16++ and intermediate CD14+CD16+ cells was first defined at the vertical of the classical CD14+CD16+ monocytes that showed the lowest CD14 expression. Following this positioning of the two CD16+ monocyte subset gates, we checked also that intermediate monocytes had the highest mean fluorescence intensity (MFI) for MHC-II (defined using an anti-human HLA-DR Ab). No isotype control was used in place of the anti-CD11c Ab because this molecule was not used to define cell subsets or a percentage of positivity for CD11c. It was initially put into our multicolor panel because human mDC express CD11c, and macaque mDCs were previously thought to be CD11c−.

We used the following Abs for mDCs and NK cells: biotinylated anti-human CD1c monoclonal antibodies that bind human XCR1 (50 μg/ml; E. Fossum and B. Bogen, manuscript in preparation). As negative controls, we used a mutated version of XCL1 in which cystein 11 is mutated to alanine [XCL1(C11A)-mCherry] 50 μg/ml, as well as anti–NIP-mCherry (a single-chain Fv Ab binding the hapten NIP previously described) (Supplemental Fig. 1) (52–54). Construction of the vaccibody gene construct using cloning of the anti-NIP has been previously described (53). Human XCL1 and XCL1(C11A) were ordered from Genscript and cloned into the vaccibody gene construct using BsmI and BsiWI. mCherry was cloned into the construct using SfiI, as previously described (55). To express the vaccibodies, the gene constructs were stably
transfected into NS0 cells, which were grown in rollerbottles. mCherry vaccibodies were purified by harvesting supernatants and applying them onto a Sepharose 4 Fast Flow column (GE Healthcare) conjugated with an anti-mCherry Ab (clone 1) (55). Eluted vaccibodies were dialyzed twice in PBS, concentrated using a 50-Kd cutoff Vivaspin column (Sartorius Stedim Biotech), aliquoted, and stored at –80°C until use.

PBMC or SMC (2.10^6 cells/tube) were thawed in the presence of 20 IU/ml DNase to prevent aggregates immediately before labeling. Cells were washed and incubated with Live/Dead blue dye (30 min, 4°C; Invitrogen) or 100 ng/ml LPS (Sigma-Aldrich) in polypropylene tubes. For CD40 intracellular FACS analyses, cells were cultured for 5 h, and brefeldin A (10 μg/ml; Sigma-Aldrich) was added during the last 4 h. For CD40 expression, cells were cultured for 18 h and labeled as above with addition of anti–CD40-APC clone 5C3 (1/10; BD Biosciences). For intracellular TNF-α detection, following membrane labeling as above without anti-CD11c, cells were fixed, permeabilized (BD Cytofix/Cytoplperm kit; BD Biosciences), and incubated with anti–TNF-α–Alexa Fluor 700 clone MAB11 (1/10, BD Biosciences; 45 min, 4°C) and incubated with anti–CD40-APC clone 5C3 (1/10, BD Biosciences; 45 min, 4°C) and incubated with anti–CD40-APC clone 5C3 (1/10, BD Biosciences) set for high-purity sorting. Cells were labeled with the following fluorescently labeled Abs or vaccibodies: CD45-V500, CD20-FITC, HLA-DR–allophycocyanin-Cy7, CD14-Qtod655, CD16–Alexa Fluor 700, CD1c (BDCA-1)–Pacific Blue, chicken IgY anti-human CADM1 followed by a donkey Ig anti-chicken IgY F(ab’)-PerCP, and human XCL1-mCherry vaccibodies, which were added concomitantly with the donkey Ig anti-chicken IgY F(ab’)-PerCP secondary Ab. Purification was obtained to at least 98%.

**Flow cytometric cell sorting**

Freshly isolated SMC were incubated (15 min, 4°C) with PBS-5% human blood) of mononuclear cells (monocytes plus lymphocytes) to the periphery. Hemocytometer complete blood count (performed independently on whole blood from acutely infected macaques was calculated by multiplying the percentage of cells among CD45hi events.

**Real-time RT-PCR quantitative analysis of transcript expression by sorted cellular subsets**

Purified cell populations were lysed in 350 μl RTL buffer (Qiagen) containing 1% of 2-ME (Sigma-Aldrich) and stored at –80°C. Total mRNA were purified using the RNAeasy kit (Qiagen), according to the manufacturer’s instructions: briefly, lysates were homogenized using QIAshredder (Qiagen), and residual DNA was removed by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). mRNA were eluted using 2 × 40 μl RNase-free water and immediately subjected to a reverse transcription step with the Quantitect Reverse Transcription Kit (Qiagen). cDNA were kept at –20°C.

Primers specific for each of 12 specific genes (CLEC9A, RAB7B, TLR3, BATF3, XCR1, CLEC4C, IL7, TLR7, TLR9, CD1E, TLR4, and TLR8) and 4 housekeeping genes (hypoxanthine phosphoribosyl transferase [HPRT], GAPDH, β-actin [ACTB], and β2-microglobulin [B2M]) were defined from macaque cDNA sequences (accession numbers NM_001194661.1, XM_001082679.2, NM_001366851.2, XM_001107737.2, XM_001142981.2, XM_0002798473.1, XM_0001083251.2, NM_001130426.1, NM_001130431.1, NM_001042688.1, NM_001370291.2, and NM_001304271.2; respectively; Table II). To detect small amounts of DNA from low numbers of FACS-sorted cells, we developed a specific nested PCR assay as currently performed in our laboratory (56, 57). cDNA were first PCR amplified (final volume 50 μl) using 2 × 40 μl RNase-free water and immediately subjected to a reverse transcription step with the Quantitect Reverse Transcription Kit (Qiagen). cDNA were kept at –20°C.

**Table I. Blood and spleen samples and clinical data from macaques**

<table>
<thead>
<tr>
<th>Macaque</th>
<th>Symptoms in Fig. 5A</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Infection Stage</th>
<th>Time Postinfection</th>
<th>CD4+ T (Cells/μL)</th>
<th>VL (Log_{10} RNA Copies/ml)</th>
<th>Spleen</th>
<th>Blood</th>
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*Viral load obtained 2 to 3 mo before euthanasia. F, female.

**Symbols in Fig. 5A**

- Open circle: Female
- Gray circle: Male
- Gray square: Acute
- Gray triangle: Chronic
- Black circle: Clinical
- Black square: Uninfected
- Gray diamond: Uninfected
- Open circle: Uninfected

**Fig. 5A** Age (y); Sex; Infection Stage

- Postinfection
- CD4+ T (Cells/μL)
- VL (Log_{10} RNA Copies/ml)
- Spleen
- Blood

- F: Female
- M: Male
- B: Both
- C: Chronic
- A: Acute
- U: Uninfected
- ND: Not determined
- Y: Yes
- N: No

**Table II.** The Journal of Immunology**
GAPDH, ACTB, and B2M. The number of cycles (25) for this first amplification step was adjusted to ensure that all amplifications remained in the exponential phase of the reaction. PCR products were quantified using LightCycler 480 Real-Time PCR Technology with LightCycler 480 SYBR Green I Master Mix (Roche Applied Science), performed on 1/190th of the initial PCR products: 10 min initial denaturation at 95°C, followed by 45 cycles of 10 s at 95°C, 10 s at 64°C, and 15 s at 72°C. The 16 genes were quantified in duplicate for the 8 purified cell populations. Results are displayed for each gene in each population as fold differences compared with the most weakly expressed housekeeping gene, HPRT, using the 2ΔΔCp method (ΔΔCp corresponding to the number of amplification cycles between the detectability of HPRT amplicons and that of the studied gene).

Statistical analysis

Results are given as medians. Mann–Whitney U test was used to compare controls and infected groups and Wilcoxon rank-sum test was used to compare cell subsets (Fig. 2). Correlations were evaluated by Spearman test. Differences were defined as statistically significant when \( p < 0.05 \). The \( p \) values were shown when \( < 0.15 \). All these nonparametric tests were performed using GraphPad Prism 5 (GraphPad).

Results

Definition of a simian mDC subset phenotypically homologous to human XCR1+ CD141 (BDCA-3)+ DC

To study all DC and monocyte subsets simultaneously in macaques, we carried out 12-color flow cytometric analysis. PBMC from nine healthy macaques were studied. The gating strategy is shown for a representative uninfected macaque (monocyte and DC subsets outlined in red, Fig. 1A). In these analyses, among live (Live/Dead) singlet (side scatter width/side scatter area and forward light scatter height/forward light scatter area dot plots) CD45+ cells, MHC-II+CD20+ B lymphocytes, monocyte subsets (classical CD14++CD16−, intermediate CD14−CD16+, and nonclassical CD14−CD16++) (Table II), and lin−neg MHC-II−CD123+ pDC were simultaneously defined. Among MHC-IIlin−neg (CD20−CD14−CD16−) cells, comcomitant detection of CD1c+, CADM1, and CD205 allowed us to distinguish the previously described CD1c+ mDC from the yet overlooked CADM1+CD205+ mDC (Fig. 1A). CADM1+ mDC also strongly expressed CD162-like molecules on different subsets were evaluated in the blood from the rhesus macaque defined them like in humans as lin−negMHC-II+CD11c+ but not of mDC in macaques. Consequently, the XCR1+ DC nomenclature will be used through this report. In the absence of specific anti-simian XCR1 Abs, CADM1 or CD205 Abs were also used to distinguish these cells from conventional CD1c+ DC.

The median proportion of circulating XCR1+ DC characterized as in Fig. 1 was 0.06% (range 0.04–0.09%; SEM 0.007; \( n = 9 \)). XCR1+ DC were also characterized in the spleens from three healthy macaques using a similar gating strategy (Supplemental Fig. 2), and their median proportion among CD45+ mononuclear cells was 0.14 (0.08–0.39%; SEM: 0.09; \( n = 3 \)).

High CD11c expression is characteristic of CD16+ monocytes and not of mDC in macaques

Most previous studies of mDC during SIV infection in macaques defined them like in humans as lin−negMHC-II−CD11c+ but without an anti-CD16 Ab in the lineage mixture (41, 45). We confirmed the observation by Autissier et al. (59) that CD1c+ mDC only weakly express CD11c, therefore most CD20+ CD14− MHC-II−CD11c+ cells correspond to CD16+ monocytes, and the population expressing the highest levels of CD11c corresponds to nonclassical CD14−CD16++ monocytes (Fig. 1D, Supplemental Fig. 3).

Simian XCR1+ DC displayed the same molecular signature as in humans and mice

We next evaluated, in FACS-sorted spleen lin−CD14/CD16/CD20−MHC-II−CADM1+XCL1+ mDC, CD1c+ mDC, lin−MHC-II−CD123+ pDC, and classical CD14+CD16+ monocytes from two uninfected macaques, the expression level of genes known to be strongly expressed either in human and murine XCR1+ mDC homologs or in human pDC, CD1c+ mDC, and monocytes (7, 13, 14, 52) (Fig. 3, Supplemental Fig. 2). As a control, we checked that all of the housekeeping genes tested had a similar expression level in all cellular subsets (Fig. 3D). We confirmed at the RNA level that, like in humans or mice, simian XCR1+ mDC expressed the highest levels of the genes encoding CLEC9A, TLR3, the transcription factor BATF3, and the small GTPase RAB7B (Fig. 3A). CLEC9A mRNA was strongly expressed in both macaques studied, in contrast to the failure of surface labeling using anti-human CLEC9A Ab in most macaques including these two macaques (staining of XCR1+ mDC failed of surface labeling using anti-human CLEC9A Ab in most macaques, whereas XCR1+ mDC were the only cells expressing XCR1, TLR3, and the anti-human CLEC9A Ab. Therefore, this Ab is not suitable to label XCR1+ DC in rhesus macaques.

In agreement with data in humans, the CLEC4C, ILT7, TLR7, and TLR9 genes were all expressed at higher levels in FACS-sorted macrophage pDC (Fig. 3B), whereas CD1E, TLR4, and TLR8 were strongly expressed by FACS-sorted CD14++CD16+ monocytes, and to a lesser extent by CD1c+ mDC, from the two uninfected macaques tested (Fig. 3C). Therefore, XCR1+ DC displayed the same specific molecular signature in rhesus macaques as in humans or mice.
Simian XCR1⁺ mDC had stronger responses to TLR3 stimulation than the other APCs

To assess whether simian XCR1⁺ mDC were functionally homologous to human and mouse XCR1⁺ mDC, we evaluated whether they had stronger responses to TLR3 triggering than other mDC or DC populations. Freshly purified PBMC from healthy macaques were stimulated using the TLR3 agonist poly(I:C) or, as a control, the TLR4 agonist LPS. TNF-α production was quantified by intracellular FACS analysis in XCR1⁺ (defined in this study as CADM1⁺) or CD1c⁺ mDC, pDC, and CD14hi or CD14lo monocytes, as well as in B lymphocytes, which do not express TLR3 (60) (Fig. 4). Representative results are shown for the different cell subsets from one macaque (Fig. 4A). A strong TNF-α production was detected following poly(I:C) stimulation only in CADM1⁺ mDC in response to 5 μg/ml (CADM1⁺ mDC versus pDC or CD14hi monocytes, p = 0.008; versus B lymphocytes and CD14hi monocytes, p = 0.012; and versus CD1c mDC, p = 0.016) or 20 μg/ml (CADM1⁺ mDC versus CD1c⁺ mDC, pDC, CD14hi, and CD14hi monocytes, p = 0.008; and versus B lymphocytes, p = 0.012). As a positive control, monocytes had the highest response to LPS (CD14lo and CD14hi monocytes versus all other subsets, p = 0.008) (Fig. 4B, 4C). Comparatively, CADM1⁺ mDC responded weakly to LPS (8.3% TNF-α-positive cells), consistently with their low expression of TLR4. The MFI of CD40 was increased 2.4-fold (p = 0.1 using the nonparametric Mann–Whitney U test) on CADM1⁺ mDC and only 1.9-fold on CD1c⁺ mDC (p = 0.2), and it was not increased in B lymphocytes, pDC, CD14hi, or CD14lo monocytes (p > 0.4) (n = 3, Fig. 4B, 4D). Thus, XCR1⁺ mDC had a stronger cytokine response to TLR3 stimulation than the other APCs in macaques, as in humans or mice.

High circulating XCR1⁺ mDC counts during acute SIV infection, but depletion during AIDS

The SIV infection model makes it possible to evaluate immunological parameters at all stages of the infection, particularly during the first days following infection, which are nearly impossible to
study in humans, and simultaneously in the circulation and lymphoid organs. In macaques sacrificed during acute 
SIV<sub>mac251</sub> infection (Table I), we measured the percentages among PBMC and CD4<sup>+</sup> SMC of all DC and monocyte subsets (as defined in Fig. 1). In the blood, XCR1<sup>+</sup> (defined in this study as CADM1<sup>+</sup>), mDC proportions peaked at day 7 postinfection (gray circles, median: 0.42%; fold change as compared with uninfected macaques: 7.2) and were significantly increased in macaques tested during very early infection (day 3 plus day 7) compared with uninfected macaques (median: 0.27 versus 0.06%; \( p = 0.003 \), Fig. 5A). As previously described (42, 43), this was also the case for pDC (medians: uninfected, 0.09%; infected day 3 plus day 7, 0.27%; \( p = 0.006 \)). The proportions of classical CD14<sup>+</sup>CD16<sup>−</sup> monocytes (medians: uninfected, 2.57%; infected day 3 plus day 7, 8.07%; infected day 10 plus day 14, 6.71%; uninfected versus all acutely infected: \( p = 0.02 \)), intermediate CD14<sup>+</sup>CD16<sup>−</sup> monocytes (medians: uninfected, 0.07%; infected day 3 plus day 7, 0.28%; infected day 10 plus day 14, 0.49%; uninfected versus all acutely infected: \( p = 0.004 \)), and CD1c<sup>+</sup> mDC (medians: uninfected, 0.29%; infected day 3 plus day 7, 0.75%; infected day 10 plus day 14, 0.51%) were also increased transiently but for a longer period of time during acute infection (days 3–14). In the spleen, only three uninfected macaques were analyzed, making it difficult to reach significant differences (Fig. 5A). Plasma viral loads (pVL) increased over time with maximal values around day 10 postinfection, as expected. Absolute cell counts were studied in eight acutely infected macaques to assess correlations with viral loads (Fig. 5B). Interestingly, among all cellular subsets studied, only circulating XCR1<sup>+</sup> mDC absolute counts (Spearman \( r = -0.90; p = 0.005 \)) and proportions (Spearman \( r = -0.81; p = 0.02 \)) showed an inverse correlation with pVL (Fig. 5B). This was not the case in the spleen.

We then measured the proportions of DC and monocyte subsets in macaques with advanced infection (two with chronic infection and pVL >5 log, seven with AIDS; Fig. 5A, Table I) compared with uninfected macaques. The proportions of XCR1<sup>+</sup> mDC among CD45<sup>+</sup> cells from the macaques with advanced infection were reduced by 5.19 in the spleens (median uninfected: 0.14% and advanced infection: 0.03; \( p = 0.02 \)) and tended to be reduced by 2.71-fold in the blood (median uninfected: 0.06% and advanced infection: 0.02; \( p = 0.05 \)), whereas CD1c<sup>+</sup> mDC proportions were reduced by 1.81-fold in the blood only (median uninfected: 0.29% and advanced infection: 0.16; \( p = 0.04 \)). In contrast, pDC and monocyte subset proportions were not significantly affected by infection, both in blood and spleen. To overcome the impact of CD4<sup>+</sup> T lymphocyte depletion on DC and monocytes, we analyzed the proportions of mDC and monocyte subsets among total myeloid mononuclear cells (Fig. 5C). This highlighted a reduction in the proportions of XCR1<sup>+</sup> and CD1c<sup>+</sup> mDC among myeloid cells, especially in the blood, in macaques with advanced disease.

Therefore, SIV<sub>mac251</sub> infection had a clear impact on XCR1<sup>+</sup> mDC homeostasis from an early rise in the circulation to an apparently global depletion during chronic infection or AIDS.

**Discussion**

Cross-species transcriptomic, phenotypic, and functional comparison allowed the identification in rhesus macaques of a DC population homologous to human CD141<sup>+</sup>, murine CD8α<sup>+</sup>, and sheep CD26<sup>+</sup> mDC. XCR1 being a specific and conserved marker spanning all of these species. In addition, we show that these cells had increased circulating numbers during the first week of SIV infection in inverse correlation with viral loads and, conversely, reduced during advanced infection.

### Table II. Primers used for real-time PCR on FACS-sorted DC subsets and CD14<sup>+</sup>CD16<sup>−</sup> monocytes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>5' In Primer</th>
<th>5' Out Primer</th>
<th>3' In Primer</th>
<th>3' Out Primer</th>
</tr>
</thead>
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<tr>
<td>CLEC9A</td>
<td>TACACCTCTCTTCAGTGGGA CAGCGTGGAACCTTCCTTTA AGGAGCATGGTGTCTTGTGA GACTGCAGTTATGGGCTGAA</td>
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<tr>
<td>RAB7B</td>
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<td>RAB7B</td>
<td>RAB7B</td>
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<td>BATF3</td>
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<td>BATF3</td>
<td>BATF3</td>
<td>BATF3</td>
</tr>
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<td>XCR1</td>
<td>XCR1</td>
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<td>ILT7</td>
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<td>ILT7</td>
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<tr>
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<td>CLECs</td>
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<td>TLR8</td>
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<tr>
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<tr>
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**Note:** Primers are used for real-time PCR on FACS-sorted DC subsets and CD14<sup>+</sup>CD16<sup>−</sup> monocytes.
For the identification of the macaque mDC population homologous to mouse CD8α+ and human CD141+ mDC, CADM1 was found to be the cell-surface marker with the best combination of selectivity, expression reproducibility, and commercial reagent accessibility (3, 6, 7, 11, 12, 15). CADM1 binds to the class I–restricted T cell–associated molecule, a surface receptor primarily expressed on activated cytotoxic lymphocytes (8). This is thought to participate in the cross talk between XCR1+ mDC and activated CD8+ T lymphocytes. None of the commercial anti-human XCR1 Abs could label specifically XCR1 in our hands, even on human primary CD141+ DC. However, we labeled very reproducibly and specifically macaque XCR1+CADM1+ cells using custom-made fluorescently coupled recombinant human XCL1, the ligand for XCR1, as previously performed in mice using labeled mouse XCL1 (52). Labeling was specific, as assessed using mutant molecules, and exclusive for this cell subset among mononuclear cells. Conversely, CD141 was expressed by macaque XCR1+ mDC, but also by CD1c+ mDC and all monocyte subsets (59), and therefore is not as distinctive of this cell subset as in humans. In addition, CLEC9A, which is expressed at the surface of human and mouse XCR1+ mDC and allows them to bind necrotic cells and cross-prime their Ags to naive CD8+ T lymphocytes (61), was labeled using anti-human Abs in only 2 of the 28 macaques analyzed for CLEC9A expression by flow cytometry. However, CLEC9A mRNA was specifically and highly expressed by XCR1+ mDC in the two macaques analyzed for RNA expression, these two macaques being both negative for CLEC9A by flow cytometry. This suggests an interesting polymorphism in the simian CLEC9A molecule affecting the epitope recognized by the anti-human CLEC9A Ab, which therefore is not suitable to identify XCR1+ DC in macaques. Hence, XCR1 seems currently to be the only cell-surface conserved specific marker in sheep, mice, macaques, and humans, and commercial Abs will be needed, but CADM1 or CD205 Abs can also be used to distinguish these cells from conventional CD1c+ DC.

The strong coexpression of CADM1, CD162, CD205, and XCR1, in combination with quantitative analysis of genes specific for the different myeloid and DC subsets, confirmed the homology among simian, human, and murine XCR1+ mDC. Particularly, in the three species, these cells expressed selectively high levels of the transcription factor Batf3, which specifically drives the development of XCR1+ mouse (CD8α+) and human (CD141+) mDC at steady state (1, 62). Macaque XCR1+ mDC strongly expressed the TLR3 gene and strongly responded to the TLR3 ligand poly(I:C) by producing TNF-α and upregulating CD40, whereas all of the other APCs responded weakly or not. XCR1+ mDC also strongly expressed genes encoding proteins involved in cross presentation, including CLEC9A and the small GTPase RAB7B, which is essential for the retrograde transport from endosomes to the trans-Golgi network, and has been hypothesized to promote assembly of the machinery required for cross presentation (52). Further functional studies

Table III. MFI of mCherry coupled NIP, mutant XCL1(C11A), and XCL1 at the surface of monocyte and mDC subsets defined in Fig. 1D

<table>
<thead>
<tr>
<th>mCherry MFI</th>
<th>NIP</th>
<th>XCL1(C11A)</th>
<th>XCL1</th>
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<tbody>
<tr>
<td>CD14+CD16- monocyte</td>
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<tr>
<td>CD14+CD16+ monocyte</td>
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<td>CD14+CD16+ monocyte</td>
<td>160</td>
<td>294</td>
<td>429</td>
</tr>
<tr>
<td>CD1c+ mDC</td>
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<td>193</td>
<td>354</td>
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<tr>
<td>CADM1+ mDC</td>
<td>337</td>
<td>439</td>
<td>1619</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>55</td>
<td>103</td>
<td>196</td>
</tr>
</tbody>
</table>

FIGURE 2. Expression levels of CADM1, CD205, MHC-II, and XCR1 molecules at the surface of different cell populations in rhesus macaques. (A) Graphical representation of the MFI of CADM1, CD205, and MHC-II labeling on CD20+ B lymphocytes, CD14+CD16+, CD14+CD16-, or CD14+/CD16- monocytes, CD1c+ and CADM1+ mDC, and pDC in the blood from nine uninfected macaques. (B) mCherry MFIs for CD20+ B lymphocytes, CD14+CD16+, CD14+CD16-, or CD14-/CD16+ monocytes, and CD1c+ and CADM1+ mDC isolated from the blood (open circles) or spleens (open diamonds) from different macaques stained with mCherry-coupled XCL1 [PBMC, n = 2; SMC, n = 2 (top panel)], mutant XCR1 (C11a) [PBMC, n = 1; SMC, n = 1 (middle panel)], or nonspecific [PBMC, n = 1; SMC, n = 1 (bottom panel)] are shown. MFIs were from the entire population, as depicted in Fig. 1C for XCR1 labeling.
must ensure that like their human and murine counterparts, simian XCR1+ mDC do perform cross presentation. This will require effector CD8+ T cells specific for SIV epitopes, which can be obtained and characterized from infected or vaccinated macaques, and purified XCR1+ cells to cross present the corresponding epitopes from an uninfected macaque expressing the same Mamu class I molecule restricting the response to these epitopes.

Most studies performed in macaques, in particular in the context of SIV infection, have defined mDC as being phenotypically similar to human mDC as lin(CD3/CD14/CD20) neg MHC-II+CD11c+ cells, without excluding CD16+ cells, because some macaque mDC express CD16 and low levels of CD11b (41, 45). Intriguingly, in these studies, and contrary to human and mouse mDC that express high levels of MHC-II molecules in the blood and spleen, macaque mDC only expressed intermediate levels of MHC-II (tested with anti–HLA-DR Ab), whereas most CD11c neg events among lin negCD123 neg cells had strong MHC-II expression levels but were not considered as mDC (41).

**FIGURE 3.** Quantification of mRNA from genes characteristic for human or murine XCR1+ mDC, pDC, and monocytes in sorted rhesus macaque spleen cell populations. (A–D) Total cellular RNA from FACS-sorted lin MHC-II+CD11c+XCR1+ mDC, lin MHC-II hiCD11c+ mDC, lin MHC-II+CD123+ pDC, and CD14+CD16+ monocytes from two uninfected macaques were reverse transcribed and analyzed for expression by real-time PCR. Results are displayed for each gene as fold differences compared with the weakly expressed housekeeping gene HPRT using the 2eΔCp method. Results obtained for genes known to be differentially expressed by human and mouse XCR1+ mDC (A), human and mouse pDC (B), human and mouse monocytes and conventional mDC (C), and for housekeeping genes (D) are displayed.
We confirm in this study the previous observation that macaque CD1c+ mDC express low levels of CD11c (59) and found that CD16+ monocytes, and particularly the minor nonclassical CD14+/2 CD16++ subset, expressed the highest CD11c levels among PBMC and SMC. Therefore, previous studies analyzing linnegCD11c+ mDC counts and proportions in the blood and spleen most probably included some CD16+ monocytes, particularly nonclassical monocytes that weakly express CD14 and therefore fall into the linneg gate. It remains possible that macaque mDC might still express highly CD11c in other tissues. This precise delineation of monocyte and DC subsets will help settle down previous discrepancies about their variations during physiological or pathological conditions.

As an illustration, our cross-sectional analysis during acute infection, a stage that cannot be examined in humans, especially in lymphoid organs, shows a sharp increase of the absolute numbers of circulating XCR1+ mDC during the first week of infection. The negative correlation of this increase with viral loads may indicate a specific susceptibility of CADM1+ mDC to this viral infection and direct cytotoxicity, and not homing to lymphoid organs, because their proportions in the spleens also tended to correlate negatively with pVL. Alternatively, inflammation, in particular IL-12 (63) and/or IL-18, might promote the development of XCR1+ DC, as recently reported in BATF3−/− mice, through the induction of other transcription factors with a partially redundant role for cell-type specification (64). Indeed, CD1c+ mDC, pDC, and monocyte proportions also tended to be elevated in the blood during the first week of infection, as previously described (43, 45, 46, 48). Conversely, during chronic infection or AIDS, the proportions of XCR1+ mDC were lower than in controls in blood and spleen, confirming our observations in viremic, chronically HIV-infected patients (35). A consequence of the CD1c+ and XCR1+ mDC number elevation during acute infection may be the induction of the CD8+ T cell responses that are known to occur early; it may also be their destruction, and that of CD4+ T cells, by these specific CD8+ T cells as well as by other cell death effectors induced by HIV-1 infection (65). The consequence of the loss of CD1c+ and XCR1+ mDC from blood as well as from lymphoid organs during chronic infection may be that Ag presentation, and particularly cross-presentation, is compromised, unless pDC or inflammatory DC relay this function (66, 67).
Future studies will define XCR1+ mDC in other organs such as gut-associated tissues and evaluate their functions in vivo in the context of SIV infection. Most importantly, identification of the equivalent of human XCR1+CD141+ and murine XCR1+CD8α+ mDC will allow preclinical vaccinal studies targeting this population in a primate model close to humans, possibly in the bivalent vaccibody format (i.e., Ags linked to the chemokine ligand of XCR1, XCL1) (53, 54), and potential immunotherapies designed to compensate their loss during chronic HIV-1 infection.

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Disclosures
E.F. and B.B. are inventors on a patent application for Xcl1-targeted vaccines.

The authors have no financial conflicts of interest.

References


