THE ROLE OF DENDRITIC CELLS AND MONOCYTES IN HIV INFECTION

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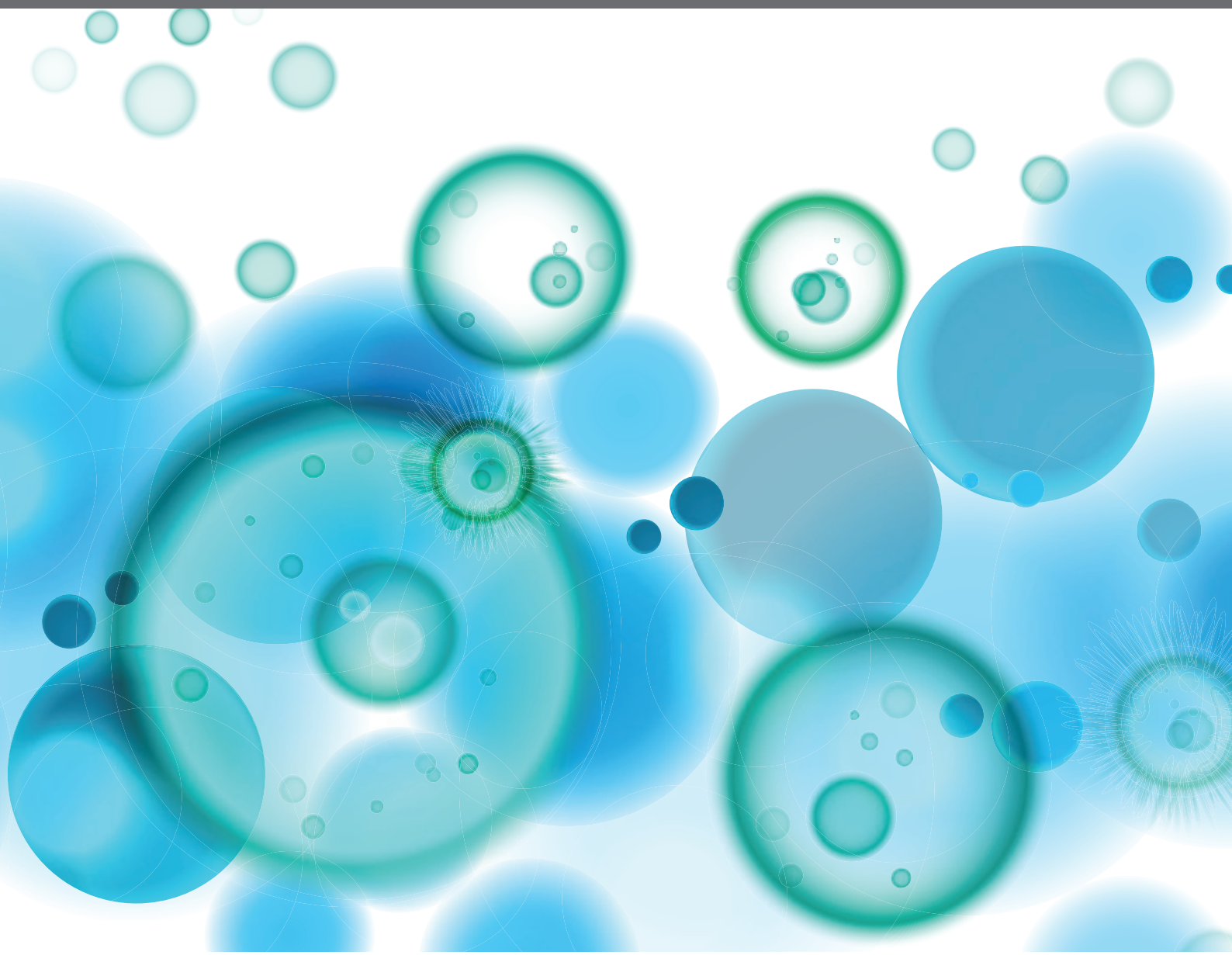

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THE ROLE OF DENDRITIC CELLS AND MONOCYTES IN HIV INFECTION

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Dendritic Cells From the Cervical Mucosa Capture and Transfer HIV-1 via Siglec-1

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Antigen presenting cells from the cervical mucosa are thought to amplify incoming HIV-1 and spread infection systemically without being productively infected. Yet, the molecular mechanism at the cervical mucosa underlying this viral transmission pathway remains unknown. Here we identified a subset of HLA-DR$^+$ CD14$^+$ CD11c$^+$ cervical DCs at the lamina propria of the ectocervix and the endocervix that expressed the type-I interferon inducible lectin Siglec-1 (CD169), which promoted viral uptake. In the cervical biopsy of a viremic HIV-1$^+$ patient, Siglec-1$^+$ cells harbored HIV-1-containing compartments, demonstrating that *in vivo,* these cells trap viruses. *Ex vivo*, a type-I interferon antiviral environment enhanced viral capture and *trans*-infection via Siglec-1. Nonetheless, HIV-1 transfer via cervical DCs was effectively prevented with antibodies against Siglec-1. Our findings contribute to decipher how cervical DCs may boost HIV-1 replication and promote systemic viral spread from the cervical mucosa, and highlight the importance of including inhibitors against Siglec-1 in microbicidal strategies.

Keywords: cervix, Siglec-1, HIV-1, *trans*-infection, myeloid cells

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INTRODUCTION

Women account for 51% of people living with HIV worldwide, and in 2017 this represented 18.8 million of females (1). Since HIV-1 infection is mostly acquired by sexual transmission (2), understanding the female genital tract immunobiology is imperative not only to halt novel infections, but also to design strategies that will limit HIV-1 spread within the mucosa and contain the virus during the early stages of infection. HIV-1 acquisition requires a series of orchestrated events that lead to systemic infection, beginning with viral entry through the genital epithelium and followed by the productive infection of distinct CD4$^+$ target cells that reside within the mucosa. Local infection is early disseminated to draining lymph nodes, aiding to spread HIV-1 systemically and boost viral replication (3). While current antiviral agents are potent inhibitors of viral infection in the mucosa, efficacy of preventive methods is also critically dependent on effective blockade of all potential receptors involved in HIV-1 dissemination from the genital mucosa to the lymphoid tissues (4–6). Yet, the precise molecular mechanisms underlying viral dissemination routes from the genital mucosa that boost systemic HIV-1 infection remain unknown.

Although productive infection during the first days after vaginal SIV exposure is low and mainly restricted to the cervicovaginal tissues (3), evidence for rapid dissemination to the draining lymph nodes has been demonstrated as soon as 24 h post-infection (7–9). These studies suggest a critical role for cervical myeloid cells and, particularly, dendritic cells (DCs) in the early dissemination of mucosal viruses to lymphoid tissues. Importantly, viral spread does not only rely on *de novo* productive infection of myeloid cells (10–12), but can be triggered upon activation of mucosal myeloid cells via the capture and storage of large amounts of HIV-1 particles that are later transferred to target cells, as previously reported for monocyte-derived DCs (13–15). Once mucosal myeloid cells migrate to secondary lymphoid tissues for induction of antiviral immune responses, trapped viruses can be efficiently transferred to CD4$^+$ T cells (10, 11), which become productively infected and fuel systemic viral dispersion. This highly infectious process is known as *trans*-infection, and pioneering work already identified that migratory HLA-DR$^+$ CD3$^-$ cervical cells efficiently captured and transmitted HIV-1 in *trans* (5). Recent data also demonstrated that among all myeloid cell subsets, vaginal and cervical DCs capture and transport transmitted/founder viruses through the cervicovaginal mucosa and facilitate infection of target cells (16, 17).

Although *trans*-infection was initially attributed to the capacity of C-type lectin receptors such as DC-SIGN to specifically bind to the viral envelope glycoprotein of HIV-1 (11), viral capture in the female genital mucosa is independent of this particular receptor, as the majority of cervical DCs capturing HIV-1 do not express DC-SIGN (18) and neutralizing antibodies against DC-SIGN cannot block viral transmission (5). In addition to C-type lectin receptors, HIV-1 capture by myeloid cells is mediated by the sialic acid binding immunoglobulin-like lectin-1 (Siglec-1/CD169) (19–21). This receptor potently enhances HIV-1 capture and storage in virus containing-compartments (22), that are later released from DCs to infect target cells via virological synapse formation (23). Siglec-1 is present on the surface of myeloid cells and its expression is potently enhanced by type-I interferon signaling (21, 24), which is triggered by viral immune sensing or bacterial lipopolysaccharide exposure, both factors associated to HIV-1 infection (25). However, it does not bind to the envelope glycoprotein of HIV-1 as C-type lectin receptors do (11), but recognizes sialylated gangliosides that are anchored on the viral membrane of enveloped retroviruses (19, 26). *In vivo*, Siglec-1 is required for robust infection and early dissemination of a retrovirus within the lymphoid tissue of a murine model (27), but its potential role during HIV-1 infection in the female genital tract remains unexplored.

Here we sought to clarify if *trans*-infection mediated by Siglec-1 could impact the early stages of HIV infection in women, and performed a comprehensive analysis of Siglec-1 expression on human cervical anatomical compartments. In all samples studied, we identified mucosal DCs expressing this receptor, whose expression was boosted by interferon alfa (IFNα) antiviral signaling. Moreover, we also found that cervical DCs enhanced viral capture and *trans*-infection, and that this mechanism was effectively prevented with antibodies against Siglec-1 receptor.

RESULTS

Myeloid Cells From Human Cervical Submucosa Express Siglec-1

We first analyzed Siglec-1 expression by flow cytometry on human cervical mononuclear cells obtained from tissues from benign hysterectomies processed immediately after prescribed surgery. Gating on hematopoietic CD45$^+$, single and viable cells allowed identifying myeloid CD3$^-$ HLA-DR$^+$ cells (**Figure 1A**), which represented a mean of 6 and 7.5 % of cells at the ectocervix and endocervix, respectively (red gate and bar graph, **Figure 1B**). Of note, HLA-DR$^-$ cells did not express Siglec-1 (gray and brown gates, **Figure 1C**). However, among the myeloid HLA-DR$^+$ fraction, a mean of 24.5 and 11.5 % of cells expressed Siglec-1 in the ectocervix and endocervix, respectively (blue gate and bar graph, **Figure 1D**). Moreover, these cells were predominantly CD11c$^+$ CD14$^+$ (pink gate and bar graph, **Figure 1E**) and CD11b$^+$ (orange gate, **Figure 1F**). In contrast, myeloid HLA-DR$^+$ cells lacking Siglec-1 expression represented three distinct subsets: CD11c$^-$ CD14$^+$, CD11c$^+$ CD14$^-$ and CD11c$^+$ CD14$^+$ cells (**Figure 1G**). Of note, Siglec-1$^+$ cells displayed a higher activation status than Siglec-1$^-$ cells as seen by HLA-DR expression levels (**Supplemental Figure 1**). These results identify Siglec-1$^+$ myeloid cells at the ectocervix and endocervix as a subset that displays typical markers of interstitial cervical DCs, such as HLA-DR, CD11c, CD14, and CD11b (28).

Siglec-1$^+$ Cells Accumulate in the Submucosa of the Ectocervix and Endocervix

To analyze the localization of Siglec-1$^+$ cells within the cervix, we next performed immunofluorescence of mucosal tissues from women undergoing benign hysterectomies. Siglec-1$^+$ cells predominantly located in the lamina propria or submucosa and, in agreement with flow cytometry data, were also positive for CD14 or CD11c (**Figure 2A**). In contrast, no Siglec-1$^+$ cells were found in the lower region of the epithelium, where CD207/langerin$^+$ Langerhans cells are commonly found (29). Immunohistochemistry staining of cervicovaginal tissues further identified Siglec-1$^+$ cells displaying myeloid cell morphology mostly accumulated within the submucosa of the ectocervix, which is lined by a stratified squamous epithelium (**Figure 2B**, left). Siglec-1$^+$ cells were also found in the endocervix, which is covered only by a single-layer columnar epithelium (**Figure 2B**, right), although the frequency was lower than at the ectocervix. However, in those endocervical tissues classified as highly inflamed based on histopathological detection of inflammatory infiltrates, we found a higher number of Siglec-1$^+$ cells (**Figure 2C**) that were comparable to those observed at the ectocervix (**Figure 2B**, left graph). Of note, the reasons for a higher inflammatory infiltrate could not be attributed to the HIV-1 infection status, the level of viral load, age or any particular clinical indication for surgery (**Table 1**). These results identified the presence of myeloid cells expressing Siglec-1 in the steady state just beneath the mucosa of the ectocervix and endocervix, where cervical DCs usually accumulate (16).

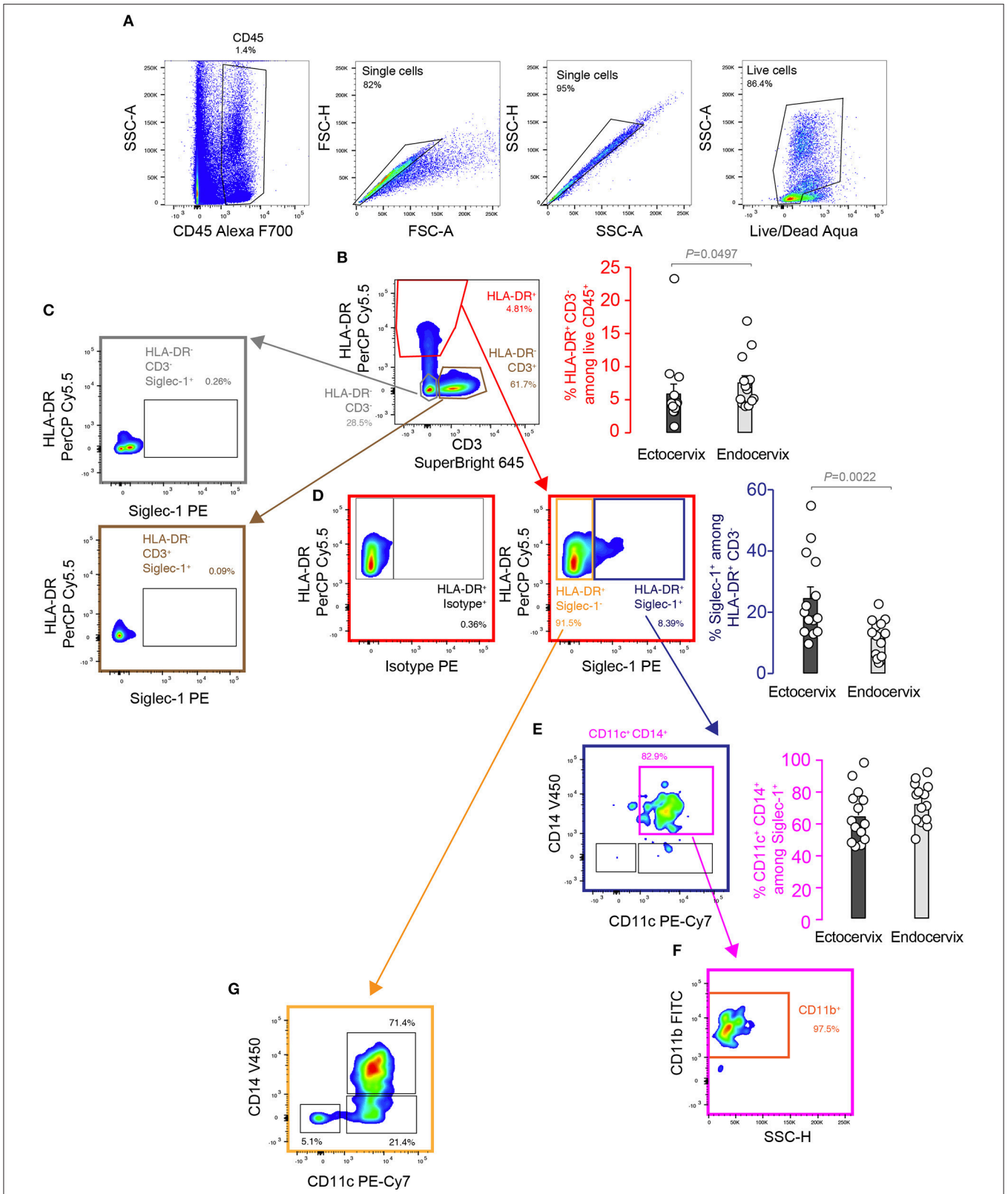

FIGURE 1 | Myeloid cells from human cervical mucosa express Siglec-1. FACS analysis and representative gating strategy of the cervicovaginal myeloid cell subsets. Colored gates and arrows indicate populations analyzed, and corresponding matching colors are used to identify bar graphs showing frequencies of those

(Continued)

FIGURE 1 | populations. **(A)** Hematopoietic cells were identified by their CD45 expression, and single-live cells were selected by doublet discrimination and live/dead staining. **(B)** Representative dot plot and frequency of HLA-DR$^+$ and CD3$^-$ on hematopoietic cells. **(C)** Representative dot plot showing that HLA-DR$^-$ cells do not express Siglec-1. **(D)** Representative dot plot and frequency of Siglec-1 expression analyzed in HLA-DR$^+$ myeloid cells compared to matched isotype control. **(E)** Representative dot plot and frequency of CD11c$^+$ CD14$^+$ cells among myeloid HLA-DR$^+$ cells expressing Siglec-1. **(F)** Representative dot plot of CD11b expression analyzed in CD11c$^+$CD14$^+$ Siglec-1$^+$ myeloid cells. **(G)** Representative dot plot of CD14 and CD11c among the myeloid HLA-DR$^+$ cells that do not express Siglec-1. All bar graphs show mean values and SEM from 14 donors. Statistical differences were assessed with a Mann-Whitney test.

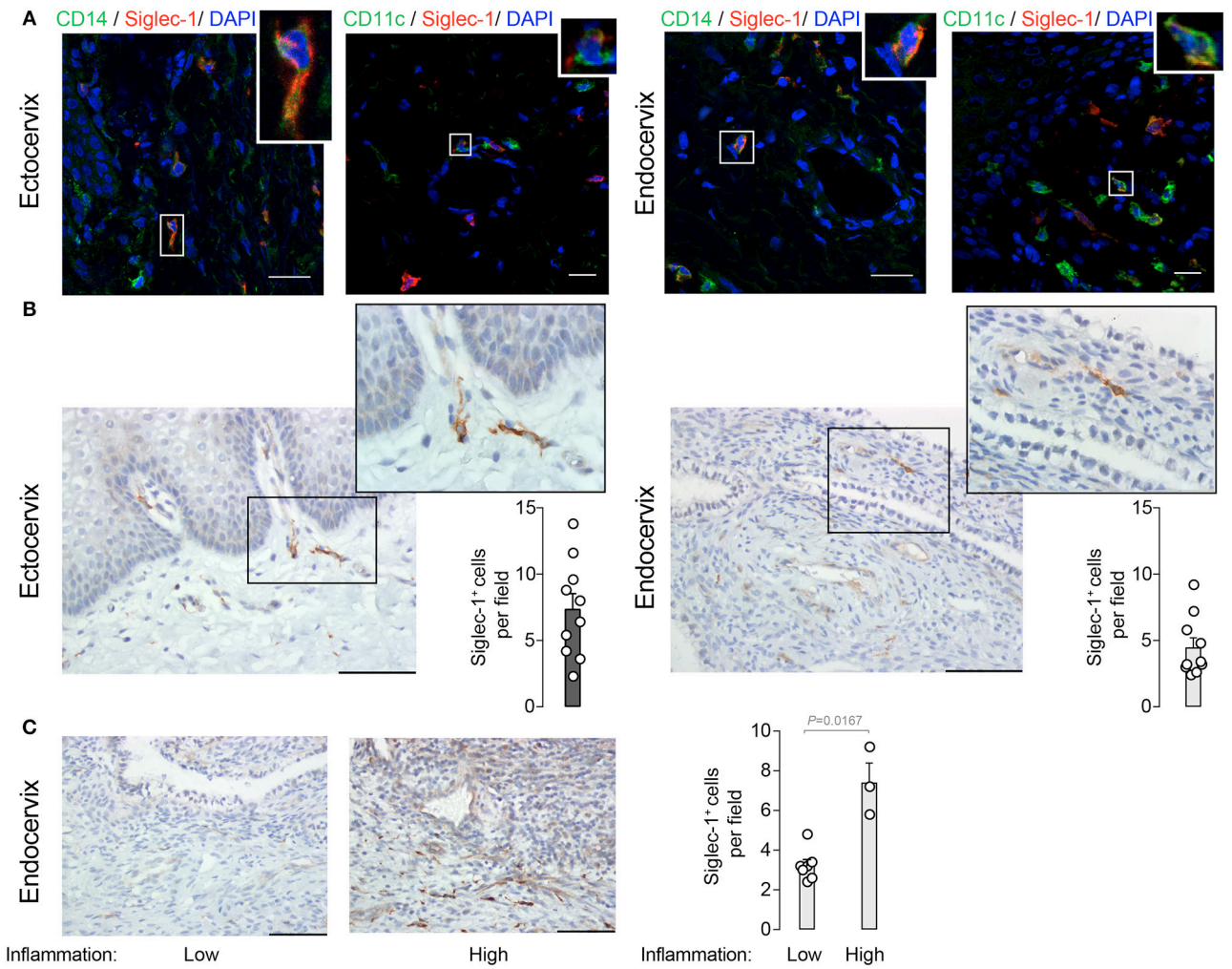

FIGURE 2 | Siglec-1$^+$ cells accumulate in the submucosa of the ectocervix and endocervix. **(A)** Representative immunofluorescent stainings on four ectocervix and endocervix of CD14 (AF488) and Siglec-1 (AF647), or CD11c (AF488) and Siglec-1 (AF647). Scale bars 20 μm. **(B)** Representative images of Siglec-1 immunostaining (40x) on 10 ectocervix and endocervix. Scale bars 100 μm. Insets show Siglec-1$^+$ cells magnification (100x). Bar graph shows the mean values and SEM of Siglec-1$^+$ cells per field counted in 5 consecutive fields. **(C)** Representative images of Siglec-1 immunostaining (40x) on 10 endocervix displaying a low and high grade of inflammation. Scale bars 100 μm. Bar graph shows the mean values and SEM of Siglec-1$^+$ cells per field counted in 5 consecutive fields of 10 endocervix with different inflammation grades. Statistical differences were assessed with a Mann-Whitney test.

DCs From Cervical Mucosa Mediate Viral Uptake via Siglec-1 and Are Detected *in vivo*

To investigate whether Siglec-1$^+$ cervical myeloid cells could capture and store HIV-1 via the interaction with this particular I-type lectin receptor, we next pulsed cervical cell suspensions processed immediately after prescribed surgery with fluorescent viruses. Cells were incubated with HIV-1 Virus-Like Particles (HIV-1$_{Gag-eGFP}$ VLPs), which lack the viral envelope glycoprotein but carry sialyllactose-containing gangliosides recognized by Siglec-1 receptor and recapitulate HIV-1 uptake results obtained with wild type viruses in myeloid cells (30, 31). After VLP exposure, cervical mononuclear cells were extensively washed and assessed by flow cytometry. Among the single-live CD45$^+$ myeloid HLA-DR$^+$ fraction, a mean of

TABLE 1 | Clinical data associated to cervical tissues where Siglec-1 was quantified by IHQ.

Patient	Siglec-1$^+$ cells per field at the Ectocervix	Siglec-1$^+$ cells per field at the Endocervix	Age (yr)	Indication for surgery	HIV status	Viral Load (copies/ml)
1	9,6	2,6	52	Prolapse	-	NA
2	2,3	3,4	53	Prolapse	-	NA
3	4,2	7,2*	45	Prolapse	-	NA
4	13,8	9,2*	52	Prolapse	-	NA
5	3,6	5,8*	60	Prolapse	-	NA
6	8,8	4,8	59	Unknown	+	<50 (ART)
7	6,4	2,4	45	Prolapse	+	<50 (ART)
8	11,6	3,2	57	Cystocele	+	140 (ART)
9	5,4	3	41	Uterine fibroids	+	400 Controller (naïve)
10	8	3,2	52	Prolapse + Cystocele	+	1.164 (naïve)

*Higher levels of inflammation were observed in these tissues**

11.8 and 3.6 % of cells expressed Siglec-1 in the ectocervix and endocervix (blue gate and bar graph, **Figure 3A**), while a 14.1 and 7 % of cells captured HIV-$1_{Gag-eGFP}$ VLPs (green gate and bar graph, **Figure 3B**). Moreover, cells not capturing VLPs lacked Siglec-1 expression (gray gate, **Figure 3C**), while cells that captured viruses were predominantly Siglec-1$^+$ in most of the tissues (purple gate and bar graph, **Figure 3D**). Viral uptake by Siglec-1$^+$ cells was further confirmed by Amnis-imaging FACS technology, which allowed microscope inspection of acquired cells and screening of Siglec-1/VLP co-localization. Amnis viral capture assays revealed that fluorescent HIV-$1_{Gag-eGFP}$ VLP localized with Siglec-1 within a virus-containing compartment (**Figure 3E**), resembling to the sac-like structures previously described in monocyte-derived DCs and activated tonsillar myeloid cells (19, 31). As HIV-$1_{Gag-eGFP}$ VLP had no viral envelope glycoprotein, observed uptake could only rely on Siglec-1 recognition of sialylated gangliosides anchored on viral membranes (26, 30). Thus, Siglec-1$^+$ myeloid cells found in the ectocervix and endocervix capture HIV-$1_{Gag-eGFP}$ VLP in a virus-containing compartment via Siglec-1 recognition of viral membrane gangliosides.

To further assess if Siglec-1$^+$ cervical cells are capable of capturing wild type HIV-1 during the natural course of infection, we next investigated the cervical biopsy of a viremic HIV-1$^+$ patient by immunostaining. Confocal microscopy analysis revealed that distinct Siglec-1$^+$ cells found in the cervical submucosa harbored viral p24 antigens (**Figure 3F).** Three-dimensional z-stack reconstructions of Siglec-1$^+$ cells from distinct tissue areas showed virus-containing compartments that were different from the p24 staining of productively infected cells lacking Siglec-1 expression **(Figure 3G** and **Movie 1**). Thus, Siglec-1$^+$ cervical cells can trap viruses throughout the course of HIV-1 infection *in vivo*.

pDCs Exposed to HIV-1 Induce Siglec-1 Expression on DCs via IFNα Secretion

Although the basal expression of Siglec-1 on cervical DCs already allows viral uptake, the level of expression of this type-I interferon inducible receptor (21) correlates with viral capture and transmission efficiency of DCs (20). Hence, right after HIV-1 infection and once the antiviral type-I IFN responses are triggered, we hypothesized that IFNα could up-regulate Siglec-1 expression on cervical DCs and enhance their viral uptake capacity. In the cervical mucosa, resident myeloid cells quickly induce type-I IFN responses after HIV/SIV infection (32). Moreover, plasmacytoid dendritic cells (pDCs), the most potent producers of IFNα, are soon recruited to the site of initial retroviral infection (33). Thus, we next assessed if IFNα secreted by HIV-1-exposed pDCs could trigger Siglec-1 expression on DCs, mimicking early events of HIV-1 pathogenesis once infection has been established and type-I IFN antiviral immunity is mounted. Blood derived pDCs were cultured alone, co-cultured with an uninfected T cell line or with an HIV-1-infected T cell line that chronically produces R5-tropic BaL viruses in the presence or absence of anti-CD4 or isotype monoclonal antibodies (mAb). HIV-1-exposed pDCs released higher IFNα compared to non-exposed pDCs (**Figure 4A**), while blockage of pDC infection with a mAb against CD4 reduced IFNα secretion compared to an isotype control (**Figure 4A**). Accordingly, Siglec-1 expression was increased on monocyte-derived DCs incubated with supernatants from HIV-1-exposed pDCs as compared to supernatants from uninfected pDCs (**Figure 4B**), and further inhibited by an anti-CD4 mAb (**Figure 4B**). Thus, HIV-1 sensing via viral fusion on pDCs induced IFNα secretion that triggered Siglec-1 expression on monocyte-derived DCs as potently as recombinant IFNα (**Figure 4C**), and was specifically inhibited by the type-I interferon blocking receptor B18R (**Figure 4C**). Moreover, HIV-1-exposed pDCs derived from pre-menopause women released significantly higher amount of IFNα than pDCs derived from men (**Figure 4D**). We also induced Siglec-1 expression with equal amounts of recombinant IFNα on monocyte-derived DCs from men and women and determined the absolute number of Siglec-1 antibody binding sites per cell (**Figure 4E**). Under these experimental conditions, however, DCs derived from women did not significantly express higher amounts of Siglec-1 than DCs derived from men (**Figure 4E**). All together, these results highlight how upon HIV-1 infection, pDC accumulation in the female genital tract could lead to an increased antiviral IFNα secretion that could limit viral infection

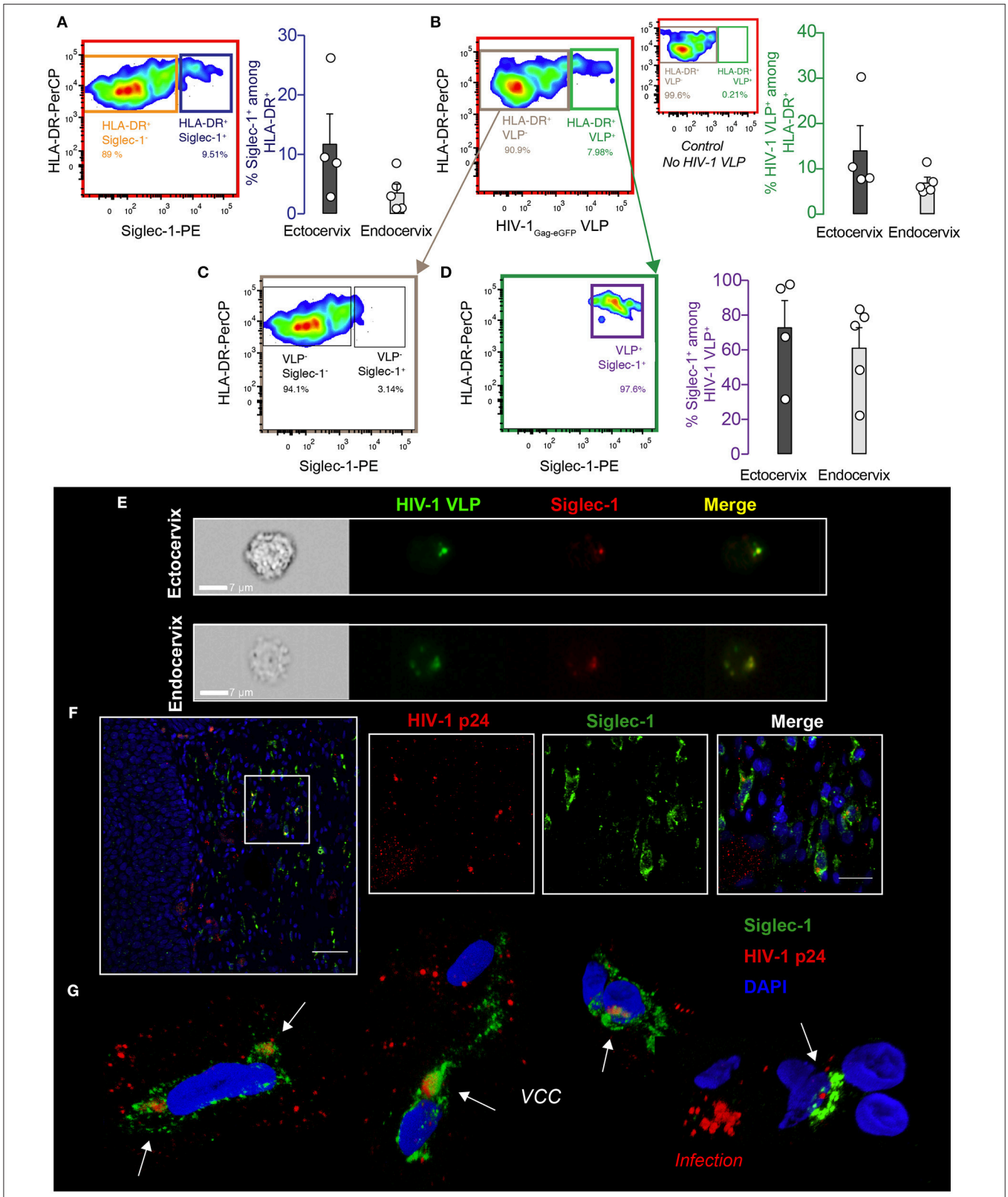

FIGURE 3 | DCs from cervical mucosa mediate viral uptake via Siglec-1 and are detected *in vivo*. **(A)** Cervical mononuclear cells isolated from the ectocervix and endocervix of benign hysterectomies were pulsed with VLPs for 18 h at 37°C, extensively washed, labeled with the indicated mAbs and assessed by FACS. Colored

(Continued)

FIGURE 3 | gates and arrows indicate populations analyzed, and corresponding matching colors are used to identify bar graphs showing frequencies of those populations. Representative dot plot and frequency of HLA-DR$^+$ and Siglec-1$^+$ cells on hematopoietic cervical cells. **(B)** Representative dot plot and frequency of cells capturing HIV-$1_{Gag-eGFP}$ VLPs among the myeloid HLA-DR$^+$ fraction. Smaller dot plot in between depicts the control without VLPs. **(C)** Representative dot plot showing reduced expression of Siglec-1 in the myeloid HLA-DR$^+$ cells not capturing HIV-$1_{Gag-eGFP}$ VLPs. **(D)** Representative dot plot of Siglec-1$^+$ cells among the cells capturing HIV-$1_{Gag-eGFP}$ VLPs. Bar graphs show mean values and SEM from the ectocervix and endocervix of 4 to 5 donors. **(E)** Images of Siglec-1$^+$ cervical cells pulsed and labeled as in **(A)**. Cells were acquired by Amnis-imaging FACS, and showed green fluorescent HIV-$1_{Gag-eGFP}$ VLPs accumulation within a sac-like virus-containing compartment enriched in Siglec-1 (labeled in red). **(F)** Paraffin-embedded cervical tissue from one viremic HIV-infected woman stained for HIV-1 p24 antigen (labeled in red), Siglec-1 (in green), and nucleus (in blue). Scale bar 50 μm. (Inset panels) zoom in of squared region with distinct fluorescences (scale bar 20 μm). **(G)** 3D volumetric x-y-z data fields reconstruction of Siglec-1$^+$ cells from four distinct areas of the cervical tissue of the viremic HIV-infected woman. Opacity representation of DAPI stained nuclei and fluorescence of the sac-like virus-containing compartment (VCC; white arrows). Right bottom image displays a characteristic cell pattern with p24$^+$ dots reflecting viral production (Infection).

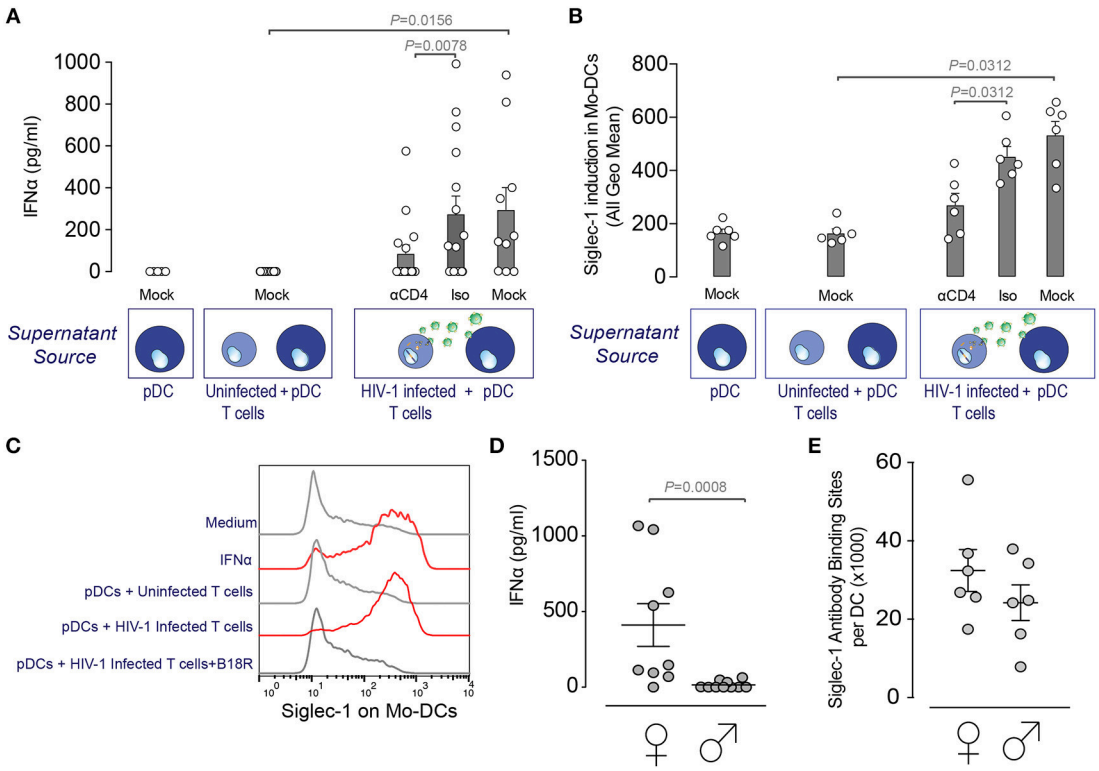

FIGURE 4 | pDCs exposed to HIV-1 induce Siglec-1 expression on DCs via IFNα secretion. **(A)** IFNα release measured by ELISA on supernatants from blood derived pDCs co-cultured 24 h alone, with an uninfected MOLT CD4$^+$ T cell line or an HIV-1 infected MOLT CD4$^+$ T cell line that chronically produces R5-tropic BaL viruses in the presence or absence of 10 μg/ml of an anti-CD4 or an isotype mAb. Bar graph shows mean values and SEM from at least 6 donors and 3 independent experiments. Statistical differences were assessed with a Wilcoxon matched-pairs signed rank test. **(B)** Induction of Siglec-1 on monocyte-derived DCs incubated with supernatants isolated from pDCs co-cultured as in **(A)** and assessed by FACS. **(C)** Representative histograms of Siglec-1 expression on monocyte-derived DCs exposed to medium, recombinant IFNα, supernatants from uninfected pDCs, supernatants from HIV-1-exposed pDCs on mock treated DCs or on DCs previously incubated with the type-I interferon blocking receptor B18R. **(D)** IFNα release measured by ELISA on supernatants of pDCs isolated from women or men and co-cultured 24 h with an HIV-1 infected MOLT CD4$^+$ T cell line. Bar graph shows mean values and SEM from 19 donors and 4 independent experiments. Statistical differences were assessed with a Mann-Whitney test. **(E)** Quantification of Siglec-1 expression levels on monocyte-derived DCs from men and women assessed by FACS. Bar graph shows mean values and SEM from 12 donors and 3 independent experiments. Prentice Rank Sum Test was used to assess statistical differences, which did not reach statistical significance ($P = 0.073$).

initially, but also promote viral capture of cervical myeloid cells via Siglec-1 induction.

IFNα Enhances Viral Capture and *Trans*-infection on Siglec-1$^+$ Cervical DCs

We next investigated if IFNα could trigger Siglec-1 expression on cervical myeloid cells and enhance their Siglec-1-mediated viral capture capacity. Small pieces of ectocervix or endocervix were cultured overnight (34), in the presence or absence of increasing concentrations of IFNα and analyzed by flow cytometry after enzymatic digestion. IFNα induced a dose-response increase in the percentage of Siglec-1$^+$ cells among the myeloid HLA-DR$^+$ CD14$^+$ CD11c$^+$ DC fraction (**Figure 5A**). Moreover, on cellular suspensions obtained from cervical tissues immediately

processed after surgery, IFNα treatment also increased the percentage of Siglec-1$^+$ cells (**Figure 5B**). This effect was more prominent in the endocervix, where basal expression of Siglec-1 was lower (**Figures 1D**, **5B**). Accordingly, IFNα treatment increased the percentage of cells capturing HIV-$1_{Gag-eGFP}$ VLP among the myeloid HLA-DR$^+$ CD14$^+$ CD11c$^+$ DC population, especially at the endocervix (**Figure 5C**). Thus, endocervical myeloid cells could mediate *trans*-infection more potently once antiviral type I IFN responses are mounted and Siglec-1 expression is triggered on DCs.

Our results suggested that α-Siglec-1 mAbs could offer protection against HIV-1 uptake and prevent dissemination mediated by cervical DCs. We therefore pre-incubated cells from the ectocervix and endocervix with an α-Siglec-1 mAb or isotype control before viral exposure (**Figure 5D**). While isotype control had no inhibitory effect, pre-treatment with α-Siglec-1 mAbs led to a reduction of HIV-$1_{Gag-eGFP}$ VLP uptake even after IFNα treatment (**Figure 5D**). Moreover, this blocking effect was further confirmed using a wild type R5-tropic HIV-1_{NFN-SX} to pulse mock treated or IFNα-treated CD3$^-$HLA-DR$^+$ cervical DCs, which were sorted and co-cultured with a reporter CD4$^+$ cell line to measure viral transfer via *trans*-infection (**Figure 5E**). Of note, at the steady state, the expression of Siglec-1 on these cells is shown on **Figure 1D**. In seven independent cervicovaginal tissues, we consistently observed a significant decrease in the levels of HIV-1 transmitted in the α-Siglec-1 mAb treated cells compared to the isotype control, which accounted for blocking a mean of 70% of the transferred infectivity by myeloid cells. Thus, at the basal state but also upon Siglec-1 induction by IFNα, the α-Siglec-1 mAb could most likely block viral binding and uptake, preventing subsequent viral transmission from myeloid cells to target cells. These results highlight that specific inhibitors against Siglec-1 can halt viral transfer and dispersion mediated by Siglec-1$^+$ cervical DCs.

DISCUSSION

A preventative strategy aimed at protecting against HIV infection must block several steps during the earliest stages of infection, avoiding not only the productive infection of primary target cells, but also the viral dissemination toward distal tissues. In both scenarios, antigen-presenting cells residing in different compartments of the female genital mucosa may play a prominent role (7, 9, 24, 35). DC-T cell conjugates represent an optimal milieu for productive HIV infection, which may boost initial viral replication of CD4$^+$ T cells (10, 36). Moreover, uninfected DCs could promote HIV dissemination to draining lymph nodes after mucosal challenge (8, 9). Yet, the precise molecular mechanism mediating these processes remained unexplored in the cervicovaginal tissue.

Here we have shown the ability of Siglec-1 to mediate viral capture and transmission to target cells on a population of cervical myeloid cells expressing HLA-DR, CD11c, CD14, and CD11b, all classical markers known to be present on DCs of the submucosa at the lower female genital tract (18, 28, 29, 37). Our findings concur with prior studies where the migratory CD3$^-$ HLA-DR$^+$ fraction or the cervicovaginal CD11c$^+$ DCs were found to be the myeloid cell subset that preferentially captured and transported HIV-1 out of the cervicovaginal mucosa (5, 16, 17). Nevertheless, we now show that on DCs of the ectocervix and endocervix, alternative receptors beyond DC-SIGN operate in HIV-1 viral uptake and transfer, as previously reported for monocyte-derived DCs (13, 15, 38–43). Our results provide a plausible mechanism for previous reports where the predominant cervical myeloid cell population that captured HIV-1 was found to be DC-SIGN negative (18), or where there was a lack of inhibition of neutralizing antibodies against DC-SIGN on viral transmission mediated by cervical myeloid cells (5). The discovery of Siglec-1 expression on myeloid cervical DCs and the capacity of Siglec-1$^+$ cells to capture viruses *in vivo* help to understand how this particular receptor can facilitate boosting of HIV-1 replication and dissemination from the genital mucosa to the corresponding draining lymph nodes in the absence of DC productive infection.

Baseline levels of Siglec-1 on myeloid cells in the lamina propria of all cervical tissues examined herein already allowed viral uptake, demonstrating that Siglec-1 could act as a viral attachment factor even in the absence of prior viral infection. However, as tissues with a high level of inflammatory infiltrate showed an increased number in Siglec-1$^+$ cells, ongoing inflammatory events triggered upon infection could magnify Siglec-1 mediated HIV-1 uptake and *trans*-infection. Indeed, in a cervical biopsy of an HIV-1 viremic woman, we found Siglec-1$^+$ cells with HIV-1-containing compartments, demonstrating that these cells actually trap viruses and form these compartments *in vivo*.

In the female genital tract, type I IFN responses from tissue resident cells represent a potent first-line of defense against many pathogens, including lentiviruses (44–47). Soon after infection, recruited pDCs sensing incoming viruses will strongly contribute to IFNα secretion (33). Here we confirmed that HIV-1 fusion on pDCs induced IFNα secretion (48, 49) and that this mechanism, which is subjected to hormonal regulation (50), was enhanced in pDCs derived from pre-menopausic women. However, gender did not affect the IFNα response that triggered Siglec-1 induction on DCs, as it had been previously reported for other interferon-stimulated genes during chronic HIV-1 infection (51). Further, we demonstrated that CD14$^+$ CD11c$^+$ DCs up-regulate Siglec-1 expression in a dose response manner after IFNα stimulation of the cervical tissue, especially at the endocervix. In contrast, higher basal expression of Siglec-1 was found on ectocervical myeloid cells at the steady state. These data suggest that while pre-existing basal immune activation at the ectocervix could already favor Siglec-1 capture of early invading viruses, endocervical cells will most likely mediate viral uptake at later time points, once antiviral type I IFN responses are mounted and Siglec-1 expression is boosted on DCs. Indeed, this could explain why we only observed an increased number of Siglec-1$^+$ cells on biopsies from the endocervix that had a high inflammatory score. Overall, detection of Siglec-1 on cervical DCs contributes to understand why the IFN system is not more effective against HIV-1 despite its substantial up-regulation early upon infection (4, 33, 52). Similarly, it may also explain why treatments inducing

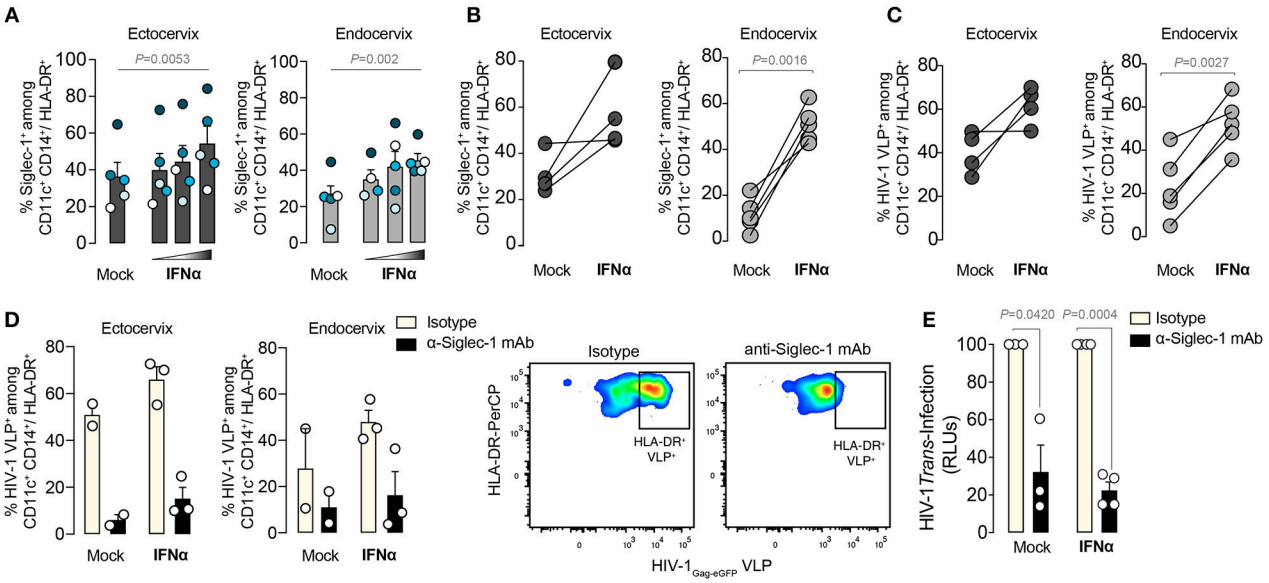

FIGURE 5 | IFNα enhances viral capture and *trans*-infection on Siglec-1$^+$ cervical DCs. **(A)** Percentage of Siglec-1$^+$ DCs within the myeloid HLA-DR$^+$ CD14$^+$ CD11c$^+$ fraction from small pieces of ectocervix or endocervix cultured in the presence or absence of increasing concentrations of IFNα (1,000; 10,000; and 100,000 IU/ml) and assessed by FACS as in **Figure 3**. Bar graph shows mean values and SEM from 5 donors and 4 independent experiments. Colors depict each particular donor. Statistical differences were assessed with a one-way repeated measures ANOVA test. **(B)** Percentage of Siglec-1$^+$ DCs within the myeloid HLA-DR$^+$ CD14$^+$ CD11c$^+$ fraction from cellular suspensions obtained from tissue digestion and cultured in the presence or absence of IFNα. Statistical differences were assessed with a paired *t*-test. **(C)** Percentage of cells capturing HIV-1$_{Gag-eGFP}$ VLPs among the myeloid HLA-DR$^+$ CD14$^+$ CD11c$^+$ fraction from cellular suspensions obtained from ectocervix or endocervix digestion and cultured in the presence or absence of IFNα. Statistical differences were assessed with a paired *t*-test. **(D)** Percentage of cells capturing HIV-1$_{Gag-eGFP}$ VLPs as in **(C)** on cells that had been previously pre-incubated with 20 μg/ml of 7D2 anti-Siglec-1 mAb or isotype control. Bar graph shows mean values and SEM from 2-3 donors. Dot plots showing representative inhibition are also depicted. **(E)** Relative R5 tropic HIV-1$_{NFN-SX}$ transmission to CD4$^+$ target cells from cervical CD45$^+$CD3$^-$CD19$^-$ HLA-DR$^+$ sorted cells pre-incubated with 20 μg/ml of isotype or anti-Siglec-1 mAbs before viral exposure. Values are normalized to isotype-treated cells (set at 100%). Statistical differences were assessed with a one sample *t*-test. Mean values and SEM from two experiments include cells from 3 or 4 donors.

pDC recruitment (53) and a strong type I IFN response (54) before vaginal challenge in macaques are incapable of limiting viral infection beyond the infected mucosa and can even enhance viral replication.

As other sexually transmitted infections such as herpes virus or chlamydia infection trigger type I IFN responses in mucosal tissues via pDC recruitment and/or bacterial lipopolysaccharide exposure (55, 56), it would be important to explore the role of Siglec-1 in favoring HIV-1 acquisition, replication and dissemination in women with pre-existing sexually transmitted infections (2). Moreover, since inflammatory CD14$^+$ CD11c$^+$ DCs are known to induce Th17 T-cell differentiation (57), and these are the preferential targets of viral infection in the cervix right after retroviral invasion (58), it would also be critical to evaluate the role of this Siglec-1$^+$ DC subset on susceptibility to HIV-1 infection.

In summary, myeloid DCs expressing a key molecule involved in HIV-1 *trans*-infection, namely Siglec-1, are found in the cervical tissues of women in the basal state. IFNα-treated myeloid DCs up-regulate Siglec-1 expression, which increases viral capture and *trans*-infection, providing an explanation of how the virus may succeed in an otherwise antiviral environment. We propose that Siglec-1$^+$ cervical DCs may facilitate HIV-1 transfer to bystander CD4$^+$ T cells and favor the nascent infection within the cervical mucosa, but also facilitate early dissemination to secondary lymphoid tissues. These findings highlight the importance of including Siglec-1 inhibitors along with potent antiretroviral agents in forthcoming microbicidal strategies, to stop not only the productive cellular infection in the cervix, but also the systemic viral dissemination from the female genital tract.

METHODS

Cervical Tissue Digestion and Immunophenotype

Human cervical tissue was obtained from women (age range 39–82 years) undergoing hysterectomy for non-neoplasic indication at either HUGTiP or Hospital Municipal de Badalona. After confirmation of healthy tissue status by the Pathology Service, a piece from ectocervix and endocervix separated by anatomical localization was delivered to the laboratory in tissue media [RPMI 1640 supplemented with 15 % FBS, 500 U/mL penicillin, 500 μg/mL streptomycin, 2.5 μg/mL Fungizone (Life Technologies), 50 μg/mL gentamicin, MEM-non essential aminoacids (Gibco) and 1 mM MEM-sodium pyruvate (Gibco)]. Tissue was processed within the next 24 h after surgery. The mucosal epithelium and the underlying

stroma of both ectocervix and endocervix were separated from muscular tissue and dissected into approximately 8-mm^3 blocks. Depending on the experiment, tissue blocks were cultured, digested or included in optimal cutting temperature compound for immunofluorescence as previously described (34).

For tissue digestion, five to eight pieces from ectocervix or endocervix were separately placed into 1.5 ml-tubes containing 5 mg/ml collagenase IV (Invitrogen) in RPMI 1640 supplemented with 5% fetal bovine serum (FBS, Gibco). Tubes were shaked at 400 rpm at 37°C for 30 min. After enzymatic digestion, tissue blocks were dissociated manually with a disposable pellet pestle in two series of 20 rotations while pulling it up and down. The suspension was filtered through a 70 μm cell strainer (SPL Life Sciences), and cells were collected at 16°C after washing with PBS. Cells were then stained with Live/Dead Aqua (Invitrogen) for 30 min at RT. Pellet was suspended in staining buffer (1% mouse serum, 1% goat serum in PBS) and stained with CD14-V450 (MØP9), CD11c-PE-Cy7 (B-ly6), HLA-DR-PerCP-Cy5.5 (G46-6), (all from BD Biosciences), CD3-eVolve 655 (OKT3) (eBiosciences), CD45-Alexa Fluor 700 (Hl30), CD11b-FiTC (M1/70), Siglec-1-PE (7-239) (all from BioLegend). Mouse IgG1-PE (BioLegend) was used as isotype control. Cells were acquired using a BD LSRFortessa SORP flow cytometer (Flow Cytometry Platform, IGTP) and analyzed with FlowJo vX.0.7 software (TreeStar).

Immunofluorescence

Cervical 5-μm cryosections were dried at RT, fixed in 3.7% formaldehyde (Sigma Aldrich) diluted in PHEM buffer as previously described in (34), permeated with 0.2% Triton X-100 (Sigma Aldrich) in PHEM buffer and blocked with 0.2% cold fish gelatin (Sigma Aldrich), 0.1% Triton X-100 and 10% normal goat serum (Gibco) diluted in PBS. Sections were first incubated with the following primary mAbs: mouse anti-Siglec-1 7-239 Ab (Abcam), rabbit anti-CD11c EP1347Y Ab (Abcam) or rabbit anti-CD14 EPR3653 Ab (Abcam). Samples were then washed extensively with PBS and incubated with the secondary mAbs Alexa 488-conjugated donkey anti-rabbit or Alexa 647-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). Sections were covered with mounting medium (ProLong™ Gold Antifade Mountant with DAPI, Life Technologies, Invitrogen) and a coverslip. Images were obtained by confocal microscopy using a Zeiss LSM 710 microscope and the Zen Blue Image acquisition software.

Immunohistochemistry

Ten cervical formalin-fixed, paraffin-embedded samples from the Pathology Department of HUGTiP were analyzed. Of note, we chose samples from five HIV-1 infected and five seronegative women, but results were equivalent regardless of the HIV status. Immunohistochemical stains were performed using a Ventana Benchmark Ultra (Ventana Medical Systems) in accordance with the manufacturer's protocol, with standard antigen retrieval (pH 9.0; Ventana) and the mAb against α-Siglec-1 (clone SP213, LS Biosciences, dilution 1/100) for 12 min. A pathologist carried out a blind quantification of Siglec-1$^+$ cells and images were captured using a DP71 digital camera (Olympus, Center Valley, PA, USA) attached to a BX41 microscope (Olympus). Siglec-1$^+$ cells in five consecutive fields in the subepithelial area were counted for ectocervix and endocervix separately. Tissues where a significant inflammatory infiltrate was detected were considered as highly inflamed.

Cell Lines

HEK-293T cells obtained from ATCC (CRL-11268) and TZM-bl (obtained through the US National Institutes of Health [NIH] AIDS Research and Reference Reagent Program) were maintained in Dulbecco's Modified Eagle Medium (DMEM). The human T cell lines MOLT CD4$^+$ uninfected and MOLT CD4$^+$ HIV-1$_{BaL}$ infected have been described elsewhere (59) and were grown in RPMI. All media contained 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin (all from Invitrogen).

HIV-1$_{Gag\text{-}eGFP}$ VLP and HIV-1 Generation

Fluorescent HIV-1 virus-like particles (HIV-1$_{Gag-eGFP}$ VLP) were generated transfecting HEK-293T cells with plasmid pGag-eGFP obtained from the NIH AIDS Research and Reference Reagent Program. Replication-competent HIV-1 stock was generated by transfecting the proviral construct NFN-SX, an HIV-1$_{NL43}$ provirus that expresses the HIV-1$_{JRFL}$ envelope glycoprotein (kindly provided by W. O'Brien). Thirty micrograms of plasmid DNA were added to cells in T75 flasks, and transfection was performed using a calcium phosphate kit (CalPhos, Clontech). Forty-eight hours post-transfection, supernatants were harvested, filtered (Millex-HV, 0.45 μm; Millipore) and frozen at −80°C until use. The p24^{Gag} content of the VLP and HIV-1 stock was determined by ELISA (PerkinElmer). TCID$_{50}$ or 50% tissue culture infective doses of HIV$_{NFN-SX}$-infection used for *trans*-infection assays were determined by end-point dilution culture on the cell line TZM-bl, which contains an HIV long terminal repeat linked to a luciferase reporter gene.

VLP Uptake Assays

1×10^6 digested ectocervical and endocervical cells were pre-incubated for 15 min at RT with 20 μg/ml of an anti-Siglec-1 mAb (7D2) (Abcam), a murine IgG1 κ isotype control (BD Pharmigen) or left untreated. Cells were pulsed overnight with 2.7- 20 ng p24^{Gag} HIV-1$_{Gag-eGFP}$ VLP at 37°C in a 5% CO_2 incubator in the presence or absence of 1,000 IU/ml of recombinant Interferon-2α (Sigma-Aldrich) in 10% FBS RPMI with antibiotics. After extensive washing, cells were stained as previously described and acquired using a BD LSRFortessa SORP flow cytometer (Flow Cytometry Platform, IGTP) and analyzed with FlowJo v10.3 software (TreeStar).

For imaging flow cytometer analyses, 1×10^6 digested ectocervical and endocervical cells were pulsed with HIV-1$_{Gag-eGFP}$ VLP as previously described. After extensive washing, cells were resuspended in PBS with 1:250 Live/Dead Aqua (Invitrogen) and incubated for 30 min at RT. Cells were then fixed and permeabilized (Fix & Perm; Invitrogen), and stained with an anti-Siglec-1-PE mAb (7-239) (BioLegend). Cells were acquired with an Amnis ImageStreamX imaging flow cytometer (Merck), and analyzed using IDEAS v6.1 software. A gradient root mean

square or RMS value > 40 was established as the best focus threshold, and single cells were selected in the Area vs. Aspect Ratio dot plot of the bright field channel.

HIV-1 p24 Immunostaining

A cervical formalin-fixed, paraffin-embedded sample from a viremic patient diagnosed with sexually transmitted HIV-1 infection a decade ago was obtained from the Pathology Department of the University Hospital Vall d'Hebron. The sample was collected when the patient had between 5,160 and 10,400 HIV-1 RNA copies/ml in blood. Samples were de-waxed and placed in decreasing ethanol concentrations. Heat-induced epitope retrieval was performed in EDTA buffer pH 9 (Abcam) in a water bath at 100°C during 10 min. Slides were permeabilized with Tris-bufferd saline 1X (TBS) (Fisher scientific) with 0.1% Triton X-100 and 1% BSA (Sigma-Aldrich) for 10 min. Subsequently, blocking was performed with TBS 1x supplemented with 10% donkey serum (Jackson Immunoresearch) and 1% BSA for 2h. Mouse anti-p24 mAb (Kal-1; Dako-Agilent) was incubated overnight at 4°C. Later, rabbit anti-Siglec-1 mAb (SP213; Lifespan Bioscience) was incubated for 15 min at RT. Samples were then stained with secondary antibodies Donkey AlexaFluor 647 anti-mouse (Invitrogen) and Donkey AlexaFluor 488 anti-rabbit; counter stained with DAPI (Thermo Fisher) and mounted with Fluoromount G (eBioscience). Samples were imaged on an Olympus Spectral Confocal Microscope FV1000 using a 20x and 60x phase objective and sequential mode to separately capture the fluorescence at an image resolution of 800 × 800 pixels. Image J software was used for image processing. Alternatively, samples were acquired in z-stacks every 0.2 μm on a Zeiss LSM 780 confocal inverted microscope with an apochromatic 63x oil (NA = 1.4) and processed with Volocity software using the 3D Opacity module for reconstruction.

Primary Cell Cultures

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of the *Banc de Sang i Texits* of Barcelona by Ficoll-Hypaque density gradient centrifugation (Alere Technologies AS). Plasmacytoid DCs were negatively isolated using magnetic beads from the Plasmacytoid Dendritic Cell isolation kit (Miltenyi Biotech) and immediately used for experiments. Monocytes were isolated using CD14$^+$ selection magnetic beads (Miltenyi Biotech) and differentiated into monocyte-derived DCs with 1,000 IU/ml of granulocyte-macrophage colony-stimulating factor plus 1,000 IU/ml of Interleukin-4 (both from R&D) during 5 days before supernatant exposure. Cells were maintained in RPMI supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

IFNα Release on Supernatants From pDCs and Siglec-1 Induction

A total of 0.1×10^6 pDCs were co-cultured with 0.1×10^6 HIV-1_{BaL}-infected MOLT-4 cells for 24 h at 37°C. Before co-culture, some pDCs were also pre-treated with 10 μg/ml of anti-CD4 mAb (clone RPA T-4) to avoid viral fusion or with an isotype mAb control (both from Beckton Dickinson) for 10 min at RT. As a negative control, pDCs were co-cultured with an uninfected MOLT-4 cell line. After 24 h of co-culture, supernatants were collected and assessed for IFNα production with VeriKine Human IFN Alpha Elisa Kit (pbl Assay Science). Alternatively, supernatants from these co-cultures were transferred to 0.2×10^6 DCs to assess Siglec-1 induction 24 h later with a FACSCalibur, labeling cells with a mAb anti-Siglec-1-PE or a matched isotype-PE control (both from AbD Serotec). Of note, these supernatants were also added to DCs that had been previously incubated with 2 μg/ml of carrier-free recombinant B18R protein (eBioscience) to block type I IFN receptor. DCs were also cultured in the presence of RPMI media or 1,000 IU/ml of recombinant Interferon-2α. The mean number of Siglec-1 Ab binding sites per monocyte-derived DC from men and women was obtained with a Quantibrite kit (Becton-Dickinson) as previously described (13).

Cervical Tissue Stimulation With IFNα

After dissection of the tissue as previously described, five pieces from ectocervix or endocervix were separately placed into a 12-well plate containing 1 ml of tissue culture medium. Interferon-2α was added to the medium at 1,000; 10,000 or 100,000 IU/ml. After 24 h at 37°C in 5% CO_2, tissue was digested and the remaining culture plate was treated with accutase (Thermo Fisher Scientific) for 30 min at 37°C to detach adherent cells. Finally, tissue and adherent cells were pooled together and stained for flow cytometry as previously described.

Trans-infection Experiments

Ectocervical and endocervical blocks from HIV-1 non-infected donors were left untreated or incubated overnight with 10,000 U/ml of IFNα and 100 ng/ml of CCL19 (Mip-3β). Tissue was then digested, pooled together and stained with mAbs as previously described to sort single CD45$^+$CD3$^-$CD19$^-$HLA-DR$^+$ live cells by FACS. Recovered cells were pre-incubated with anti-Siglec-1 7D2 or isotype control mAbs for 10 min at RT. Cells were subsequently incubated with 185 ng of p24/ml of an R5 tropic HIV-1_{NFN-SX} (with an estimated $TCID_{50}$ of 116.824) in the presence of 20 μg/ml of the indicated mAbs for 4 h at 37°C. After extensive washes, myeloid cells were co-cultured with the reporter TZM-bl cell line at a 1:1 ratio for 48 h. Luciferase activity was measured with Britelite plus (Perkin Elmer) in a Synergy MX luminometer (Biotek).

Statistical Analysis

Data are reported as the mean and the standard error of the mean (SEM) for each condition. We analyzed mean changes using Mann-Whitney test, Wilcoxon matched-pairs signed rank test, paired *t*-test, and one-way repeated measures ANOVA test as indicated, which were considered significant at $P < 0.05$. Significant mean changes from 100% of the data normalized to percentages were assessed with a one sample *t*-test, considered significant at $P < 0.05$. Gender main effect inference across multiple experiments was assessed using the Prentice Rank Sum Test, a generalized Friedman rank sum test with replicated blocked data. All analyses and figures were generated with the GraphPad Prism v7 Software and R v3.5.

ETHICS STATEMENT

Informed written consent was obtained from all participants and the study protocols were approved by the University Hospital Germans Trias i Pujol (HUGTiP, Badalona, Spain) and the Vall d'Hebron University Hospital (HUVH, Barcelona, Spain) Clinical Research Ethics Committee (reference numbers PI-14-070 and PR (IR)294/2017). The study was undertaken in accordance with the Declaration of Helsinki and the requirements of Good Clinical Practice.

AUTHOR CONTRIBUTIONS

DP-Z, JC-P, NI-U, and MG conceived and designed the experiments. AH-G, JC, GT, JG, and AT obtained, processed, and analyzed tissues. DP-Z, JC-P, IE, SB, MP, CS-P, and VA-S performed the experiments. DP-Z, JC-P, IE, SB, MP, CS-P, JC, GT, MJB, JM-P, NI-U, and MG analyzed and interpreted the data. DP-Z, JC-P, NI-U, and MG wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00825/full#supplementary-material

Movie 1 | 3D volumetric x-y-z data field reconstruction of a Siglec-1$^+$ cell from the cervical tissue of a viremic HIV-infected woman. Opacity representation of DAPI stained nuclei and fluorescence of the sac-like virus-containing compartment along with Siglec-1 staining.

Supplemental Figure 1 | Representative histograms of HLA-DR expression comparing the Siglec-1 positive and negative populations on CD3$^-$ CD11c$^+$ CD14$^+$ cells from the ectocervix and endocervix. Bar graphs show the geometric mean fluorescence values and SEM of HLA-DR expression from 14 donors. Statistical differences were assessed with a Wilcoxon matched-pairs signed rank test.

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Myeloid Cell Crosstalk Regulates the Efficacy of the DNA/ALVAC/gp120 HIV Vaccine Candidate

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Vaccination with DNA-SIV + ALVAC-SIV + gp120 alum results in inflammasome activation, high levels of IL-1β production, emergency myelopoiesis, and the egress of $CXCR4^+$ $CD14^+$ pre-monocytes from bone marrow. Previously we have shown that this vaccine-induced innate monocyte memory is associated with decreased risk of SIV_{mac251} acquisition. Because IL-1β also promotes the propagation of monocyte-derived suppressor (M-MDSC)-like cells, here we extended our analysis to this negative regulator subset, characterizing its levels and functions in macaques. Interestingly, we found that DNA prime engages M-MDSC-like cells and their levels are positively associated with the frequency of $CD14^+$ classical monocytes, and negatively with the levels of $CD16^+$ monocytes, correlates of decreased and increased risk of SIV acquisition, respectively. Accordingly, M-MDSC frequency, arginase activity, and NO were all associated with decrease of CD8 T cells responses and worse vaccination outcome. DNA vaccination thus induces innate immunity by engaging three subsets of myeloid cells, M-MDSCs, $CD14^+$ innate monocyte memory, and $CD16^+$ monocytes all playing different role in protection. The full characterization of the immunological space created by myeloid cell crosstalk will likely provide clues to improve the efficacy of HIV vaccine candidates.

Keywords: MDSC, trained immunity, myeloid cells, HIV/SIV, vaccine

INTRODUCTION

Immature myeloid cells with a potent inhibitory effect on immunity, including granulocytes, macrophages, and dendritic cells, have been described in humans, macaques, and mice. In recent years, myeloid-derived suppressor cells (MDSCs) have emerged as a major immunosuppressive non-lymphoid population, often linked to immune evasion and unfavorable disease outcome in tumors and infections including HIV (1, 2).

MDSCs are a highly heterogeneous population that includes cells that are morphologically and phenotypically similar to monocytes (monocytic M-MDSCs) and neutrophils (polymorphonuclear PMN-MDSCs) (3). While the nomenclature and phenotypes used to categorize these cell populations vary, human MDSCs are generally defined as cells negative for the expression of MHC class-II HLA-DR and positive for CD33 and CD11b expression. The CD14 or CD15 phenotypic markers are, respectively, used to differentiate between MDSCs derived from monocytes or neutrophils (4).

MDSCs regulate the homeostasis of inflammatory processes (5) and accumulate during unresolved inflammation (6). It is currently unknown whether MDSCs are immature myeloid precursors whose differentiation is blocked during emergency myelopoiesis, or if they are the product of monocyte and neutrophil reprogramming following TLR-signaling and cytokine stimulation (7). The induction of MDSCs is thought to require a combination of long-lasting antigen presentation and strong signals such as growth factors GM-CSF, G-CSF, and other cytokines including IFN-γ, IL-1β, IL-4, IL-6, IL-13, and TNF-α (8–11). The best-known transcription factor regulating MDSC expansion and activity is the signal transducer and activator of transcription 3 (*STAT3*). *STAT3* promotes MDSC survival and blocks their differentiation into mature myeloid cells (12, 13).

MDSCs use a variety of immunosuppressive mechanisms in which the metabolism of the conditionally essential amino acid L-arginine (L-arg) plays a central role. L-arginine can be metabolized by arginase (ARG1 and ARG2), which expression is controlled by *STAT3* (14), and by nitric-oxide synthase 2 (NOS2/iNOS). Both ARG and NOS compete for L-arginine and generate either urea, or citrulline and nitric oxide (NO), respectively (15). In turn, the depletion of extracellular L-arginine and urea production affect the function of the CD3 TCR zeta chain (16). Nitric oxide is one of the most versatile components of the immune system, and numerous immune cells produce and respond to NO (17). NO increases MDSC recruitment in inflammatory sites, inhibits cell proliferation by nitrosylation of receptors, promotes T cell death, and, in the presence of IL-1β, IL-6, IL-23, and TGF-β, favors the development of $CD4^+$ T helper producing IL-17 (Th17) and T regulatory cells (Tregs) (18, 19). In addition, MDSCs mediate immunosuppression through reactive oxygen species (ROS), and other mediators such as IL-4 receptor-α (IL-4Rα), programmed death-ligand 1 (PD-L1), interleukin-10 (IL-10), tumor growth factor-β (TGF-β), and phosphorylated *STAT3* (14, 20). While the role of MDSCs in the modulation of T cell responses has been extensively studied, their role in B cell suppression remains poorly understood. Studies have shown MDSCs to both directly regulate B lymphopoiesis (21) and indirectly modulate B cells by generating B regulatory cells (Bregs) (22).

During viral infections, MDSCs or MDSC-like cells suppress $CD4^+$ and $CD8^+$ T cells proliferation, migration, and function. In addition, a few reports have also described the ability of M-MDSCs to suppress B cell responses (23). MDSCs act as a double-edged sword in HIV/SIV infection (24, 25) by suppressing anti-viral specific immune responses (1, 26), while also antagonizing immune activation (27–29). *Ex vivo* MDSCs derived from HIV-infected patient blood inhibited polyclonal and antigen-specific $CD4^+$ and $CD8^+$ T cell proliferation and IFN-γ production, but increased $FoxP3^+$ $CD4^+$ Treg differentiation (18). Interestingly, stimulation of PBMCs with the purified HIV envelope glycoprotein 120 (gp120) *in vitro* induced functional MDSCs capable of suppressing T-cell proliferation (30).

Less is known of the role that vaccination plays in inducing MDSCs, or what effect these cells have on protection. Two recent studies in macaques have shown that MDSCs are induced by influenza and HIV vaccines. Indeed, an mRNA vaccine encoding for influenza hemagglutinin administered in macaques induced both suppressive M-MDSCs (HLA-DR^- $CD14^+$ cells) and non-suppressive myeloid cells in blood and at the injection site (31). Moreover, a peptide-prime/modified vaccinia Ankara (MVA) boost vaccine regimen induced MDSC-like cells ($CD33^+$ $CD11b^+$ $CD14^+$ DR^{low} cells) and was associated with set-point viral load, suggesting a negative role for M-MDSCs in protection against high viral replication (26).

We previously demonstrated that innate monocyte memory mediated by classical monocytes (HLA-DR^+ $CD14^+$ $CD16^-$ cells) is central to the protection elicited by a DNA-SIV + ALVAC-SIV + gp120 alum vaccine administered in macaques (32). While the levels of vaccine-induced classical monocytes and *NLRP3* inflammasome activation were correlated with reduced risk of SIV_{mac251} acquisition (protective), $CD16^+$ monocytes and *STAT3* were correlates of increased risk of SIV acquisition (harmful). Given that *STAT3* and IL-1β all result in MDSC accumulation, we studied the kinetics and function of this immunosuppressive subset and its role in protection in macaques vaccinated with the DNA-prime + ALVAC + gp120 boost strategy. Due to the considerable diversity of phenotypic markers used to define human MDSCs (33), we extended the characterization of these cells to include HLA-DR^- $CD14^+$ monocytes in addition to the canonical $CD33^+$ $CD11b^+$ HLA-DR^-CD14^+ cell subset. Indeed, circulating monocytes expressing the monocytic $CD14^+$ marker but lacking the expression of MHC class II cell surface receptor HLA-DR have also been identified as major mediators of tumor-induced immunosuppression (13, 34).

Our results demonstrate that the DNA-SIV + ALVAC-SIV + gp120 alum regimen increases the levels of M-MDSC-like cells (HLA-DR^- $CD14^+$ cells) that are associated with an increased risk of SIV_{mac251} acquisition. The frequency of MDSCs and their transcriptome were associated with a reduction of interferon-stimulated genes (ISGs) and T and B cell pathways. Moreover, we found that an increase in arginase activity was inversely associated with protective classical monocytes and *NLRP3*. Arginase activity was instead positively associated with harmful $CD16^+$ monocytes and, in turn, with a decrease in gag-specific IFN-γ^+ and TNF-α^+CD8^+ T cell responses, and increased risk of SIV_{mac251} acquisition. These results unravel complex mechanisms of vaccine-induced protective immunity through the crosstalk between activating and suppressive myeloid cells.

MATERIALS AND METHODS

Animal Study and Challenge

The study was conducted as previously described (32). All animals used in this study were colony-bred rhesus macaques (*Macaca mulatta*) provided by Covance Research Products. Monkeys were housed and handled in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International, and the care and use of the animals complied with all relevant institutional (U.S. National Institutes of Health) guidelines. The protocol (AUP 491) was approved by the Advanced BioScience Laboratories Institutional Animal Care and Use Committee.

Twelve juvenile macaques were immunized intramuscularly twice with DNA-SIV at weeks 0 and 4 (**Figure 1A**) as previously described (35). Each immunization contained a total of 6 mg of DNA in 1.5 ml PBS. DNA primed animals were given the following DNA constructs: 206S SIV p57gagmac239 (1 mg); 209S MCP3-p39gagmac239 (1 mg); 221S $SIV_{macM766}$ gp160 (2 mg); 103S LAMP-Polmac239 (2 mg). At weeks 12 and 24, all macaques were boosted with intramuscular inoculations of 10^8 p.f.u. of ALVAC recombinants (vCP2432), expressing SIV_{mac251} gag-pro and gp120TM (Sanofi Pasteur), and with 200 μg each of $SIV_{mac251-M766}$ and $SIV_{smE660-CG7V}$ gp120-gD proteins adjuvanted in alum alhydrogel (InvivoGen), as previously described (36). The proteins were administered intramuscularly in the thigh opposite the one of the ALVAC injection site. In addition to the 12 vaccinated animals, 6 concurrent control animals were treated with the alum adjuvant at weeks 12 and 24. Four weeks after the last immunization (week 28), the 12 immunized macaques and 6 control animals were challenged intrarectally with 10 repeated low-doses of pathogenic SIV_{mac251} (120 TCID50, 50% tissue culture infective dose) once a week. Thirty-five non-contemporaneous controls, challenged with the same virus stock in the same facility and following the same procedures, were added to the 6 concurrent controls as previously described (32). The time of acquisition was identified as the number of exposures to SIV_{mac251} prior to the detection of SIV-RNA in plasma.

Measurement of SIV Viral DNA in Rectal Tissue

SIV_{mac251} DNA was quantified in mucosal tissues collected 2–3 weeks after viral infection. Genomic DNAs were isolated from tissues and the absolute quantitation of pro-viral DNA load was assessed by a real-time qPCR assay with sensitivity up to 10 copies $\times 10^6$ cells, as previously described (37).

FACS Staining

Identification of M-MDSCs was performed together with monocyte subsets. PBMCs (5–10 $\times 10^6$ cells) were stained with the following antibodies: CD3 (clone SP34-2; BD Biosciences; Catalog #563916, 1.0 μl) and CD20 (clone 2H7; BD Biosciences; Catalog #560735, 1.0 μl), both in PE-Cy7, and NHP-CD45-BV786 (clone D058-1283; BD Biosciences; Catalog #563861, 3.0 μl), CD14-APC (clone M5E2; BD Biosciences; Catalog# 561390, 7.5 μl), CD16-FITC (clone 3G8; BD Biosciences; Catalog #555406, 5.0 μl), HLA-DR-APC-Cy7 (clone L243; BioLegend; Catalog #307618, 4.0 μl), CD11b-Pe-Cy5 (clone ICRF44; BioLegend; Catalog #301308, 0.0625μl), CD33-PE (clone AC104.33; Miltenyi Biotec; Catalog #130-091-732, 5.0 μl), CD192 (CCR2)-BV421 (clone 48607; BD Biosciences; Catalog #564067, 3.0 μl), and CD184 (CXCR4)-PE-CF594 (clone 12G5; BD Biosciences; Catalog #562389, 5.0 μl). Aqua LIVE/DEAD kit (Invitrogen; Catalog #L34966, 3.0 μl) was used to exclude dead cells. For this identification panel, myeloid cells were gated as $CD45^+Lin^-$ (CD3 & CD20). Monocyte populations were identified and classified by the expression of CD14 and CD16 as Classical monocytes: $Lin^-CD45^+CD14^+CD16^-HLA\text{-}DR^+$; Intermediate: $Lin^-CD45^+CD14^+CD16^+$ HLA^-DR^+; and Non-Classical: $Lin^-CD45^+CD14^-CD16^+HLA\text{-}DR^+$. Flow cytometry acquisition was performed on an LSRII (BD Biosciences) with a minimum of 500,000 events recorded. Marker expression was examined using FACSDiva software (BD Biosciences) and further analyzed using FlowJo v10.1 (Treestar, Inc., Ashland, OR).

Kynurenine and Tryptophan Plasma Levels

Tryptophan and Kynurenine plasma concentrations were measured by using the Tryptophan ELISA (Rocky Mountain Diagnostics, Colorado Springs, CO, USA, Catalog #BA E-2700) and Kynurenine ELISA commercial kits (Rocky Mountain Diagnostics, Colorado Springs, CO, USA, Catalog #BA E-2200). For tryptophan measurement, 20 μl of plasma were precipitated, the recovered supernatants were derivatized, and the product was used to perform the ELISA according to manufacturer instructions. For kynurenine assay, 10 μl of plasma were acylated and used to perform the ELISA according to manufacturer instructions. The data are presented as the ratio between kynurenine and tryptophan (Krn/Try) levels.

Intracellular Staining

PBMCs (1–3 $\times 10^6$ cells) were stimulated with 2 μg ml^{-1} of the cognate peptide pools for 6 h in RPMI containing 10% human serum in the presence of 5 μg ml^{-1} of GolgiPlug (10 μg ml^{-1}, BD Biosciences). Negative controls received an equal concentration of DMSO. Cells were stained with the following surface marker-specific antibodies for 30 min at 4°C: APC Cy7 anti-CD3 (SP34.2; BD Biosciences), BV421 anti-CD4 (OKT4; BioLegend), and CD8-BV570 (clone RPA-T8; BioLegend). Following fixation and permeabilization, cells were stained with ECD anti-CD69 (clone TP1.55.3; Beckman Coulter), PE anti-IL-2 (MQ1-17H12; BD Biosciences), IFNγ-APC (B27; BD Biosciences), and FITC anti-TNFα (Mab11; BD Biosciences). The Aqua LIVE/DEAD kit (Invitrogen) was used to exclude dead cells. Samples were acquired on an LSRII flow cytometer and analyzed using FlowJo version 9.6.3 (Treestar, Inc.).

Luminex

Cryopreserved supernatants were analyzed using three MILLIPLEX Non-Human Primate Multiplex assays (EMD Millipore). The following targets were assayed following the manufacturer's instructions: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, IFN-γ, MCP-1, MIP-1a

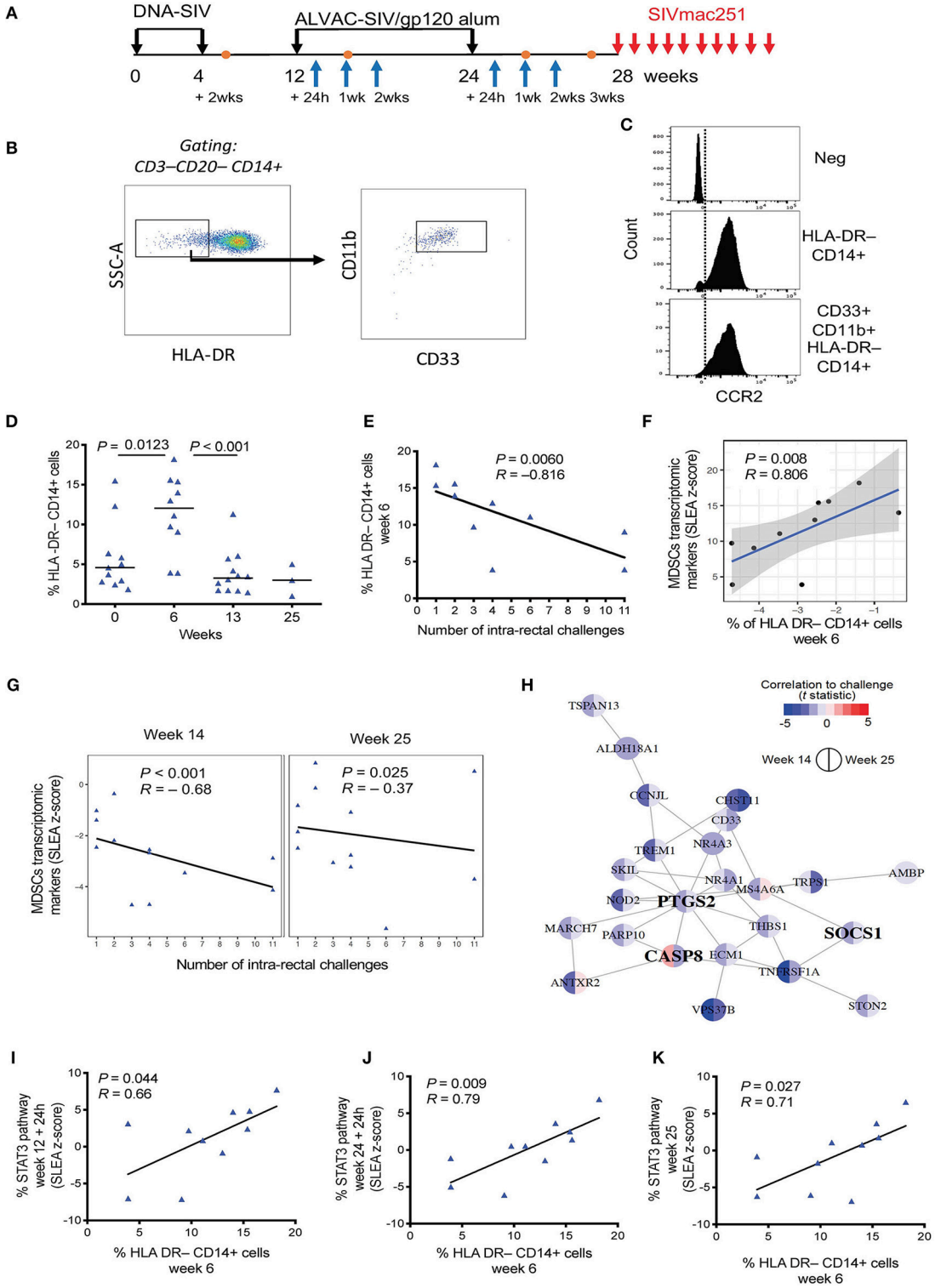

FIGURE 1 | (A) Vaccine strategy. Orange dots indicate the collection of PBMCs, and blue arrows indicate collection of whole blood for microarray analysis. **(B)** Phenotypic identification of M-MDSC (CD33$^+$ CD11b$^+$ HLA-DR$^-$ CD14$^+$) and MDSC-like cell (HLA-DR$^-$ CD14$^+$) subsets in the blood of macaques. Data obtained from a naïve, non-vaccinated animal is shown. **(C)** Histogram showing CCR2 positivity in the HLA-DR$^-$ CD14$^+$ population and CD33$^+$CD11b$^+$ HLA-DR$^-$ CD14$^+$ cells. The isotype control is shown in the first panel. **(D)** Percentage of HLA-DR$^-$ CD14$^+$ cells in the blood of vaccinated macaques collected before vaccination and

(Continued)

FIGURE 1 | after each immunization (week 0, $n = 11$; week 6, $n = 10$; week 13, $n = 12$; week 25, $n = 4$). Mean and standard error are shown for each group. **(E)** Correlation between the level of HLA-DR$^-$ CD14$^+$ cells at week 6 in 10 vaccinated animals and the number of challenges to infection. **(F)** Scatterplot showing the levels of MDSC transcriptomic markers at week 14 as a function of HLA-DR$^-$ CD14$^+$ measured at week 6. Sample enrichment analysis was used to average the expression of MDSC transcriptomic markers. Spearman correlation and *t*-test was used to find the correlation between MDSC transcriptomic markers and HLA-DR$^-$ CD14$^+$ cells. **(G)** Scatterplot representing the average expression of MDSC transcriptomic markers at 2 weeks after the 1st ALVAC boost (week 14) and 1 week after the second ALVAC boost (week 25) as a function of the number of SIV challenges to infection for all 12 vaccinated animals. Linear regression (black line) and the Spearman correlation are shown on each plot. **(H)** MDSC transcriptomics markers associated with increased risk of SIV acquisition following challenge (i.e., leading edges of the GSEA analysis) were used as input to infer gene-to-gene network using the GeneMANIA application. Each node corresponds to an MDSC marker correlated with acquisition at week 14 or/and week 25. The color of the node is proportional to the t statistic testing that the correlation between the gene and challenge is different from zero. Edges are inferred by GeneMANIA based on co-expression, co-localization, genetic interaction or physical protein-protein interactions. MDSC markers that are interferon-stimulated genes (ISG) are labeled in bold. **(I–K)** Sample level enrichment analysis of *STAT3* pathways showing positive correlation with the levels of HLA-DR$^-$ CD14$^+$ cells at week 6.

(PRCYTOMAG-40K-11), IL-21, IL-22, IL-23, RANTES (PRCYT2MAG-40K-04), and TGF-β (TGFBMAG-64K). After thawing the samples on ice, 25 μl of each supernatant was briefly loaded into the well and mixed with 25 μl assay buffer and 25 μl magnetic beads. The plates were incubated under agitation at 4°C for 18 h. After washing, 25 μl of detection antibody were added to each well and incubated for 1 h at room temperature (RT). Next, 25 μl streptavidin-PE was added to each well and incubated for 30 min at RT. Finally, wells were washed and 150 μl sheath fluid was added. Samples were acquired on a Bio-Plex 200 System (Bio-Rad).

Arginase Activity

Arginase activity was analyzed on Plasma using the Arginase Activity Assay Kit (MAK112, Sigma-Aldrich, St. Louis, MO) following the manufacturer instructions. Briefly, samples were thawed on ice and, in order to deplete the urea, 50 μl of plasma were loaded in an Amicon®Ultra 10K centrifugal filter (UFC501096 EMD Millipore), diluted with pure water to 500 μl, and centrifuged at 13,000 × g for 30 min at 4°C. Following centrifugation, the eluted solution was discarded. Filtered samples were then diluted with pure water to 500 μl, and centrifuged at 13,000 × g for 30 min at 4°C. At the end of centrifugation, the remaining volume of each sample was measured, and ultra-pure water was added to reach a final volume of 40 μl. Each sample was loaded into 2 wells of a 96-well plate (20 μl/well), representing the sample well and the sample blank well, and 20 μl/well of ultra-pure water were added to each well. Together with samples, the plate was loaded with urea standard and water as positive and negative controls, respectively. Samples were loaded in singlicate , whereas controls were loaded in duplicate. Ten microliter of 5X substrate buffer, composed of Arginine Buffer and Mn Solution, were added to the wells except for sample blank wells, and they were incubated for 120 min at 37°C. Following the incubation, 200 μl of Urea Reagent, composed of Reagents A and B, was added to each well to stop the reaction. Finally, 10 μl of 5X Substrate Buffer was added to the sample blank wells to have the same reagents proportion of reagents as the Sample Wells. After mixing, the plate was incubated for 60 min at RT, and finally acquired with microplate spectrophotometer Power Wave XS2 (BioTek Instruments, Winooski, VT) to measure the absorbance at 430 nm (A_{430}) of each well. The arginase activity was determined per the following equation.

$$Arginase\ Activity = \left[\frac{(A_{430}\ sample\ well) - (A_{430}\ sample\ blank\ well)}{(A_{430}\ urea\ standard) - (A_{430}\ water)}\right] \times \left[\frac{1\text{mM} \times 50 \times 10^3}{40\mu l \times 120\ \text{min}}\right]$$

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) Analysis

The total free radical contents were analyzed on Plasma and mucosal cell supernatants using the OxiSelect™ *in vitro* ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA, Catalog #STA-347) following manufacturer instructions. Briefly, cryopreserved samples were thawed in ice, and insoluble particles were removed by centrifuging at 10,000 g for 5 min. Following this, 50 μl of standards, plasma diluted 1:5 with PBS, or undiluted mucosal cell supernatants were single-loaded in a 96-well plate suitable for fluorescent measurement. To each well was then added 50 μl of Catalyst, incubated for 5 min at room temperature, and then 100 μl of dichlorodihydrofluorescein (DCFH) solution. The plates were incubated at RT for 30 min in the dark and the fluorescence was read using a plate reader at 480 nm excitation/530 nm emission (VictorX4, Perkin Elmer, Inc., Waltham, MA, USA). The ROS/RNS content of each sample was determined by interpolation of unknown samples with a standard curve generated with hydrogen peroxide. For plasma samples, the standard curve was generated by diluting the standards with PBS. For mucosal cell supernatants, the standard curve was generated by diluting the standards with R10 media.

Gene Expression Array Analysis

Twelve macaques vaccinated with DNA prime and ALVAC/gp120 alum boost were included in a gene expression profiling study. PreAnalytiX tubes (#762165) were used to collect 2.5 ml of whole blood from these animals at 24 h and 2 weeks after the 1st boost or 1 week after the second boost. Paxgenes were gently rocked for 2 h and then stored at −80°C. Total RNA was extracted using Agencourt RNAdvance Blood Kit (Beckamn Coulter #A35604). The isolated total RNA was checked for quantity and quality using a NanoDrop 2000c (Thermo Fisher Scientific) and an automated electrophoresis

system (Experion, Bio-Rad). Samples with an RQI classification ≥7.0 were selected to proceed downstream to amplification. Samples were normalized at 50 ng for input and amplified using Illumina TotalPrep RNA amplification kits (Ambion) according to the manufacturer's protocol. Microarray analysis was conducted using biotinylated cRNA hybridized to Human HT-12 version 4 BeadChips (Illumina). The arrays were scanned using iSCAN (Illumina) and quantified using Genome Studio (Illumina). Analysis of the Genome Studio output data was conducted using R/Bioconductor software packages. Bead arrays were read, and missing values (>0.01%) were imputed using the nearest-neighbor method as implemented in the R package impute. Quantile normalization and log2 transformation for variance stabilization were then applied to raw intensities.

For each gene, a linear regression model with the number of SIV challenges to infection as an independent variable and gene expression as a dependent variable was fit using the R package LIMMA. A moderated *t*-test was used to test that the coefficient of regression was statistically different from 0. The Benjamini–Hochberg method was used to correct the *P*-values for multiple testing (adjusted *P*-values). Genes with an adjusted *P*-value below 5% were defined as differentially expressed genes. GSEA was used to evaluate the gene sets (pathways) associated with the number of SIV challenges to infection and frequency of HLA-DR$^-$ CD14$^+$ measured at week 6. In GSEA, the most variable probes across samples were used to remove redundant probes annotated to the same gene. Genes were pre-ranked by LIMMA t statistic, and GSEA was used to assess the enrichment of gene sets from the Molecular Signatures Database gene sets (version 5.1) and transcriptomic markers of MDSCs (38). The GSEA Java desktop program was downloaded from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp) and used with GSEA Pre-Ranked module parameters (number of permutations: 1,000; enrichment statistic: weighted; seed for permutation: 111; 15 ≤ gene set size ≤2,000). Sample-level enrichment analysis was used to investigate the enrichment of pathways in the different samples. Briefly, the expression of all the genes in a specific pathway was averaged across samples and compared to the average expression of 1,000 randomly generated gene sets of the same size. The resulting Z score was then used to reflect the overall perturbation of a pathway in a sample.

Network Analysis

GeneMANIA version 3.5.1 was used to identify relations (co-expression, co-localization, genetic interactions and physical interactions) between MDSC transcriptomic marker. To that end, the human orthologs and homologs of the macaque's genes included in the classifier were obtained from the NCBI gene and homologene portal. The human homologs were then imported into GeneMANIA, and a network was generated with default parameters (equal weight of network) except no (0) inferred nodes were used to consolidate the network.

Statistical Analysis

The Mann-Whitney-Wilcoxon test was used to compare continuous factors between the two groups. Correlation analysis was performed using the Spearman rank correlation method using exact permutation *P*-values. Multiple comparison analysis were performed to include all the time points analyzed using the Benjamini-Hochberg or the Tukey's multiple comparison analysis when no association between the frequency of these cells was found at different timepoints.

RESULTS

The DNA-SIV Prime Induces HLA-DR$^-$ CD14$^+$ Cells That Correlate With an Increased Risk of SIV$_{mac251}$ Acquisition

CD14$^+$ cells with low or absent HLA-DR expression have been linked to suppressive monocytic function (34), and they have recently been characterized as myeloid-derived suppressor cells in rhesus macaques (31). The DNA-prime ALVAC + gp120 alum boost strategy demonstrated a significant 52% vaccine efficacy in protecting macaques against SIV$_{mac251}$ (32). Here we assessed the kinetics of monocytic-MDSCs and their role in this protection. Blood was collected pre-vaccination, 2 weeks after the prime (2xDNA, week 6), and after each immunization with ALVAC + gp120 alum (boosts at weeks 13 and 25; **Figure 1A**). Circulating monocytic MDSCs were identified as live HLA-DR$^-$ CD14$^+$ cells that were negative for CD3 and CD20 molecules (lineage). Although conflicting reports have arisen on the validity of including CD33 as a marker for macaques MDSC (31), we also took into consideration the CD33$^+$ CD11b$^+$ HLA-DR$^-$ CD14$^+$ cell population (referred to as M-MDSCs). The gating strategy used to identify M-MDSCs and HLA-DR$^-$ CD14$^+$ cells in the blood of a non-vaccinated animal is shown in **Figure 1B**. Both identified subsets were highly positive for the CCR2 marker, in line with phenotypic markers used to define MDSCs in humans (**Figure 1C**).

We could not detect significant changes in the levels of circulating CD33$^+$ CD11b$^+$ HLA-DR$^-$ CD14$^+$ cells during the course of immunization, possibly due to the high variability observed in this subset in addition to the relatively small number of animals in this group (**Supplementary Figure 1A**). Interestingly, the frequency of HLA-DR$^-$ CD14$^+$ cells in blood was significantly increased by the DNA-prime (baseline vs. week 6: $P = 0.0123$, one-way ANOVA, Tukey's multiple comparisons), while no differences were detected between the frequencies pre-vaccination and after the 2nd ALVAC + gp120 boosts (**Figure 1D**). Of note, there was no association between the frequency of these cells at different timepoints. Strikingly, we observed a significant association between the frequency of HLA-DR$^-$ CD14$^+$ cells after the DNA prime (week 6) and the number of challenges to infection ($P = 0.0006$, $R = -0.816$, Spearman test (**Figure 1E**). Significance was retained when the *P* value was adjusted for the 4 time points analyzed (Benjamini-Hochberg test, $P = 0.0160$).

Total blood was collected for microarray analysis before and at 24 h, 1 or 2 weeks after the first boost (week 12 + 24 h and week 13 and 14), and 24 h and 1 and 2 weeks after the second boost (week 24 + 24 h, and weeks 24 and 25) with the ALVAC-SIV + gp120-alum (**Figure 1A**). Transcriptomic signatures of vaccine-induced immune responses were identified

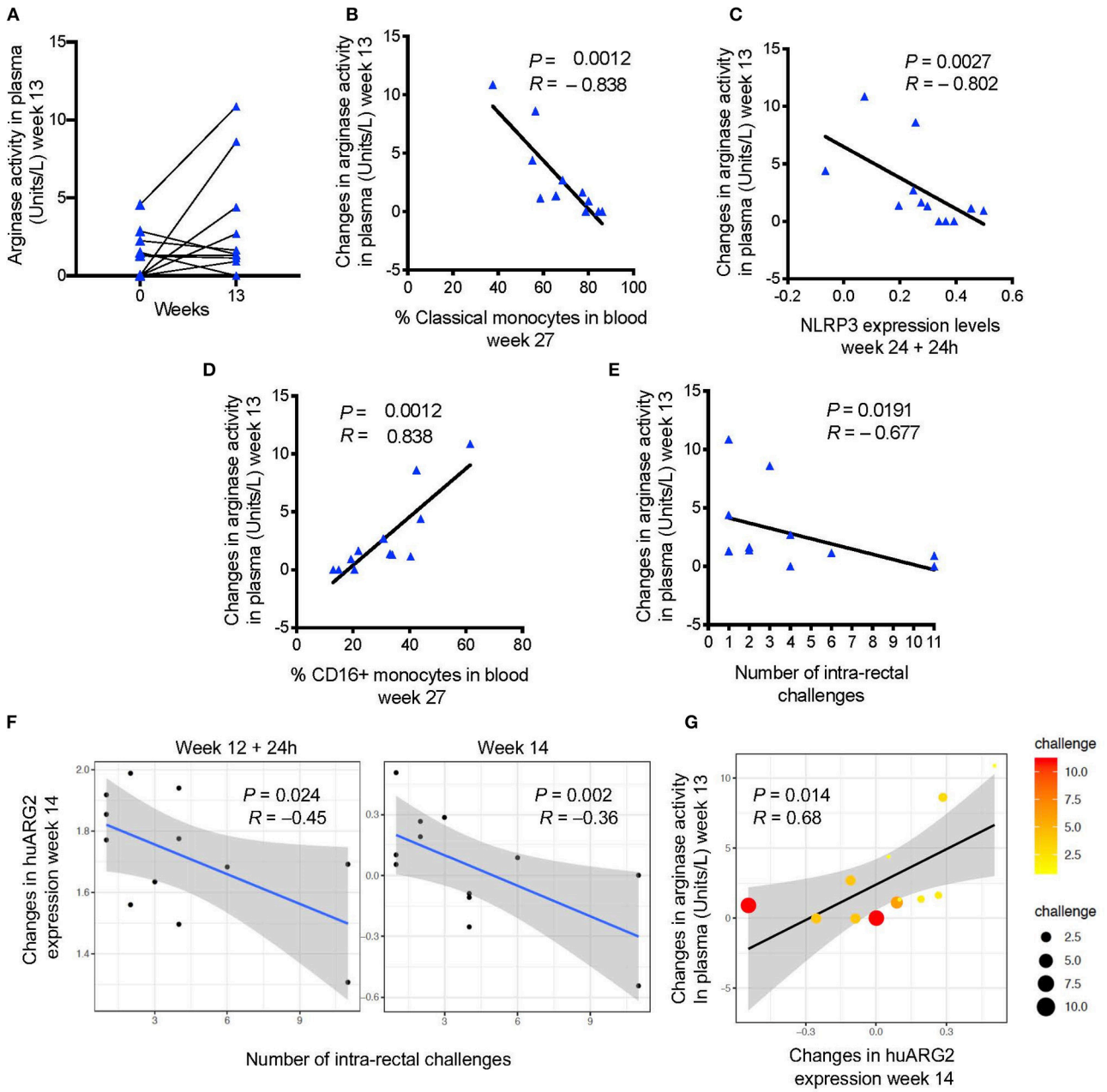

FIGURE 2 | (A) Arginase activity measured before and after immunization (baseline or week 0 and week 13). **(B)** Changes in arginase activity (week 13—pre-vaccination) in plasma are negatively associated with **(C)** the frequency of classical monocytes in blood (week 27), and with *NLRP3* expression (24 h after the last immunization), and **(D)** positively associated with the frequency of $CD16^{+}$ monocytes. **(E)** Vaccine induced levels of arginase activity and **(F)** probe annotated to human *ARG2* are negatively associated with the number of challenges to infection (increased risk of SIV acquisition). **(G)** A positive correlation was found between vaccine induced arginase activity and *ARG2* gene expression. Scatter plot shows both gene and plasma levels in relation to the number of challenges (dot size and color). In all the plots, 12 animals are shown.

as changes in gene expression after the vaccination compared to the pre-vaccination timepoint. To determine whether our vaccine induced MDSCs associated genes, Gene set enrichment analysis (GSEA) was used and vaccine induced genes were compared to a MDSC-associated genset previously identified by Heim et al. (38) (**Supplementary Figure 1B**). Transcriptomic markers of MDSCs were significantly induced at 24 h after each boost with ALVAC + gp120, as shown in **Supplementary Table 1**. Vaccine-induced MDSC markers included *PTGS2*, the gene coding for the enzyme cyclooxygenase 2 (*COX2*). Of note, the vaccine-induced transcriptomic markers of MDSCs measured at 2 weeks following the 1st boost (week 14) were positively

associated with the frequency of HLA-DR$^-$ CD14$^+$ cells ($P = 0.008$, $R = 0.806$; adjusted $P = 0.19$; **Figure 1F**). Association between transcriptomic markers of MDSCs induced by vaccination and the number of SIV challenges was then assessed (**Supplementary Table 2**). In addition to the enrichment of MDSC transcriptomic markers among genes associated with SIV_{mac251} acquisition, the average expression of MDSC transcriptomic markers measured after each boost was significantly negatively correlated with the number of challenges (week 14: $P < 0.001$, $R = -0.68$; week 25: $P = 0.025$, $R = -0.37$ by the Spearman test, and $P = 0.049$ when the Benjamini-Hochberg correction is applied; **Figure 1G**). *CD33* and of cyclooxygenase-2 (*COX-2 or PTGS2)* were among the MDSC genes associated with an increased risk of SIV_{mac251} acquisition after the 1st boost (week 14), as shown in the network analysis (**Figure 1H**) and in **Supplementary Table 2**. Together with PGE_2, the expression of *COX-2* may represent a critical step for redirecting dendritic cell development toward functionally stable MDSCs (39). Indeed, PGE_2 together with MDSC-inducing factors IL-1β and IFNγ induce high levels of *COX-2* in differentiating MDSCs and stabilizing their suppressive functions (39).

All together, these results suggest that the DNA-SIV-induced HLA-DR$^-$ CD14$^+$ cell population may be enriched with M-MDSCs. Thus, we will refer to this population as M-MDSC-like cells.

In line with this observation, the frequency of M-MDSC-like cells was also positively correlated with activation of the *STAT3* signaling pathway at 24 h after the first (week 12 + 24 h: $P = 0.044$, $R = 0.66$) and second boosts (week 24 + 24 h: $P = 0.009$, $R = 0.79$), and at week 25 ($P = 0.027$, $R = 0.71$; **Figures 1I–K**).

Plasma Arginase Level Is Associated With an Increased Risk of SIV_{mac251} Acquisition

Arginine metabolism plays a central role in the regulation of immune cell function (40). MDSCs expressing arginase and an increase in arginase activity have been described in trauma, cancer, and in certain infections (41). We measured arginase activity in the plasma of non-vaccinated macaques and macaques vaccinated after the first ALVAC + gp120-alum boost (week 13). Arginase activity levels were increased in some animals, though the overall increase was not significant (**Figure 2A**). However, changes in the arginase activity levels after the first boost (week 13 levels—pre-vaccination levels) were negatively associated with the frequency of classical monocytes, and with the *NLRP3* inflammasome pathway, two previously identified correlates of HIV vaccine protection (32) (arginase vs. classical monocytes: $P = 0.0012$, $R = -0.838$; arginase vs. *NLRP3* expression: $P = 0.0027$; $R = -0.802$; **Figures 2B,C**). On the contrary, vaccine-induced arginase activity was positively associated with the frequency of CD16$^+$ monocytes, a previously identified correlate of increased risk of SIV_{mac251} acquisition (32) (arginase vs. CD16$^+$ monocytes: $P = 0.0012$, $R = 0.838$; **Figure 2D**). Accordingly, the arginase activity levels were also associated with increased risk of SIV_{mac251} acquisition (Spearman test: $P = 0.019$; $R = -0.67$; **Figure 2E**).

In MDSC, L-arginine is metabolized by two enzymes: a cytoplasmic arginase I (ARG1), and a mitochondrial arginase II (ARG2) that is widely expressed and associated with control of NO production (41). While no probe matched the arginase genes for rhesus macaques on the microarray platform, probes annotated to human *ARG2* (*huARG2*) there was negative association with acquisition (**Figure 2F**) in vaccinated animals at week 14 ($P = 0.078$, $R = 0.658$, Benjamini-Hochberg correction), and with arginase levels at week 13 ($P = 0.014$; $R = 0.68$; **Figure 2G**), but did not withstand correction for multiple comparisons on the 4 point analyzed was included ($P = 0.693$).

Nitric Oxide-Related Genes Correlate With Plasma Arginase Level and ARG2 Expression

The regulation of arginine availability is a mechanism that can potentially lead to the control of NO production (42). Indeed, through arginine depletion, MDSCs may control NO production and regulate other arginine-dependent biological processes. We attempted to measure NO and intracellular iNOS expression levels by ELISA and FACS analysis, respectively, but we were unable to find antibodies that cross-react with rhesus macaques. Hence, we performed pathway enrichment analysis on total blood collected at 24 h and 1 week or 24 h and 2 weeks after the two ALVAC boosts (weeks 12, 14, 24, and 25; **Figure 3A**). Genes implicated in synthesis and signaling pathways of nitric oxide were associated with SIV_{mac251} acquisition (**Supplementary Table 3**; GSEA: nominal *P*-value ≤ 0.05) at 4 time points: at 24 h and 1 or 2 weeks after the 3rd and 4th immunization (**Figure 3B**), however there was no significance when all 4 time points were considered. NO-related genes associated with SIV acquisition include *NOS1AP* (the adaptor protein of the NO synthase), *NOSTRIN* and *AGTR2* (coding for the inhibitors of endothelial NO synthase), and *AKT1* (coding for a kinase regulated by NO). Interestingly, NO-related genes induced by vaccination at week 14 (2 weeks after the 1st ALVAC + gp120 alum boost) were positively associated with the changes in arginase levels in plasma following the same immunization (week 13) ($P < 0.001$, $R = 0.85$). NO biosynthesis may be at least partially regulated by *Arg2* (43), and the NO pathway was also significantly associated with *huARG2* expression (**Figure 3D**). All together, these results suggest that vaccination-induced changes in NO related genes and plasma arginase levels affected protection against SIV acquisition.

HLA-DR$^-$ CD14$^+$ Cells Are Associated With Decreased Expression of Interferon-Stimulated Genes and T Cell Pathways

Because MDSCs have been implicated in the suppression of interferon stimulating genes (ISGs) and adaptive immune responses, we first looked for possible associations between their levels and specific T cell responses (**Supplementary Table 3**). Transcriptomic analysis revealed a negative correlation between the level of M-MDSC-like cells measured at prime, and ISGs that underwent a log2-fold change (**Figure 4A**). Following the second boost with ALVAC + gp120 alum, the expression of ISGs correlated with a decreased risk of SIV_{mac251} acquisition ($P = 0.0136$, $R = 0.69$, **Figure 4A**), and was negatively associated

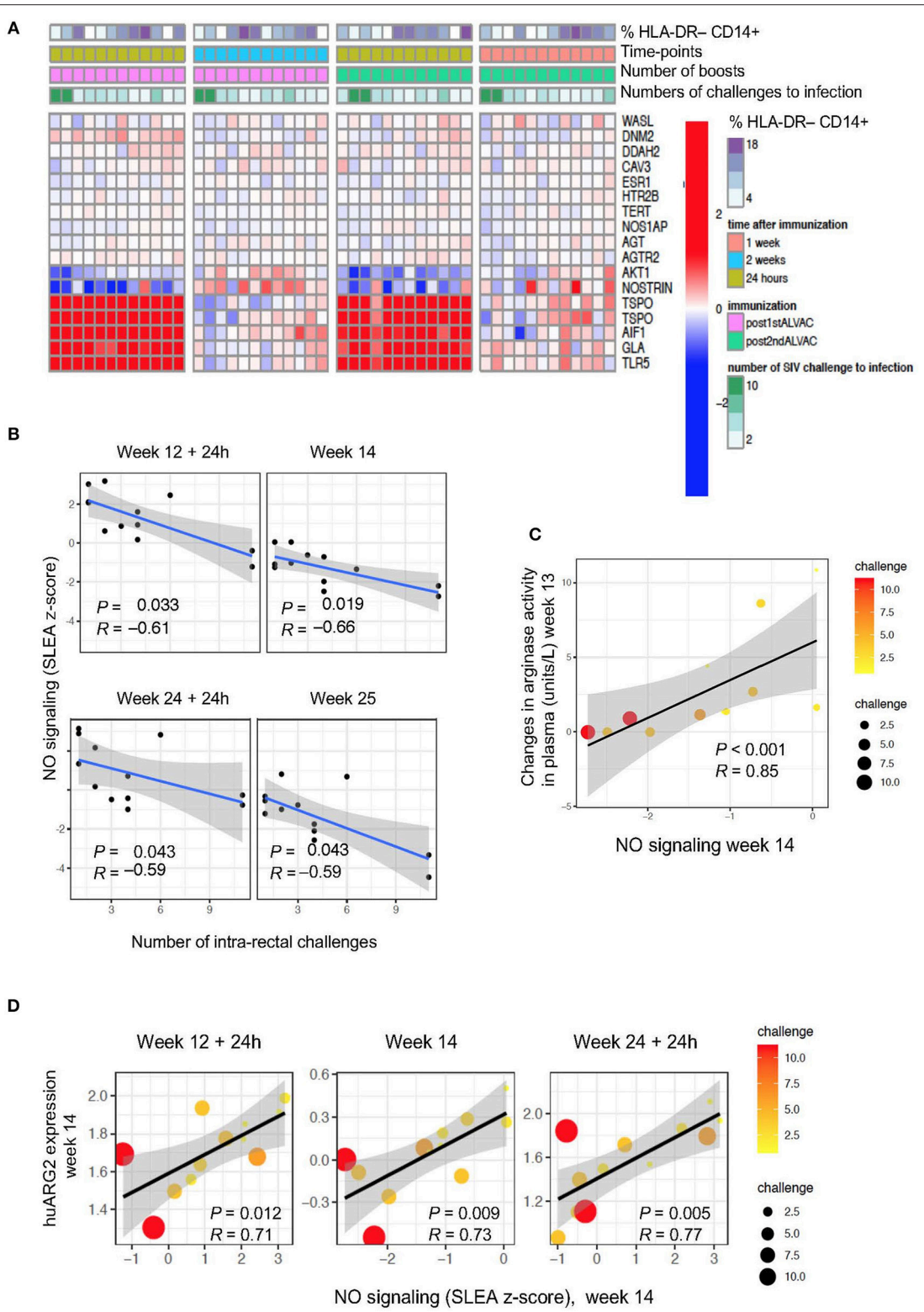

FIGURE 3 | (A) Heat map showing gene changes in NO pathways significantly associated with virus acquisition. GSEA revealed an enrichment of NO-related genes correlated with challenge. The log2-fold difference (post-vaccination—pre-vaccination) of the NO-related genes (leading edge genes of the gene sets GO_REGULATION_OF_NITRIC_OXIDE_SYNTHASE_ACTIVITY and GO_REGULATION_OF_NITRIC_OXIDE_BIOSYNTHETIC_PROCESS) is presented in the heatmap. **(B)** NO pathway association with number of challenges to infection. Sample enrichment analysis was used to average the expression of the NO-related genes for each

(Continued)

FIGURE 3 | sample collected post-vaccination (y-axis) and is presented as a function of the number of SIV challenge to acquisition (x-axis). A linear regression model was fitted (blue line), and its 95% confidence interval is presented (gray zone). A Spearman correlation and *t*-test were used to assess the significance of the association between NO gene expression and challenge. **(C)** Scatterplot of arginase activity as a function of NO gene expression at week 14. The size of the dots is proportional to the number of SIV challenges to acquisition for each animal. **(D)** Scatterplot of the gene expression of hu*ARG2* as a function of NO gene expression at different timepoints post-boosts. The size of each point is proportional to the number of SIV challenges to acquisition for each animal.

with the frequency of HLA-DR$^-$ CD14$^+$ cells at the prime ($P = 0.0394$, $R = -0.673$, sample-level enrichment analysis (SLEA) method, **Figure 4B**). Genes included the kinases *JAK1*, *JAK2*, the transcription factor *STAT1*, and the suppressors of cytokine signaling SOCS1, as shown by the network analysis in **Figure 1H**, which inhibit receptor signaling by directly inhibiting both JAK kinases and cytokine receptors (44, 45).

T cell pathways induced by vaccination were also found to be associated to protection (defined as increased number of challenges to infection) (24 h after the 1st boost $P = 0.0007$, $R = 0.836$, and 2nd boost $R = 0.683$, $P = 0.0143$, **Figure 4C**). At the same time, T cell pathways measured at 24 h after the 2nd boost were associated with the frequency of HLA-DR$^-$ CD14$^+$ cells at the prime ($P = 0.0005$, $R = -0.915$, **Figure 4D**). Of note, the NO pathway had a significant negative correlation with the same T cell activation pathways at 24 h after the 1st and 2nd boosts (**Figure 4E**, $P = 0.00412$, $R = -0.78$).

HLA-DR$^-$ CD14$^+$ Cells Are Associated With Decreased Expression of B Cell Pathways

We then asked the question whether an association could be found with B cell pathways (**Supplementary Table 3**). Indeed, B cell pathways were negatively correlated with M-MDSC-like cell frequencies at 24 h after the first boost ($P = 0.035$, $R = -0.68$, data not shown) and second boost ($P = 0.0068$, $R = -0.818$), and positively associated with protection (1st boost: $P = 0.003$, $R = 0.78$; 2nd boost: $P = 0.065$, $R = 0.55$; **Figures 5A**,**B**). Thus, these results suggest a harmful long-term effect of the prime on monocytic myeloid suppressive cells that decreases vaccine-induced protection. The NO pathway had also a significant negative correlation with the B cell activation pathway at 24 h after the 1st and 2nd boosts (**Figure 5C**: $P = 0.022$, $R = -0.66$).

Arginase and ROS Levels Correlate With Reduced SIV-Specific CD8$^+$ T Cell Responses

Recent research showed that MDSCs could inhibit HIV-specific CD8$^+$ T cell responses in macaques vaccinated with an MVA-based HIV vaccine strategy (24). Priming with DNA-SIV resulted in low, but detectable, Envelope- and Gag-specific CD8$^+$ T cells producing IFN-γ, IL-2, and TNF-α measured in blood at week 6 by intracellular staining (**Figures 6A,B**). We did not find a direct correlation between the frequency of these cytokine-producing T cells and the levels of MDSCs or M-MDSC-like cells at any timepoint during vaccination (**Supplementary Table 3**). However, IFN-γ^+ and TNF-α^+ CD8$^+$ T cell responses to gag associated negatively with plasma arginase activity at the same time (week 6; IFN-γ: $P = 0.025$, $R = -0.79$; TNF-α: $P = 0.011$, $R = -0.87$; **Figures 6C,D**). Moreover, the level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) did not associated with the levels of HLA-DR$^-$ CD14$^+$ cell population (data not shown), however it associated with an increased frequency of the CD33$^+$ CD11b$^+$ HLA-DR$^-$CD14$^+$ cell subset ($P = 0.037$, $R = 0.67$; **Figure 6E**). In turn, the latter subset assisted with reduced levels of TNF-α^+ CD8$^+$ T cell responses to gag at the end of the immunization regimen (week 27; $P = 0.034$, $R = -0.82$; **Figure 6F**). MDSCs can activate T regulatory cells that dampen T cell responses via catabolism of the essential amino acid tryptophan (Tryp), and accumulation of the kynurenine (Kyn) metabolite. The Kyn/Tryp ratio measured in the plasma of macaques vaccinated with the DNA and ALVAC + gp120 alum regimens had no association with suppressive myeloid cells, nor with SIV-specific T cell responses or viral outcome (**Supplementary Table 4**). Hence, these results point to the catabolism of L-arginine as an important mechanism of immunosuppression involved in the low level of protection afforded by this vaccine strategy, as both arginase and NO target this essential amino acid.

DISCUSSION

In recent years, new myeloid-derived suppressor cell subsets have been identified and characterized in inflammatory conditions and tumors (12). Accumulating evidence indicates an important role for MDSCs in controlling immune responses to pathogens (46). The expansion and activation of MDSCs during viral infection have been described as both detrimental and beneficial to the host. Through their immune suppressive function, MDSCs may, in fact, hamper host immune responses but conversely also limit inflammation and collateral tissue damage following an infection (46). In the case of HIV, MDSC mediated suppression of immune activation could reduce target cells for the virus (24, 47–49). Most of the studies aimed to underscore the relative contribution of MDSCs in HIV pathogenesis have described them as harmful, as MDSCs expand during untreated chronic infection and their levels are associated with disease progression (1, 50–53).While less is known about the role of MDSCs in vaccines, non-responsiveness to immunization has also been linked to MDSC expansion. Indeed, in a peptide-prime/modified vaccinia Ankara (MVA) boost vaccine regimen the M-MDSCs-like cells frequency was positively associated with set-point viral load, suggesting a negative role in protection from high viral replication (26).

Previously, we identified different monocytic myeloid subsets as correlates of increased and decreased risk of acquisition in the

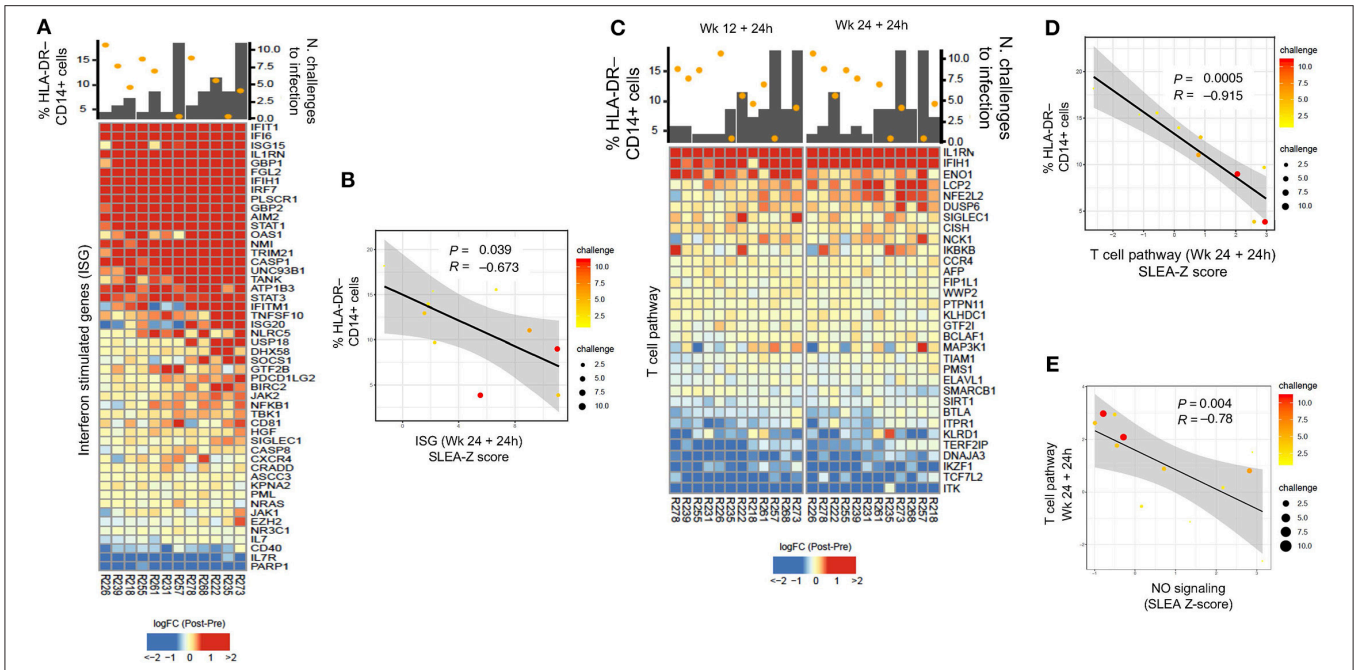

FIGURE 4 | (A) Heatmap showing log2-fold change (post-vaccination—pre-vaccination) of interferon-stimulated genes 24 h after the 2nd ALVAC boost negatively correlated with HLA-DR$^-$ CD14$^+$ cell frequencies measured 2 weeks after prime, and positively correlated with protection from SIV challenge. **(B)** Sample enrichment analysis was used to summarized the expression of interferon-stimulated genes per sample. The scatter plot shows the frequencies of HLA-DR$^-$CD14$^+$ cells as a function of the interferon-stimulated genes measured 24 h after the 2nd boost. **(C)** Heatmap showing log2-fold change (post-vaccination—pre-vaccination) T cell pathway genes that are negatively correlated with HLA-DR$^-$ CD14$^+$ cells and, at the same time, with protection from SIV acquisition. **(D)** The scatter plot shows the frequencies of HLA-DR$^-$CD14$^+$ cells as a function of the T cell pathway measured 24 h after the 2nd boost **(E)** and the T cell pathway as a function of the NO pathway. In all the scatterplots, the size of the dots is proportional to the number of SIV challenge to infection of the vaccinated animals. A Spearman correlation and *t*-test was used to test the correlation is different from 0.

blood of macaques vaccinated with the DNA-SIV + ALVAC-SIV + gp120 alum regimen (32). Further, classical monocytes (HLA-DR$^+$CD14$^+$CD16$^-$ cells) were associated with a decreased risk of SIV acquisition (32). The engagement of the myeloid compartment and the generation of a memory innate response following ALVAC immunization was most likely driven by the activation of the *NLRP3* inflammasome and the release of IL-1β. CD16$^+$ monocytes and *STAT3* activation correlated with increased SIV_{mac251} acquisition (32).

We postulated that immunosuppression by MDSCs may be playing a role in the limited vaccine efficacy (VE = 52%) afforded by the DNA-SIV + ALVAC-SIV + gp120 alum vaccine. In fact, the recombinant ALVAC vaccine vector is a known inducer of GM-CSF and CCL2 (54), and the common receptor CCR2 is expressed on virtually all classical monocytes and MDSCs. Vaccination induced myelopoiesis, and high levels of CCL2 were also detected after the DNA prime (32). Additionally, the DNA prime, the recombinant ALVAC vector, and the alum adjuvant are all known inflammasome activators, which in turn contributes to MDSC activation (9, 55, 56).

We observed that the HLA-DR$^-$CD14$^+$ cell population expanded after the DNA-prime. While the antibody panel we used was designed to detect M-MDSCs, the CD15 antibody clone we used showed limited cross-reactivity. Consequently, we cannot discount the possibility that some of the gated cells in the HLA-DR$^-$CD14$^+$ population are in fact neutrophils (31, 57). Unlike the study conducted by Lin et al., we did detect CD33$^+$ cells within the HLA-DR$^-$ CD14$^+$ cell population in macaque PBMCs, in alignment with the findings of Sui et al. (24, 31). However, this population's frequency did not change during vaccination, nor did it associate with MDSC-related genes or *STAT3*. Altogether, our data strongly suggest that HLA-DR$^-$ CD14$^+$ cells may be enriched in M-MDSCs, as we found their frequency to associate positively with transcriptomic markers of MDSCs (38).

Vaccine-induced HLA-DR$^-$ CD14$^+$ cells, MDSC gene expression, and levels of *STAT3* pathway activation (32) were all correlates of increased risk of SIV acquisition, suggesting that MDSCs harm vaccine effectiveness. Of the four MDSC-mediated immunosuppressive mechanisms we studied, we identified the arginase catabolism and NO biosynthesis as the ones primarily associated with diminished protection of the DNA-SIV + ALVAC-SIV + gp120-alum vaccine. Vaccination with this regimen induced changes in the levels of arginase activity in the plasma, and animals with increased levels proved more susceptible to infection. In addition, a heightened level of *Arg2* expression was also associated with decreased vaccine efficacy. The physiological function of Arginase 2 in humans is still poorly understood, but studies have suggested a role in regulating cell arginine concentrations by controlling substrate

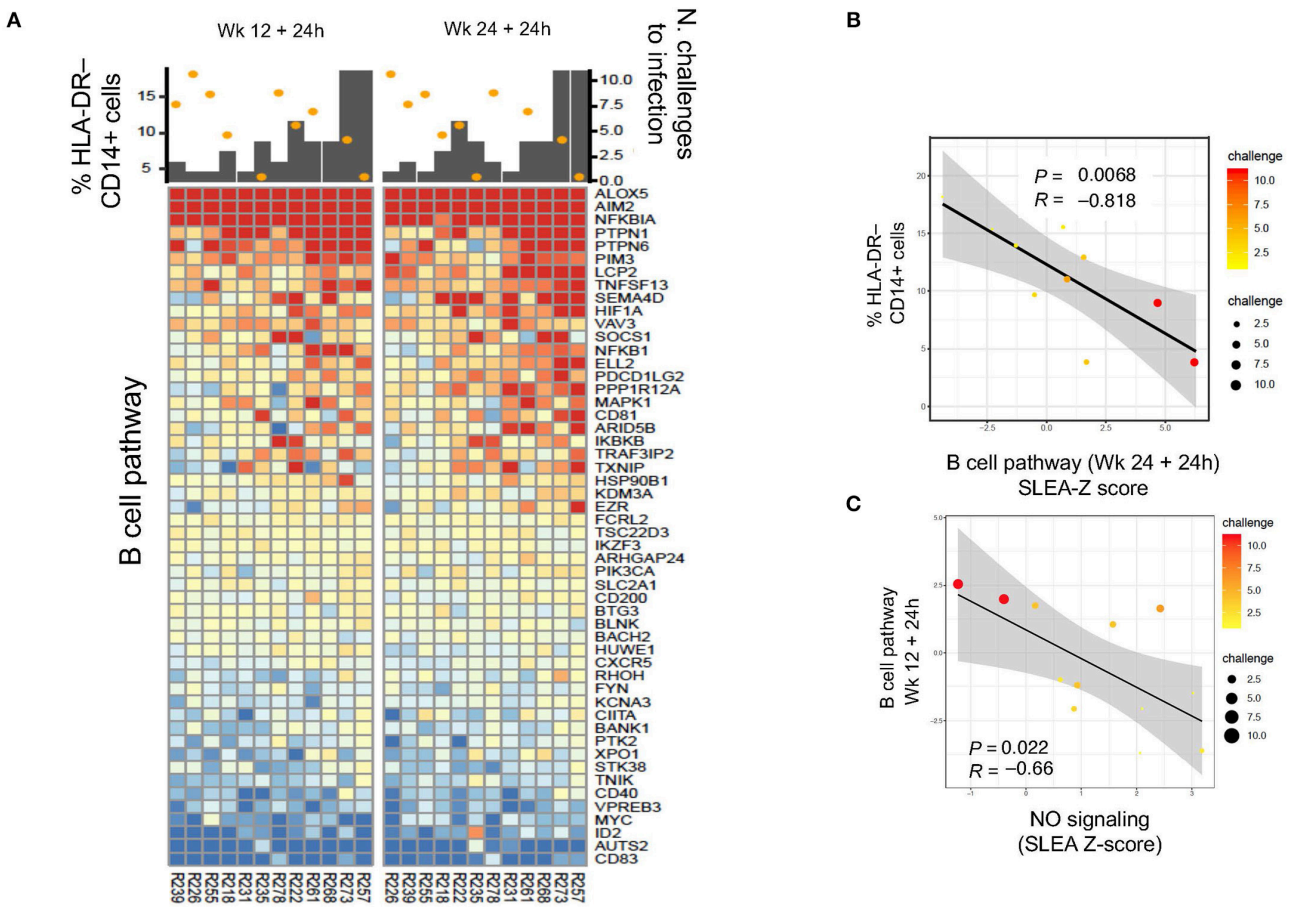

FIGURE 5 | (A) Heatmap showing log2-fold change (post-vaccination—pre-vaccination) in B cell pathways that are negatively correlated with HLA-DR$^-$ CD14$^+$ cell frequency and also correlated with protection from SIV acquisition. **(B,C)** Sample enrichment analysis was used to summarized per sample the expression of genes part of the B cell pathway. The scatter plots shows the frequencies of HLA-DR$^-$CD14$^+$ cells as a function of the B cell pathway measured 24 h after the 2nd boost **(B)** and the B cell pathway as a function of the NO pathway **(C)**. The size of the dots is proportional to the number of SIV challenge to infection of the vaccinated animals. A Spearman correlation and *t*-test was used to test the correlation is different from 0.

availability for the biosynthesis of NO, proline, and polyamines from the arginine precursor (43). In fact, *Arg2* expression levels were associated with NO-related genes encoding for NO synthesis and signaling that associated with increased virus acquisition. We could not directly link arginase activity or NO pathway activation to HLA-DR$^-$CD14$^+$ cells, nor can we exclude the possibility that other cell types including low-density neutrophils may have contributed to these immunosuppressive responses. However, the expansion of the HLA-DR$^-$ CD14$^+$ population was directly associated with the reduction of ISGs and T and B cell pathways following the ALVAC + gp120 alum boosts.

Our data point to a complex interplay between the CD14$^+$ and CD16$^+$ monocyte subsets and MDSCs, via arginase activity and inflammasome activation. The arginase activity was inversely associated with the frequency of classical monocytes, with inflammasome activation, all correlates of decreased risk of SIV acquisition, and positively associated with the frequency of CD16$^+$ monocytes. Together, these findings support the existence of a complex crosstalk between immune-activating and suppressive monocytic innate cells, in which the inflammasome activation and arginase catabolism of L-arginine are central components.

We have previously shown that classical monocytes were associated with protective Th2 cell responses (32). The levels of HLA-DR$^-$CD14$^+$ cells, arginase levels, and NO pathways all associated with decreased adaptive T and B immune responses, including SIV Gag-specific CD8$^+$ T cells.

Our findings indicate a negative role for MDSCs in protection, however given the contradictory effects of immune suppressive cells in other viral infections (52, 58), it is tempting to speculate that MDSCs may have also contribute to protection from virus acquisition, for example by decreasing inflammation, thus reducing vulnerable HIV targets, such as activated CD4$^+$ T cells. Results from HIV vaccine trials in humans and macaques suggest that inducing stronger adaptive immune responses may

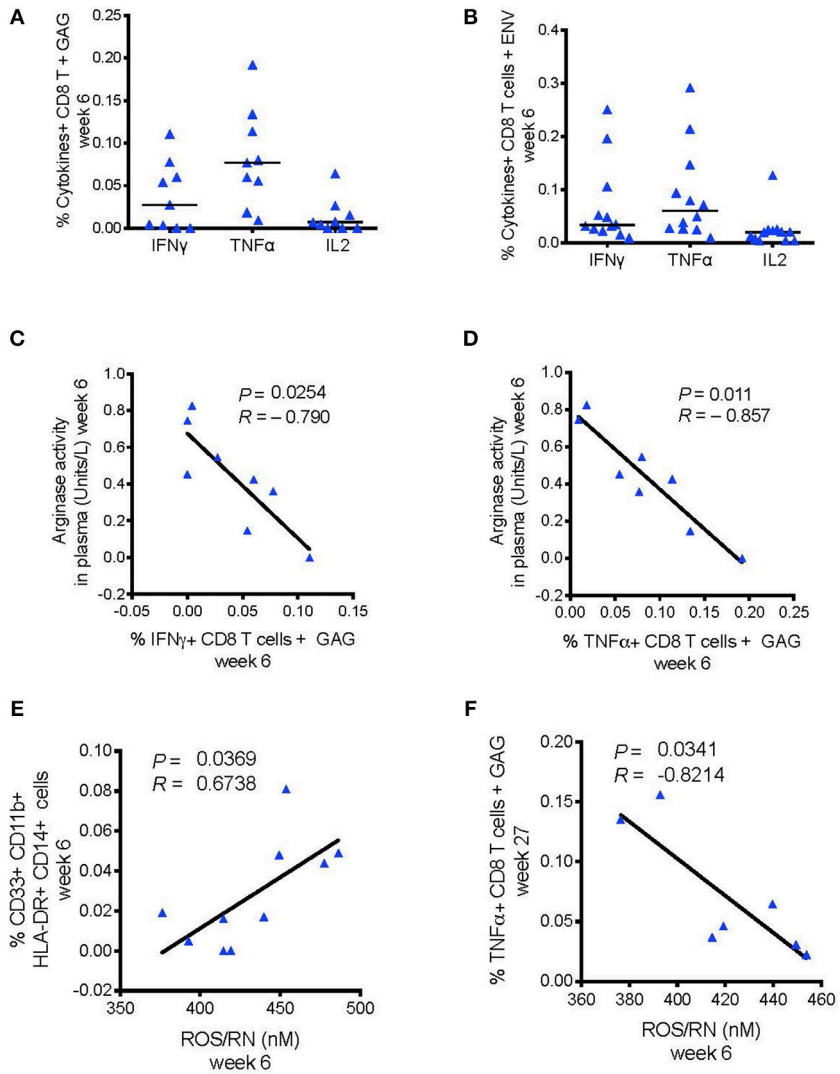

FIGURE 6 | (A) $CD8^+$ T cell responses measured in blood 2 weeks after the DNA-prime (week 6) by ICS. Cells were stimulated *in vitro* with overlapping peptides encoding for SIV_{mac251} env ($n = 12$) **(B)** or gag ($n = 9$). **(C)** Negative association between the levels of arginase activity in plasma at week 6 and the levels of $CD8^+$ T cells producing, IFN-γ, and **(D)** TNF-α responses to SIV-gag peptides. **(E)** Association between the frequency of ROS/RN levels with $CD33^+CD11b^+$ HLA-DR^- $CD14^+$ cells ($n = 10$), and **(F)** with SIV-gag specific TNF-α^+ $CD8^+$ T cell (week 27).

not be advantageous, as too much inflammation may increase HIV targets and thereby exacerbate infections (48, 59–62). Indeed, we found that the DNA-primed strategy induced fewer T cell responses and pro-inflammatory cytokines than both an Adenovirus-based vector (Ad26)-primed vaccine strategy and the MF59-adjuvanted vaccine, though the former did achieve superior protection (32, 49). In the current study, we did not observe any associations between specific $CD4^+$ T cells and MDSCs, but this could perhaps be due to the time points chosen to collect blood samples.

Given the strong immunosuppressive capacity of MDSCs on $CD4^+$ T helper cells, and the observed decrease in specific $CD8^+$ T-cell responses observed also in our study, it is nevertheless possible that MDSCs might have affected vaccine-induced Th1-cell responses. We have previously identified Th1- cell responses to be harmful in ALVAC-vaccinated macaques (48, 49, 63), and limited induction of MDSC or MDSC-like cells may thus be partially beneficial in controlling inflammation and HIV target cells, particularly at mucosa sites.

Collectively, the data presented here and those published in ref 32 demonstrate that MDSC and $CD16^+$ monocytes have an opposite effect on the efficacy of the DNA + ALVAC + gp120 HIV vaccine candidate than innate classical $CD14^+$ monocytes (**Figure 7**), underscoring the fundamental role of myeloid cells in shaping protective immune responses. A better understanding of the role of MDSCs in vaccine-mediate protection will be instrumental to improve the efficacy of HIV vaccine candidates, as well as vaccines against other human pathogens.

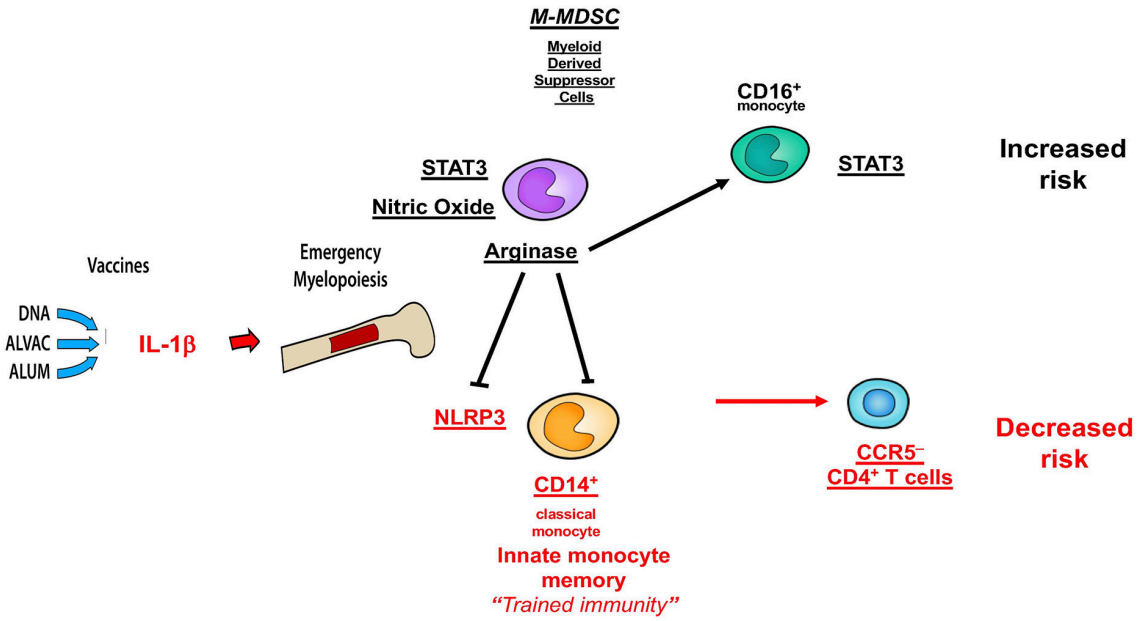

FIGURE 7 | Schematic of correlates of decreased or increased risk of SIV acquisition in the DNA-SIV + ALVAC-SIV + gp120-alum strategy and their crosstalk. Cell subsets in black are associated with an increased acquisition risk, while those in red are associated with protection from SIV acquisition.

CONTRIBUTION TO THE FIELD STATEMENT

A preventive vaccine for HIV is urgently needed. A vaccine using a Canarypox virus vector ALVAC was tested in a Thailand clinical (Thai) trial and, for the first time, resulted in significant protection from HIV acquisition. The level of protection afforded by this vaccine was limited, and this strategy must be improved. In the current study, we furthered our understanding of how this partial protective HIV vaccine candidate harnesses innate myeloid-derived cells and their role in vaccine efficacy. We show that immunosuppressive cells called MDSCs may interfere with the proper induction of T and B cell signals and specific $CD8^+$ T cell responses, that are in turn needed to clear HIV infection. We also analyzed the immune suppressive mechanisms of MDSCs that are central to their harmful role. Altogether, our results underline the complexity of the immune system and suggest ways to strengthen the effectiveness of current HIV candidate vaccines.

Code Availability

All source codes used are available https://github.com/sekalylab/mdsc.

DATA AVAILABILITY

Microarray data can be obtained at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GEO: GSE108011.

ETHICS STATEMENT

The study was conducted as previously described (32). All animals used in this study were colony-bred rhesus macaques (*Macaca mulatta*) provided by Covance Research Products. Monkeys were housed and handled in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International, and the care and use of the animals complied with all relevant institutional (U.S. National Institutes of Health) guidelines. The protocol (AUP 491) was approved by the Advanced BioScience Laboratories Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

GF designed the study and wrote the paper with MV, who also performed data analyses and prepared the figures. SF and R-PS analyzed the gene expression data, performed the correlates of risk analyses, prepared the figures, and helped write the manuscript. DB performed the flow cytometry for monocytes in blood and some correlative analyses. KF, MR, and RK performed the intracellular cytokine analysis. IS and MB performed the ELISA and Luminex assays. JB and YS provided suggestions for the identification of MDSCs by FACs. All the authors performed critical review of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01072/full#supplementary-material

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REVIEW
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Human Dendritic Cell Subsets, Ontogeny, and Impact on HIV Infection

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Dendritic cells (DCs) play important roles in orchestrating host immunity against invading pathogens, representing one of the first responders to infection by mucosal invaders. From their discovery by Ralph Steinman in the 1970s followed shortly after with descriptions of their *in vivo* diversity and distribution by Derek Hart, we are still continuing to progressively elucidate the spectrum of DCs present in various anatomical compartments. With the power of high-dimensional approaches such as single-cell sequencing and multiparameter cytometry, recent studies have shed new light on the identities and functions of DC subtypes. Notable examples include the reclassification of plasmacytoid DCs as purely interferon-producing cells and re-evaluation of intestinal conventional DCs and macrophages as derived from monocyte precursors. Collectively, these observations have changed how we view these cells not only in steady-state immunity but also during disease and infection. In this review, we will discuss the current landscape of DCs and their ontogeny, and how this influences our understanding of their roles during HIV infection.

Keywords: HIV, dendritic cells, in trans, myeloid, plasmacytoid

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INTRODUCTION

The first description of a dendritic cell originated from Paul Langerhans in 1868 based on his identification of skin-based cells (now known as Langerhans cells) that had a striking dendritic or "tree-like" morphology (1). Following the study and characterization of the mononuclear phagocyte system, seminal work by Ralph Steinman and Zanvil Cohn led to the identification of phagocytic cells in the spleen that could also induce antibody responses, which were then formally named "dendritic cells" (DCs) in 1973 (2). DCs were further shown to express high levels of surface major histocompatibility complex (MHC) molecules and potently induced proliferation of naïve T cells (3). In addition to the spleen, DCs were described across multiple peripheral tissue compartments by Hart and Fabre (4), and antigen-retaining cells also identified within B-cell follicles by Szakal and Hanna (5) and Nossal et al. (follicular DCs) (6). The DC family was subsequently expanded in 1994 to include a plasma-like cell (plasmacytoid DC) first described in 1958 (7) found to develop DC-like features upon culture by O'Doherty in the Steinman lab (8), and later identified in 1999 as the principal type I interferon-producing cell in blood by Cella et al. (9) and Siegal et al. (10). It was also discovered in the late 1980s that DCs (then described as veiled accessory cells) could be derived from cultured monocytes (11), meaning by the turn of the century the variety of DCs encompassed Langerhans cells and interstitial DCs in non-lymphoid tissue, Steinman-Cohn DCs, and follicular DCs in lymphoid tissue, Steinman-Cohn DCs, and pDCs in circulation and monocyte-derived DCs.

What defines a DC has been subject to extensive change over time but typically refers to their ability to take up antigens and present them to antigen-specific naïve T cells, leading to activation and proliferation of the T cell. As the complexity of DC subsets has grown, so too has our appreciation of their various functions outside of T cell priming and within different disease contexts, such as during infection with a viral pathogen such as human immunodeficiency virus (HIV). In this review, we attempt to summarize the current repertoire and ontogeny of DCs in peripheral blood and tissue of the anogenital tract (sites where the sexual transmission of HIV occurs) and provide an up-to-date look into their role in propagating and defending against HIV infection.

DENDRITIC CELL SUBSETS AND ONTOGENY

The ability to define and describe key immune cells has rapidly improved in recent years due to advances in single-cell technologies, which allow us to accurately discern subtle transcriptional and functional differences in the highly diverse cells of the immune system. Although microscopy and flow cytometry approaches have provided a wealth of information about immunity during steady-state and disease, they are constrained by a limited number of assessible parameters and require prior knowledge about specific antigens of interest. The advent of increasingly high-parameter techniques, particularly single-cell RNA sequencing which captures the entire transcriptome (17,000+ genes) of a single cell at a precise moment in time, has enabled rigorous and unbiased classification of immune cells (12–20) and their developmental processes (21–24). A large number of studies leveraging the power of these high-dimensional single-cell technologies have focused on the landscape of DCs given their rarity and reported heterogeneity across peripheral blood and tissue.

Dendritic Cells in Peripheral Blood

Traditionally, the DC population in peripheral blood has been classified into two lineages based on phenotypic and functional characteristics. Conventional DCs (cDCs), also known as myeloid DCs, can be defined as $CD11c^+$ $CD123^-$ and are specialized at antigen uptake and presentation to naïve T cells, thus representing the "typical" antigen-presenting DC that primes adaptive immunity. cDCs have previously been subdivided into two subsets (cDC1 and cDC2) based on homology to murine equivalents ($CD8\alpha^+$/$CD103^+$ and $CD11b^+$ DCs respectively) (25) and the differential expression of key transcription factors that drive their development; interferon regulatory factor (IRF)8, basic leucine zipper transcriptional factor ATF-like 3 (BATF3) and DNA-binding protein inhibitor 2 (ID2) for cDC1 and IRF4, Neurogenic locus notch homolog protein 2 (Notch2) and Kruppel-like factor 4 (KLF4) for cDC2 (26, 27). In contrast, plasmacytoid DCs (pDCs) are $CD11c^-$ $CD123^+$ cells best characterized for their type I interferon (IFN-I) production during viral infection but can also perform a variety of other functions including T cell stimulation and pro-inflammatory cytokine and chemokine secretion (28). Other populations of peripheral blood DC (either distinct from or further subsets of cDCs and pDCs) have also been described based on the expression of various other markers including CD2, CD5, CD16, CD34, and Slan (29–35) but have not been confirmed as distinct subsets by detailed transcriptomic or lineage analyses to date. By extensively profiling the DC population through unbiased single-cell RNA sequencing and flow/mass cytometry, several key studies have identified 6 distinct populations of blood DCs, spanning four putative cDC subsets, one subset of pDCs, and one intermediary subset that spans both cDC and pDC-like gene expression (**Figure 1A** and **Table 1**) (14, 17, 36).

Conventional DC1

Peripheral blood cDC1s are a single and discrete population of DC and can be best identified by expression of C-type lectin-like receptor (Clec)9A and cell adhesion molecule 1 (CADM1) (found almost exclusively on cDC1) (14, 36), and also express high levels of CD141 and XCR1, which is how they have commonly been identified (37, 38). cDC1s represent a rare population of DC [∼0.05% of peripheral blood mononuclear cells (PBMCs)] (39) and are mainly noted for their superior cross-presentation ability compared to other DC subsets (37, 39), efficiently priming CD8+ T cells against extracellular antigens such as bacterial and viral pathogens that DCs cannot become infected by. In addition, cDC1s can efficiently present necrotic antigens to T cells (39), in part mediated by the binding of Clec9A to extracellular actin exposed during the process of cellular necrosis (40, 41). cDC1s also express high levels of Toll-like receptor (TLR)3, TLR9, and TLR10 (14, 42, 43) which allows them to detect intracellular dsRNA and DNA, and leads to IRF3-dependent production of type I IFNs and interleukin (IL)-12 (37, 44).

Conventional DC2

In contrast to a single population of cDC1s, peripheral blood cDC2s can be further subdivided into two subsets: cDC2-A and cDC2-B [referred to as DC2 and DC3, respectively by Villani et al. (14)]. Although both cDC2 subsets can be characterized by CD1c expression, cDC2-A ($CD32b^+$) are distinguished by higher levels of CD11c, CD1c, and MHC class II genes, whilst cDC2-B ($CD36^+$ $CD163^+$) have increased expression of inflammatory genes and a similar expression signature to that of $CD14^+$ classical monocytes (14). These two subsets appear to match previous reports of cDC2s divided into $CD5^{hi/lo}$ populations (31) (corresponding to cDC2-A/B, respectively) as well as putative $CD14^+$ and $CD163^+$ subsets of CD1c-expressing cells (cDC2-B) (45, 46). Furthermore, from their mass-cytometry-based examination of human blood DCs, Hernandez et al. also describe several populations of cDC2s separated by CD163 and signal regulatory protein α (SIRPα) expression (cluster 3–5) (17), with cluster 3 ($CD11c^{++}$ $CD163^-$) and cluster 5 ($CD11c^+$ $CD163^+$) loosely corresponding to cDC2-A/B. Interestingly, cluster 4, as defined by intermediate CD163 expression and high levels of CD11c (like cDC2-A) but low CD1c (like cDC2-B), may represent innate plasticity between cDC2-A/B, which

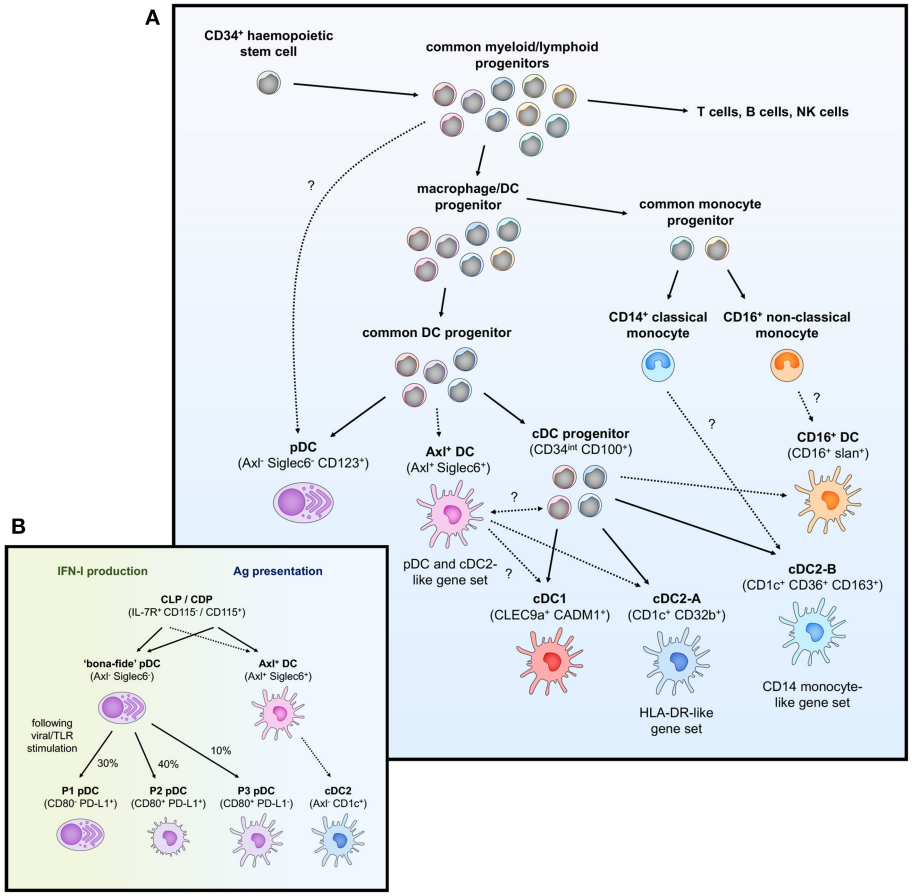


FIGURE 1 | Overview of peripheral blood DC subsets and ontogeny. **(A)** Highly proliferative and self-renewing CD34$^+$ hematopoietic stem cells produce early progenitors each primed toward distinct cell fates. These progenitors pass through several shared phenotypes to create heterogenous populations of macrophage/DC progenitors, common DC progenitors, cDC progenitors to eventually generate functional DCs (pDCs, Axl$^+$ DCs, cDC1, cDC2-A, cDC2-B, and CD16$^+$ DC). Whether these DCs represent fully-differentiated cells is contentious—Axl$^+$ DCs have been separately described to be a fully functional end-stage cell, be capable of acquiring a cDC2-like phenotype, or represent a more mature cDC progenitor that can further differentiate into cDC1 and cDC2. Their relationship to a primitive cDC progenitor (CD34^{int} CD100$^+$) is also not clear. The relationship between cDC2-B and CD16$^+$ DC with monocytes is also ambiguous at the present time. **(B)** pDCs (CD123$^+$ BDCA2$^+$) have previously been ascribed both IFN-I production and antigen (Ag) presentation properties. The identification of Axl$^+$ DCs which also express typical pDC markers has clarified these functions, which can now be separately attributed to Axl$^-$ pDCs and Axl$^+$ DCs, respectively. However, Axl$^-$ pDCs can also differentiate into Ag-presenting pDCs following stimulation (P2 and P3 pDCs) that have limited capacity for IFN-I production, creating some complication for accurately demarcating cells with IFN-I ability and T cell stimulation in the CD123$^+$ population.

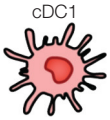
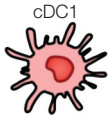
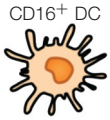
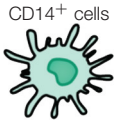
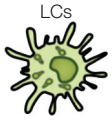
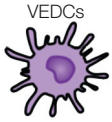
is unsurprising given the high interindividual variation in the circulating cDC2 population (17).

cDC2s represent the major subset of myeloid DC in blood and act as potent stimulators of naïve T cells. They also express a wide range of lectins such as Clec4A, Clec10A, Clec12A, and DEC205 (Clec13B) (47–50). They also express TLR2, 4–6, 8 & 9 and correspondingly produce a wide range of soluble factors in response to TLR stimulation such as tumor necrosis factor (TNF)- α, IL-1, IL-6, IL-8, IL-12, and IL-18, and chemokines such as CCL3, CCL4, and CXCL8 (14, 51). Consistent with their DC-like gene signature, CD5^{hi} cDC2-A appear to undergo increased CCR7-dependent migration, stimulate higher naïve T cell proliferation and produce higher levels of innate cytokines than cDC2-B (14, 31). cDC2-A/B also appear to induce different T helper (Th) cell polarization [Th2, Th17, Th22, and regulatory T cell (Treg) vs. Th1, respectively] (31), thus directing the immune system in quite distinct directions.

CD16$^+$ DC

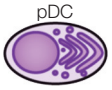
A fourth subset of CD11c-expressing DC has also been identified which lacks expression of either CD141 or CD1c and can instead be identified by CD16 expression [referred to as DC4 by Villani et al. (14)]. These DCs may be similar to the CD16$^+$ DCs described earlier by MacDonald et al. (34) which have high CD86 and CD40 expression but lower levels of HLA-DR and possess potent T cell stimulatory capacity. CD16$^+$ DCs have previously been reported to produce large amounts of inflammatory cytokines in response to TLR agonists such as TNF-α, IL-6, and IL-12 (51–53), and so appear to occupy a pro-inflammatory role. However, it is important to note

TABLE 1 | Human blood and tissue dendritic cell phenotypes.

Blood				Tissue			
Population	**Subsets**	**Markers**		**Population**	**Subsets**	**Markers**	
cDC1  (IRF8, BATF3, ID2)	cDC1	HLA-DR^{+} CD11c^{lo} CD33^{+}	**Clec9a^{+}** **CD141^{+}** **XCR1^{+}** **CADM1^{+}**	cDC1 	Dermal cDC1	HLA-DR^{+} **CD141^{++}** **CD1c$^{-/low}$** **CD11c^{low}** **CD14^{-}** Langerin^{-}	CADM1^{+} Clec9a^{+} XCR1^{+} CD103^{+} (intestinal) SIRPα^{-}
cDC2-A  (IRF4, Notch2, KLF4)	cDC2-A	HLA-DR^{++} CD11c^{++} CD33^{+} CD11b^{+} SIRPα^{+}	**CD1c^{++}** **CD32b^{+}** CD5^{hi} HLA-DR-like gene set	cDC2 	Dermal Langerin^{-} cDC2	HLA-DR^{+} **CD1a^{+}** **CD11c^{+}** **CD141^{-}** SIRPα+	**Langerin^{-}** DC-SIGN^{-} CD11b^{+} CD1c^{+} CD103^{+} (intestinal)
	cDC2-B	HLA-DR^{+} CD11c^{+} CD33^{+} CD11b^{+} CD5^{lo}	**CD1c^{+}** **CD36^{+}** **CD163^{+}** CD14-mono-like gene set		Dermal Langerin^{+} cDC2	HLA-DR^{+} **CD1a^{+}** **CD11c^{+}** **CD141^{-}** SIRPα+	**Langerin^{+}** DC-SIGN^{-} CD11b^{+} CD1c^{+} CD103^{+} (intestinal)
CD16^{+} DC 	CD16^{+} DC	HLA-DR^{+} CD11c^{+} CD141^{-} CD1c^{-} CD33^{int}	**CD16^{+}** **Slan^{+}** CD86^{hi} CD16-mono-like gene set	CD14^{+} cells 	CD14^{+} MDM	HLA-DR^{+} **CD1c$^{-/low}$** **CD14^{+}** CD16^{-}	**Autofluorescence^{-}** CD11c^{+} CD11b^{-} DC-SIGN^{+}
					CD14^{+} CD1c^{+}	HLA-DR^{+} **CD1c^{+}** **CD14^{+}** CD16^{-}	**Autofluorescence^{-}** CD11c^{+} CD11b^{-} DC-SIGN^{+}
					CD14^{+} macrophages	HLA-DR^{+} **FXIIIA^{+}** **CD14^{+}**	**Autofluorescence^{+}** CD64^{+} DC-SIGN^{+}
Axl^{+} DC  (ID2, TCF4)	CD123^{+} Axl^{+} DC	HLA-DR^{+} CD11c^{int} CD1c^{-} CD123^{+} BDCA-2^{+} BDCA-4^{int} CD2^{hi} CD5^{+}	**Axl^{++}** **Siglec6^{++}** **Siglec1^{+}** **Siglec2^{+}** CD45RA^{+} CD33^{int} pDC-like gene set	LCs 	LCs	HLA-DR^{+} **CD1a^{++}** **Langerin^{+}** CD11c^{lo} CD1c^{+}	**Birbeck granules^{+}** E-Cadherin^{+} DC-SIGN^{-} EpCAM^{+}
				CD1a^{+} VEDCs 	CD1a^{+} VEDCs	HLA-DR^{+} **CD1a^{+}** **Langerin^{+}** **CD11c^{+}**	**Birbeck granules^{-}** DC-SIGN^{-} CD14^{-}
	CD11c^{+} Axl^{+} DC	HLA-DR^{++} CD11c^{+} CD1c$^{int/+}$ CD123^{int} BDCA-2^{int} BDCA-4^{lo} CD2^{hi} CD5^{+}	**Axl^{+}** **Siglec6^{+}** **Siglec1^{int}** **Siglec2^{+}** CD45RA^{int} CD33^{+} cDC2-like gene set				
				IDECs 	IDECs	HLA-DR^{+} **CD1a$^{+/lo}$** **CD11c^{+}** FCeR1^{+}	**Birbeck granules^{-}** CD36^{+} CD32$^{+/lo}$ CD11b$^{+/lo}$

(Continued)

TABLE 1 | Continued

Blood				Tissue			
Population	**Subsets**	**Markers**		**Population**	**Subsets**	**Markers**	
pDC  (TCF4, IRF7, IRF8)	pDC	HLA-DR^{lo}	**CD123^{hi}**	Intestinal Macrophages 	MF1	**HLA-DR^{+}**	CD11b^{+}
		CD11c^{-}	**BDCA-2^{hi}**			**CD14^{+}**	CD206^{-}
		CD33^{-}	**BDCA-4^{+}**			**CD11c^{+}**	CD1c$^{+/lo}$
		CD2$^{lo/hi}$	Axl^{-}		MF2	**HLA-DR^{++}**	CD11b^{+}
		CD5^{-}	Siglec6^{-}			**CD14^{+}**	CD206^{+}
		CD45RA^{+}	CCR7^{+}			**CD11c^{+}**	CD1c^{+}
					MF3	HLA-DR^{+}	**CD11b^{-}**
						CD14^{+}	CD206^{+}
						CD11c^{-}	CD1c^{-}
					MF4	HLA-DR^{+}	**CD11b^{+}**
						CD14^{+}	CD206^{+}
						CD11c^{-}	CD1c^{-}

Known transcription factors in brackets. Key distinguishing markers highlighted in bold.

that CD16^{+} CD11c^{+} cells may not represent actual DCs but rather a subset of CD16^{+} non-classical monocyte. DC4s identified by Villani et al., (14) have marked differences in IFN-I signaling/viral response and leukocyte migration gene expression to CD16^{+} non-classical monocytes despite other similarities in transcriptional profile. Based on surface marker expression however, such as comparative levels of CD16, CD11b, CD14, and CD36, DC4s may also describe Slan^{+} cells (previously thought to be a CD16-expressing DC, but now associated with monocyte identity) (54–56). Recent work examining DC4 phenotype and T cell stimulatory function also suggests they represent a subset of Slan-expressing (73%) CD14$^{dim/-}$ CD16^{++} monocyte (57). Regardless, further studies are needed to understand the phenotypic and functional nuances of this cell subset, particularly due to the exclusion of CD14 and CD16-expressing cells in most other recent high-dimensional studies of the DC repertoire (17, 36).

Plasmacytoid DC

Traditionally defined as CD123^{+} BDCA-2^{+} cells, pDCs have also been redefined by single-cell RNA sequencing as encompassing two DC subsets. "Bona-fide" pDCs are presently defined as a single population of IFN-I-producing cells lacking the capacity to stimulate T cell responses whilst in an immature state and are distinct from a small proportion of "contaminating" CD123^{+} myeloid DCs (referred to as Axl^{+} DCs hereafter) which are unable to produce IFN-I but can potently activate T cells (14, 17, 36). Based on phenotype, Axl^{+} DCs match previous descriptions of a CD2^{hi} CD5^{+} CD81^{+} "subset" of pDC that produces little to no IFN-I but induces higher T cell stimulation than CD2^{lo} counterparts (14, 36). Although pDCs express slightly higher levels of typical pDC markers (CD123, BDCA-2, and particularly CD304/BDCA-4) than Axl^{+} DCs (14, 36), this cannot be reliably used to separate the two populations. Instead, pDCs can be gated as negative for specific Axl^{+} DC markers (Axl, Siglec6) as well as myeloid markers (CD11c, CD33, CX3CR1) expressed by the Axl^{+} DCs. pDCs can also be easily identified by their secretory morphology with a round shape, eccentric nuclei, pronounced endoplasmic reticulum, and pale Golgi zone (14, 36).

pDCs have previously been attributed a variety of functions ranging from the production of antiviral type I and III IFN, priming NK cell activation via IL-12 and IL-18 secretion, and antigen presentation and priming of T cells [reviewed by Swiecki and Colonna (28)]. With the discovery of contaminating myeloid DCs within the CD123^{+} gate used to previously define pDCs, many of these reported cDC-like properties such as T and B cell activation potential and IL-12 production can no longer truly be attributed to pDCs. "Bona-fide" pDCs still represent key drivers behind type I and III IFN responses particularly to viral pathogens through their endosomal expression of TLR7 and TLR9 (which sense ssRNA and dsDNA, respectively) and high constitutive expression of IRF7 (14, 58). Activation of TLR7/9 also induces NF-κB expression, leading to the production of TNF-α and IL-6 by pDCs, reviewed in Gilliet et al. (59). Other soluble factors produced by "bona-fide" pDCs upon TLR stimulation include the chemokines CCL3, CCL4, CCL5, IL-8, CXCL10, and CXCL11 (14, 36). In contrast to the reported inability of immature pDCs to stimulate T cells, several recent studies have described allogeneic T cell proliferation potential by Axl^{-} "bona-fide" pDCs activated with CD40L and IL-3, influenza virus or CpG oligonucleotides (17, 60), suggesting activated pDCs may be able to differentiate into cDC-like cells. Indeed, Alculumbre et al. identified three stable subpopulations of activated Axl^{-} pDCs defined by PD-L1 and CD80 expression; PD-L1^{+} CD80^{-} cells retained a plasmacytoid morphology and high IFN-I production whilst PD-L1^{-} CD80^{+} cells developed a dendritic morphology, had increased CCR7 expression and were capable of T cell activation and Th2 polarization but were unable to produce IFN-I **(Figure 1B)** (60). PD-L1^{+} CD80^{+} cells represented an intermediate state between typical pDC and cDC-like functions. Altogether, this suggests that even the Axl^{-} "bona-fide" pDC compartment has a large degree of heterogeneity and is consistent with previous reports that

only a small fraction of pDCs produce IFN-I in response to stimuli (61, 62).

Axl$^+$ DC

The newly described CD123$^+$ myeloid DCs can be defined by the expression of unique discriminatory markers (Axl, Siglec6) as well as a blend of typical pDC (CD123, BDCA-2, BDCA-4, and CD45RA) and cDC2 markers (CD11c, CD33, CX3CR1, CD1c, CD2). They have been alternatively identified as Axl$^+$ Siglec6$^+$ AS DCs by Villani et al. (14), CD33$^+$ CD45RA$^+$ CD123$^+$ pre-DCs by Zoccali et al. (36) and Axl$^+$ cells expressing either CD11c, CD123 or CD1c (or a combination thereof) by Alcantara-Hernandez et al. (17). Clustering analysis indicates that they span a continuum of pDC-like and cDC2-like states which can be identified through CD123/CD11c expression: CD123^{hi} CD11c^{lo} cells have a transcriptomic profile more similar to pDCs, and CD123^{lo} CD11c^{hi} cells appear more closely related to cDC2s (14). This is consistent with variation across the Axl$^+$ DC population in TCF4 and ID2 expression, key transcription factors responsible for maintaining pDC and cDC commitment, respectively. Despite their phenotypic similarity with pDCs, Axl$^+$ DCs cells are unable to produce IFN-I and resemble cDC2s in terms of basic function and morphology. Consistent with their potent allostimulatory potential, they express high levels of CD86 and HLA-DR, but also express a unique pattern of glycan-binding lectins (Siglec1, Siglec2, and Siglec6) and Axl, which binds apoptotic cells, indicating they have distinct functions outside of antigen presentation and T cell stimulation (14, 33). Like cDC2s, Axl$^+$ DCs express TLR4 and TLR5, as well as IRF4 and IRF8, indicative of the capacity to respond to bacterial pathogens with cytokine and chemokine production. However, they have limited expression of TLR7, IRF7 and other genes expressed by pDCs associated with IFN-I production and secretion (*DERL3*, *LAMP5,* and *SCAMP5*), which corresponds with their inability to produce IFN-I in response to TLR7/9 stimulation (14, 36). Given their recent discovery, the full spectrum of their immune functions remains to be elucidated.

Dendritic Cell Ontogeny

Under the traditional model of haematopoiesis via progressive fate "decisions," both cDCs and pDCs develop from CD34$^+$ haematopoietic stem cells (HSC) which transition into the common myeloid progenitor (CMP), excluding lymphoid lineage potential, which then differentiates into the macrophage-DC progenitor (MDP) and excludes granulocyte potential (63–68). The MDP further differentiates into the common DC progenitor (CDP), thus excluding monocyte and macrophage lineages, with the expression of zinc finger and BTB domain containing 46 (ZBTB46) and ID2 driving specification into a cDC-precursor whilst transcription factor 4 (TCF4) expression leads to pDC commitment (69–72). Further differentiation of the cDC-precursor into cDC1s and cDC2s is dependent on the expression of key transcription factors associated with each subset (BATF3 and IRF8 for cDC1 or IRF4 and KLF4 for cDC2 as previously mentioned) (73–76).

With the identification of several new DC subsets (cDC2-A/B, CD16$^+$ DCs, and Axl$^+$ DCs), the process that might generate these cells and its relation to existing notions of DC development is unclear (**Figure 1A**). Given the transcriptional relationship between cDC2-B and CD16$^+$ DCs with CD14$^+$/CD16$^+$ monocytes, respectively, it is tempting to speculate they may be derived from a monocytic origin. DC-like cells can be generated by differentiation of monocytes into monocyte-derived DCs (MDDCs), which can occur *in vitro* via IL-4 and granulocyte-macrophage colony stimulatory factor (GM-CSF) supplementation or *in vivo* at tissue sites during inflammation (77–79), but whether MDDCs form in circulation during homeostasis is unclear. CD16$^+$ MDDCs generated *in vitro* express several key genes associated with the DC4s described by Villani et al. (14), namely *SERPINA1*, *CD97*, *ITGAL,* and *TCF7L2* (80), but CD14$^+$ MDDCs appear to transcriptionally align with CD14$^+$ DCs in skin rather than CD14$^+$ blood monocytes (50). Further fate mapping and lineage tracing studies adopting the exact gating strategy used to describe these subsets would be valuable for confirming their exact ontogeny.

The origin and relationship of Axl$^+$ DCs to other DCs remains controversial, particularly as to whether they represent a fully differentiated and functional DC or whether they exist as precursor cells to cDC1/2. Villani et al. identified that AS DCs in their study had a limited capacity for further proliferation, and functionally and morphologically resembled fully differentiated cDC2s (14). In addition, AS DCs were found to transition toward a cDC2 but not cDC1 phenotype over culture, indicating they do not represent a general cDC precursor. The distribution of Axl$^+$ DCs also does not appear to correspond with previously identified cDC precursors, given Axl$^+$ DCs cannot be identified in skin but are present in secondary lymphoid organs (17). In contrast, Zoccali et al. demonstrate that CD33$^+$ CD45RA$^+$ CD123$^+$ cells (corresponding to Axl$^+$ DCs), are cDC precursors (preDCs) and can differentiate into functional cDC1 and cDC2, and further identified committed pre-cDC1 (CADM1$^+$) and pre-cDC2 (CD1c$^+$) subsets of preDC (36). All of the preDC populations were capable of IL-12 and TNF-α production in response to TLR stimulation and induced robust T cell proliferation, reflecting that a precursor status does not preclude effector DC function. Interestingly, Axl$^+$ DCs were examined in the CD141$^-$ gate by Villani et al. and so it may be that pre-cDC1s were not captured in their analysis of AS DC differentiation potential leading to the observation that these cells could not transition into a cDC1 phenotype. As suggested by Bassler et al. (81), these uncertainties in Axl$^+$ DC development and differentiation potential could be resolved by further examination of (1) whether AS DCs and preDCs completely overlap, and then using a unified sorting strategy for (2) differentiation assays and (3) comparative transcriptome and lineage mapping analysis. Finally, Villani et al. identified a CD34^{int} CD100$^+$ circulating cDC progenitor, which appears morphologically primitive and lacks the ability to respond to FMS-like tyrosine kinase 3 ligand (Flt3L) or GM-CSF (both required for pre-cDC development) but is capable of producing both cDC1 and cDC2 (14). The potential relationship between this cDC progenitor and CD34$^+$ haematopoietic stem cells remains intriguing, as is the observation that these cDC progenitors do not upregulate Axl or Siglec6 gene expression at any time

over culture and differentiation, thus further complicating our understanding of the cellular origins of Axl^+ DCs and their role in DC ontogeny.

Furthermore, recent studies have cast uncertainty over the myeloid progenitor identity of DCs, particularly pDCs given their morphological similarity to plasma B cells. pDCs have traditionally still been associated with a myeloid lineage, with evidence to show pDC commitment within common DC progenitors (82–85). However, the generation of pDCs from CDPs appears to be insufficient to account for the frequency of pDCs compared to cDCs *in vivo*, suggesting there may be other developmental pathways for pDCs. Several recent studies have described the generation of pDCs from a lymphoid lineage—Rodrigues et al. (24) demonstrate that the majority of IFN-I-producing pDCs (70–90% of immature splenic pDCs) are derived from a pDC-committed lymphoid progenitor (IL-$7R^+$ $CD115^-$) in mice that produces substantially more pDCs than CDPs (IL-$7R^-$ $CD115^+$). Furthermore, pDCs ($Bst2^+$ $CD45RA^+$) generated from these CDPs could only weakly produce IFN-I and were instead efficient antigen-presenting cells, and also expressed a combination of pDC and cDC-associated genes, leading to their description by Rodrigues et al. (24) as pDC-like cells rather than real pDCs. Indeed, these cells appear more reminiscent of Axl^+ DCs and/or P3 $CD80^+$ $PD\text{-}L1^-$ pDCs. The pDC-biased lymphoid progenitors were also shown to contain a $Ly6D^+$ $SiglecH^-$ subset capable of generating both pDCs and B cells (24), consistent with a recent study using human adult bone marrow and single-cell RNA sequencing to demonstrate pDCs share a common progenitor population with B cells (23), further confirming a possible lymphoid lineage for pDCs. In contrast to these findings, recent single-cell tracking studies have reinforced a myeloid developmental pathway for pDCs; lineage tracing of $CD115^+$ CDPs shows that most pDCs develop from this myeloid progenitor (86), and pDCs show similar development kinetics to other myeloid cells whereas pDC-biased progenitors arise before lymphoid counterparts (87, 88). Overall, increasing evidence suggests that pDCs can have a dual myelo-lymphoid origin which may dictate their functions when fully differentiated.

Studies across both humans and mice have increasingly shown that cDCs and putative Axl^+ DCs can also be derived from both myeloid and lymphoid progenitors (89–91). This collectively reflects a paradigm shift in our understanding of haematopoiesis, moving away from a model of homogenous multipotent progenitors that bifurcate into distinct but rigid cell fates (i.e., MDP bifurcation into macrophage or dendritic cell fate) and instead toward one where lineage is imprinted early during development in early progenitors, possibly prior to the emergence of distinctive phenotypes (92–99). As such, progenitors tend to follow predetermined differentiation pathways and most demonstrate uni-lineage potential, with some bi- and multi-lineage progenitors capable of multiple fates (97). Thus, stages of DC development, such as a "common DC progenitor" likely represent a mixture of progenitors following pre-determined pDC or cDC1/2 commitment **(Figure 1A)**, rather than a homogenous population that subsequently undergoes a fate decision. In summary, the complexity and subtlety in early haematopoiesis makes it difficult to accurately trace or predict DC development, and understanding the transcriptional programming that is required or sufficient to imprint DC subset-specific fates will be crucial to a complete appreciation of DC ontogeny.

Dendritic Cells in Tissue

The Human Anogenital Tracts

The human genital and anorectal tracts (hereafter collectively referred to as the anogenital tract) are made up of three distinct tissue types that sexually transmitted pathogens such as HIV may encounter upon sexual transmission; skin, type I mucosa, and type II mucosa. These physical barriers differ in their physical, chemical, and biological makeup and therefore their permeability to infection. The different subsets of mononuclear phagocytes (MNPs) within these tissues are still being characterized but will be reviewed below and are summarized in **Figure 2** and **Table 1**.

The skin is made up of two distinct layers, the outer epidermis and the underlying connective tissue layer called dermis, and covers the outer foreskin, glans penis, labia major & labia minora and the anal verge. It is made up of a thick stratified squamous epithelium with an outer layer of cornified cells, making it a formidable barrier to HIV infection. The vagina, ectocervix, inner foreskin, anal canal, and penile fossa navicularis are all covered by a type II mucosa, which similar to the skin, contains an outer epidermal layer with an underlying connective tissue layer called *lamina propria*. Unlike the skin, the type II mucosa lacks an outer layer of cornification and therefore has been shown to be more susceptible to HIV infection. The type I mucosa is considered the most susceptible tissue type of the human anogenital tract to HIV infection. Covering the endocervix, urethra, rectum, and colon, it is made up of a single layer of columnar epithelium overlaying the *lamina propria*.

Method of Extraction of Dendritic Cells From Human Tissue

It is extremely important to note that the method of extraction of immune cells from tissue can have significant effects on the isolated cells functional state and also surface expression marker profile and therefore classification, causing potential conflicts in the literature. A notable example of this has been the correct identification of human tissue cDC1 (100–103). Botting et al. recently thoroughly tested a range of extraction techniques including a number of tissue dissociation enzymatic digestions and migration assays specifically looking at mononuclear phagocytes (104). They were able to show that a number of key surface identification markers and HIV entry receptors were enzymatically cleaved (e.g., CD11c, CD1c, CD14, CD4, CCR5), up-regulated (e.g., CD80, CD83, CD141) or down-regulated [e.g., Clec9A, mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)] depending on the method of extraction. This highlights the importance of taking note of the isolation methodology when investigating tissue mononuclear phagocytes and may help explain the ever-changing classification of these cells and conflicting results in the literature.

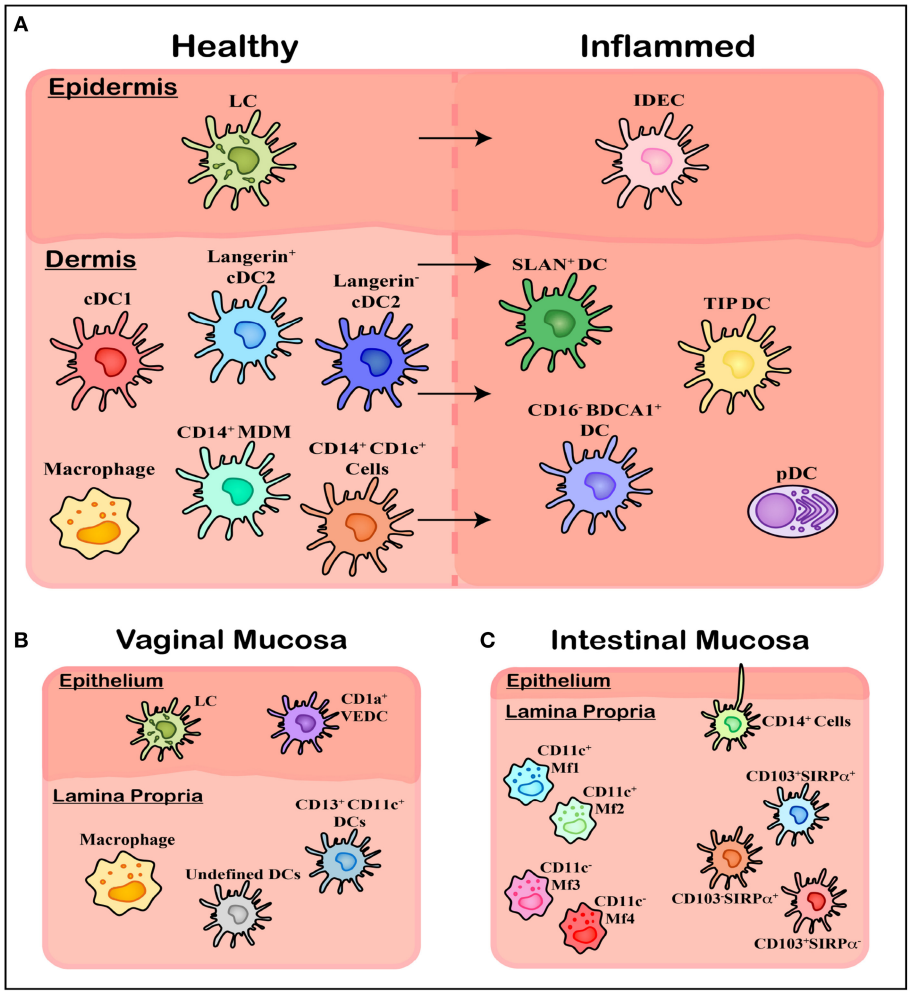


FIGURE 2 | Overview of mononuclear phagocyte subsets in human tissue. **(A)** Within healthy skin, it is believed Langerhans Cells (LCs) are the solitary population, however the underlying connective tissue of the dermis contains a range of subsets including cDC1, cDC2 (langerin expressing and langerin negative populations), and CD14 expressing cells including tissue resident Macrophages, Monocyte-Derived Macrophages (MDMs), and an uncharacterized $CD1c^+$ population. Upon inflammation these cells are also present however a number of other subsets of MNPs migrate in, including Inflammatory Dendritic Epidermal Cells (IDECs) in the epidermis, as well as pDCs, $SLAN^+$ DCs, TIP DCs, and $CD16^-$ $BDCA1^+$ cells in the dermis. **(B)** Within the vagina another novel population has been identified in epithelial layer, termed $CD1a^+$ VEDCs, while the underlying *lamina propria* has not been thoroughly characterized, with both macrophages and DCs present however exact subsets have not been extensively defined. **(C)** Within the *lamina propria* of intestinal tissue four separate $CD14^+$ Macrophage populations (MF1-4) have been characterized by their $CD11c^+$ expression, while undefined $CD14^+$ cells have been shown to extend dendrites through the epithelium to sample luminal microbes. Finally, CD103 and SIRPα can define three population of DCs which align with blood cDC1s, cDC2s, and $CD14^+$ monocytes.

Epidermal Dendritic Cells

The dendritic cells of the epidermis are one of the first cells to encounter incoming pathogens such as HIV. Historically it was believed that only a single population of human dendritic cells resided in this outer layer of tissue, however recent research has suggested this may not be the case.

Langerhans Cells

Since their discovery in 1868 the exact classification of Langerhans cells (LCs) has been controversial. While they are no longer considered to be a nerve cell as was first thought, it is still heavily debated whether LCs should be classified as DCs or macrophages. Functionally speaking LCs act as DCs, as they pick up foreign antigens, migrate out of tissue to the lymph node and present this antigen to naïve T cells, a process that macrophages do not undertake. However, ontogenically speaking LCs are more macrophage like, both sharing a common precursor with embryonic origins as well as having self-renewing abilities (105–107). Furthermore, while early *in vitro* studies reported the potential of monocytes to develop into LCs under GM-CSF and transforming growth factor beta (TGF-β) stimulation (108), it has more recently been shown that blood cDC2s have the potential to develop an LC-like phenotype under GM-CSF or thymic stromal lymphopoietin (TSLP) and TGF-β or bone morphogenetic protein 7 (BMP7) stimulation (109, 110).

LCs are characterized by their high expression of CD1a and Langerin as well as the presence of distinct Birbeck granules in their cytoplasm helping distinguish them from Langerin expressing dermal cDC2 (111) (**Figure 2A**). Being one of the first cells of the immune system coming into contact with invading pathogens, LCs are efficient at identifying foreign antigens, picking them up and presenting them to CD4 T-cells. This process is coordinated by the pattern recognition receptors (PRRs) they express including Langerin and TLRs 1, 2, 3, 5, 6, and 10 (112). Activated LCs have been shown to extend dendrites through keratinocyte tight junctions to sample and process antigens, while still maintaining barrier integrity by forming tight junctions themselves with the surrounding keratinocytes (113, 114). They have the ability to stimulate Th1, Th2, Th,17, and Th22 responses (115–118) making them highly immunogenic and adaptable to the wide range of antigens they encounter, while also showing immunosuppressive abilities under particular inflammatory conditions (119, 120).

CD1a$^+$ Vaginal Epidermal Dendritic Cells (VEDCs)

More recently, while investigating HIV and its interactions with vaginal epidermal dendritic cells (VEDCs) Pena Cruz et al. identified a DC subset within healthy human epidermis of the vagina distinct from LCs (121) (**Figure 2B**). Like LCs, these cells were characterized as CD1a$^+$ Langerin$^+$ DC-SIGN$^-$ but lacked Birbeck granules, thus distinguishing them from LCs. Given Langerin drives Birbeck granule formation (122, 123), the presence of Langerin without the latter feature is curious and supports other mechanisms for the formation of these LC-specific structures in addition to Langerin alone. Furthermore, these VEDCs expressed high levels of CCR5, CXCR4, and CD4 which are all key entry receptors for HIV infection. Due to their recent identification the ontogeny and functionality of these cells is yet to be further characterized in humans.

Inflammatory Dendritic Epidermal Cells (IDECs)

Work done by Wollenberg et al. in the late 1990's identified a novel epidermal DC subset present within inflamed skin which they termed inflammatory dendritic epidermal cells (IDECs) (124) (**Figure 2A**). Using biopsies from inflammatory skin conditions including atopic dermatitis and eczema, they phenotyped these novel cells as CD1a$^+$ Langerin$^-$, lacking Birbeck granules and having increased FcεR expression, distinguishing them clearly from the LCs which were also present (124, 125). They have also been shown to express CD11b, CD11c, MR, and DC-SIGN (CD209).

Based on the lack of Birbeck granule and surface molecule expression it is possible that these cells are similar to VEDCs, however, despite these cells being characterized two decades ago, limited work has been done to determine their ontogeny or function. While their function still remains unknown, functional differences have been reported between IDECs and LCs, with the former showing no signs of dendritic extensions through tight junctions to process antigens unlike what has been shown by LCs (126). Furthermore, both IDECs and LCs within atopic dermatitis skin have been shown to have markedly lower TLR2 expression compared to LCs in healthy skin, while IDECs and not LCs have markedly higher levels of the maturation marker CD83 and MHC class I and class II molecules (127).

Dermal/Lamina Propria Dendritic Cells

The dendritic cells in the underlying dermis/*lamina propria* are also of importance within the context of HIV infection. If the physical barrier of the epidermis is compromised such as by physical trauma or inflammation, HIV can come into direct contact with these cells. While a lot of research has focused on skin dermal DCs, recent studies in mucosal sites such as the gut and female genital tract have determined new classifications of the cells that reside there.

Tissue cDC1

As previously described in blood, cDC1s in both lymphoid and non-lymphoid tissues carry a similar phenotype suggesting a precursor-progeny relationship (103). Here they are characterized by their high expression of CD141, moderate levels of Clec9A, XCR1, and CADM1 and low to negative levels of CD11c, CD14, CD1c, CD11b, and SIRPα (103, 128). They have continuously been shown to have an increased capacity for cross presentation of antigens compared to other dermal DC subsets which is further increased with TLR3 stimulation (103). Tissue cDC1s produce high levels of TNF-α and CXCL10 following stimulation, while showing limited production of IL-1, IL-6, IL-8, IL-10, IL-12, and IL-23 (103) and have been shown to weakly promote a Treg response in intestinal tissue (128).

Previously in the literature, cDC1s have been identified by CD141 and CADM1 expression and lower expression of CD11c. However, Botting et al. recently showed that all skin mononuclear phagocytes express CADM1, and during the process of spontaneous migration cDC2s upregulate CD141 and cDC1s upregulate CD11c (104). Thus, cDC1 are almost impossible to confidently identify using this method of isolation. These findings further emphasize the importance of isolation methods to ensure correct identification of DC subsets in tissue and when analysing data in the literature.

Tissue cDC2

cDC2s in tissue express more C-type lectins (CLRs) than their blood counterparts (e.g., Mannose receptor), and have a more activated phenotype expressing higher levels of the maturation markers CD80, CD83 and CD86 (103, 128, 129). In tissue, cDC2s express CD1a, CD1c, CD11c, and SIRPα (49, 74). Similar to epidermal LCs, cDC2s have also been shown to be DC-SIGN$^-$, which helped support the idea that DC-SIGN is not a pan DC marker (130). The absence of Birbeck granules as well as higher CD11c and CD11b expression on cDC2s in the dermis helps distinguish these cells from LCs (50, 111, 131). They express TLRs 1-9 (50, 132) while producing a range of cytokines including IL-1β, IL-6, IL-8, IL-10, IL-23, CXCL-10, and TNF-α (103) and have shown to stimulate a Th17 response in intestine (128).

Recently, a Langerin-expressing subset of cDC2s were identified in the dermis of the skin distinct from LCs (50, 111). These cells are phenotypically and genotypically related to cDC2s expressing moderate levels of CD11b, CD11c, and CD13 which are all absent or lowly expressed by LCs, whilst also expressing

lower levels of CD1a and Langerin compared to LCs (50, 111). Further distinguishing them from LCs that have migrated from the epidermis is their replacement kinetics (111) and presence in sites where LCs are not found, including the lungs (111) and intestine (133). Further investigation is needed to determine if this Langerin expressing subset differs in its functionality compared to Langerin$^-$ cDC2s.

CD14-Expressing Cells

Cells expressing CD14 make up a large proportion of the mononuclear phagocyte population in human tissues. Until recently it was thought this population was made up of two distinct subsets, tissue resident autofluorescent macrophages and non-autofluorescent DCs (134). In 2014 McGovern et al. showed that the non-autofluorescent CD14$^+$ cells were actually monocyte-derived macrophages (135). Replacement kinetics and transcriptomic studies suggest a precursor-progeny relationship between these cells and CD14$^+$ monocytes (135). Furthermore, McGovern et al. showed these cells have limited induction of naïve T cells compared to tissue cDC2s, while being strong stimulators of memory CD4$^+$ T cells comparable to both tissue cDC2s and macrophages (135). However, it is of note that skin CD14$^+$ cells can be split into two subsets according to CD1c expression and it is the CD14$^+$ CD1c$^-$ cells that McGovern defined as monocyte-derived macrophages. It is still unclear whether the CD14$^+$ CD1c$^+$ cells are more macrophage or dendritic cell like. Interestingly, it is CD14-expressing cells only that express DC-SIGN, which was previously believed to be a DC marker. Thus, DC-SIGN is likely to in fact be a marker of macrophages.

Recent publications investigating CD14-expressing cells in the mucosa of the gut have shown this cell compartment to be made up of four distinct macrophage populations, termed Mf1-4 (136) **(Figure 2C)**. These four subsets cans be distinguished by their expression of CD11c, HLA-DR and CD11b: Mf1 and Mf2 are CD11c$^+$ with the former having lower expression of HLA-DR compared to the latter, while Mf3 and Mf4 are CD11c$^-$ with the former being CD11b$^-$ and the latter CD11b$^+$. Furthermore, from the transcriptomic and replacement kinetic analysis of these four subsets, Bujko et al. suggested that these cells are derived from incoming peripheral blood monocytes which progressively differentiate to Mf1s and Mf2s as an intermediate before further differentiation to either Mf3s or Mf4s. Whether this characterisation is relevant in other non-lymphoid tissues where these cells have been more thoroughly studied, such as the skin or mucosal tissue such as the cervix and vagina, has yet to be determined.

Intestinal Dendritic Cells

Using CD103 and SIRPα, Watchmaker et al. characterized three distinct DC populations within healthy human small intestine, each of which could be related to previously studied human blood DCs and mouse tissue DCs (128) (**Figure 2C**). Within this tissue the dual positive population (CD103$^+$ SIRPα$^+$) was the dominant subset and was shown to be closely related to blood cDC2s, sharing common transcription factors IRF4 and PR domain zinc finger protein 1 (PRDM1). The single positive CD103$^+$ SIRPα$^-$ population was shown to be closely related to blood cDC1s with conserved expression of IRF8 and B-cell lymphoma 6 (BCL6), and the CD103$^-$ SIRPα$^+$ subset shared common transcripts with CD14$^+$ monocytes. Watchmaker et al. then went on to show distinct differences in the functionality of these cells, with the dual positive population and the CD103$^-$ SIRPα$^+$ subset showing much higher levels of T cell proliferation compared to the single positive CD103$^+$ population. The dual positive population also induced significantly higher levels of Foxp3 expression in these proliferating cells suggestive of a Treg phenotype. Furthermore, it was shown that the dual positive and single positive CD103$^+$ population induced higher levels of IL-17-producing Th17 cells while the CD103$^-$ SIRPα$^+$ subset produced Th1 interferon-γ-producing cells.

More recently these intestinal DCs have been investigated in more detail, confirming the relationships with their blood counterparts suggested by Watchmaker et al. (128) but also highlighting the importance of using CD14 as a marker to differentiate monocyte-derived cells (CD14$^+$) from bona-fide DCs (CD14$^-$), suggesting that previous analysis of these cells likely included a mix of the two (137). This was underscored by extensive RNA sequencing analysis which showed both the CD14$^{+/lo}$ dual positive CD103$^+$ SIRPα$^+$ and CD14$^{+/lo}$ single positive CD103$^+$ cells had monocyte lineages, clustering with subsets of the CD14$^+$ Mfs described by Bujko et al. whereas the CD14$^-$ subsets aligned with a bona-fide cDC2 signature. This was further supported by their antigen uptake abilities, with the CD14$^{+/low}$ subsets showing increased ability to take up *Escherichia coli* by PHrodo analysis compared to the CD14$^-$ populations, consistent with previous findings that show human small intestine macrophages are more efficient at antigen uptake than DCs (136). Finally, the migratory kinetics of these cell were assessed, with the dual positive population showing the highest rate of CCR7-dependent migration out of the tissue, whereas the single positive population had the lowest. Richter et al. hypothesized that these results suggested the single positive CD103$^+$ population was therefore made up of a larger proportion of monocyte-related cells compared to the double positive population.

Inflammatory Dendritic Cells

In inflammatory conditions, a number of distinct tissue DCs have been identified including CD16$^-$ CD1c$^+$ DCs (138), TNF-α and inducible nitric oxide synthase (iNOS)-producing DCs (TIP-DCs) (139, 140) and Slan$^+$ DCs (141, 142) **(Figure 2A)**. CD16$^-$ CD1c$^+$ DCs have been described in inflammatory conditions including within synovial fluid of arthritis patients and tumor ascites, expressing CD14, CD11c, CD1a, CD11b, MR, SIRPα, and FcεR1. These cells were shown to have a DC like morphology and a transcriptomic approach showed this subset to be distinct from known DC subsets while still sharing common gene signatures with *in vitro* monocyte-derived DCs. Furthermore, these cells were able to produce a high Th17 response in naïve CD4$^+$ memory T cells. With their CD14$^-$ CD1c$^-$ phenotype both TIP-DCs and Slan$^+$ DCs can be distinguished from the CD16$^-$ BDCA$^+$ DCs. Both TIP-DCs and Slan$^+$ DCs have been identified in psoriatic skin, while Slan$^+$ cells have also been

identified in *lupus erythematosus* (141), steady state skin (143), and tonsil (144) and been shown to produce a range of cytokines upon stimulation including IL-6, IL-23, TNF-α, IL-12, and IL-1β (53, 141, 142, 144). Whether any of these cell subsets are present in inflamed tissue of the human anorectal tract is yet to be determined.

THE ROLE OF DENDRITIC CELLS IN HIV INFECTION

When a cell is identified as a viral substrate, often a whole continuum of phenotypes and subsets is branded in a similar manner. Whilst it is clear that $CD4^+$ T cells are the primary substrate of HIV, not all subsets are equally permissive to infection and each subset can have very different outcomes when infected. The same can also be applied to the unique continuum of mononuclear phagocytes in the form of their phenotype and what specific subset they have differentiated into. HIV interactions with DCs can rarely be described as one size fits all. Our attempts as a field to do this with the identification of DC-SIGN as a HIV receptor on *in vitro* monocyte-derived DCs is an example where we lose the bigger picture in the context of DC-HIV interactions (145, 146). Rather than one receptor-HIV envelope interaction, the interactions of HIV with DCs is complicated and informs us of a sentinel immune network that is built for distinct roles *in vivo*—that is, there is a division of labor and no one DC subset behaves the same (130, 147). As a consequence, no single DC subset interacts with HIV in an identical manner. Whilst the first contact of HIV with the virus is complicated, so too are the outcomes. Over time, many investigators have staked their position in one of two camps: the first is that DC subsets need to become infected with HIV to mediate viral spread or the second, where DC subsets simply carry HIV to enable safe passage and transfer to a secondary lymph node where a contacting resident $CD4^+$ T cell is the unfortunate recipient (**Figure 3**). In reality, both camps are correct, yet depending on the DC subset there can indeed be bias with which camp the observation sits in. As a field, we need to be open to both and appreciate the continuum of outcomes, however complex they turn out to be.

Historical Perspective of Dendritic Cells in HIV Infection

From here on, it is important we outline the initial seminal contributions of how DCs interact with HIV and how each DC phenotype (often influenced by DC isolation) plays a significant role in past observations. We will then fast forward to outline how exponentially expanding/powerful technologies have now given us further insights into how rich the DC subset landscape is and importantly how this landscape sits *in vivo* and importantly in the context of HIV pathogenesis.

The Dawn of the Interface of Dendritic Cell and HIV Biology

Soon after the discovery of HIV as the causative agent of Acquired Immunodefiency Syndrome (AIDS) were early and successive reports of HIV antigens within lymph nodes of patients with persistent generalized lymphadenopathy (148, 149). In this setting, virus was observed in the context of germinal centers and concentrated in association with follicular dendritic cells (fDC). Whilst isolation and mechanistic dissection of the role of fDCs was not initially possible, later studies on HIV-fDC dynamics highlighted their role in endocytic capture and preservation of HIV in the germinal centers of lymph nodes (150). Thus, instead of their role in the preservation of native antigens for B cell presentation, fDCs were saturated with HIV virions and facilitating the preservation of virus. Shortly following immunopathological studies of lymph nodes was the observation of HIV in association with Langerhans cells in skin biopsies of patients clinically presenting with AIDS (151). Through the use of electron microscopy, these early studies could readily pick up features of Langerhans cells through the presence of Birbeck granules (151), with virus at times in association with them (152) and evidence of viral budding and cytopathicity (151). Whilst more recent work has resolved how LCs interact with HIV, it was clear that these primary observations pointed to LCs being directly infected with HIV (as evidenced by viral budding) and capturing HIV in compartments enriched in receptors such as the CLR Langerin (as evidenced by the presence of Birbeck granules). Whilst many of these early seminal studies pointed to a role of several dendritic cell subsets in HIV pathogenesis, lack of dendritic cell markers and difficulties faced in their isolation for *in vitro* studies limited the mechanistic understanding of how each DC subset was encountering HIV.

Understanding HIV-DC Interactions Through the Early Studies of Blood Dendritic Cells

Work in the laboratory headed by Knight and Patterson (153–155) would start the journey on a preliminary understanding of how HIV can interact with DC subsets, using DCs isolated from blood. So as not to confuse the observations of early studies on blood dendritic cell subsets and more contemporary studies, it is important to outline early DC isolation techniques as they often involved *in vitro* culture steps that led to distinctly different phenotypes, as opposed to the freshly isolated DC subsets we now have the power to isolate from human blood or from tissue (104). In these early isolation methods, short-term culture of PBMCs isolated by Ficoll gradients would primarily deplete monocytes by adherence, and through subsequent and often intricate metrizamide gradient separations, buoyant blood dendritic cell populations could be isolated with purity assessed by the lamellipodia/veils on what we now recognize as partially mature DCs (maturation often resulting from the *in vitro* culture). During the same time, work in the Steinman laboratory led by Paul Cameron and colleagues further combined early immuno-depletion methods with similar gradient based enrichment but for the first time included stringent selection of dendritic cells through fluorescence-based sorting for cells without immune lineage markers; lineage being defined at the time as abundant immune markers that could readily be used to detect monocytes (CD14), NK cells (CD16), B cells (CD19), and T cells (CD3).

However, the outcome of these early studies was often not consistent across laboratories, with the work led by Knight and

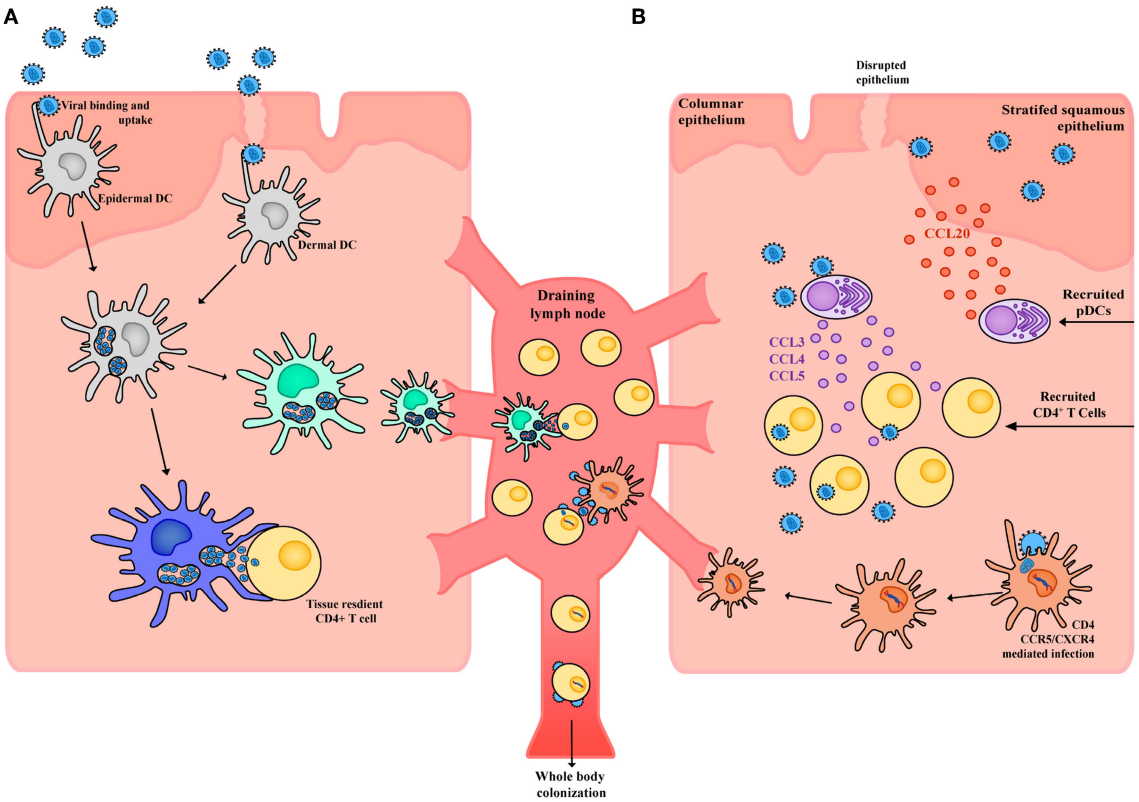


FIGURE 3 | Schematic of HIV interactions with DCs in tissue. **(A)** Within the stratified squamous epithelium of skin and type II mucosa epidermal DCs pick up incoming HIV virions potentially through a wide array of receptors including CLRs, syndecans, and CD169. Virus is taken up into open vesicular compartments that are more extensively formed in proinflammatory conditions. DCs harboring virus (yet not productively infected) can directly and immediately transfer the virus to tissue resident $CD4^+$ T cells or can migrate to the draining lymph node and transfer the virus to $CD4^+$ T cells that reside there. For the latter to occur, maturation of DC subsets will increase the half-life of HIV to enable efficient transfer. **(B)** Whilst in immature DCs, the half-life of captured HIV would not be conducive to transfer after migration in draining lymph nodes. Given immature DCs are susceptible to infection, latter viral transfer may proceed if they are infected. From here the DC migrates to the draining lymph node and productively infects $CD4^+$ T cells. Of note, whilst DCs may not be highly susceptible to HIV infection, low frequency infections can still lead to robust viral transfer when in contact with $CD4^+$ T cells. This is analogous to communication of the immune response by DCs, where only limiting numbers can still mediate a productive immunological outcome. Finally, in response to HIV infection epithelial cells can produce CCL20 which drives the migration of pDCs into the tissue. These pDCs in turn produce high levels of CCL3, CCL4, and CCL5 which drive migration of $CD4^+$ T cells into the tissue, increases CCR5 expression and in doing so further facilitates local HIV infection and spread.

Patterson highlighting the infection of dendritic cells whilst observations by Cameron et al. culminated in the seminal observation that DCs primarily capture HIV without being infected and in such an efficient manner that it produces vigorous cytopathic infection upon coculture of $CD4^+$ T cells with HIV-exposed DCs (156). This latter observation is what is now referred to as infection "*in trans*." Although the term "*in trans*" was not coined by Cameron and Steinman, they provided the first seminal evidence it could occur by culturing $lineage^-$ murine DCs and observing they could be used to capture and transfer virus to human $CD4^+$ T cells in a manner equivalent to human blood-derived DCs (156). Given the knowledge that murine cells are refractory to HIV infection, this was the most stringent example of DCs binding, capturing and later transferring virions to recipient $CD4^+$ T cells without becoming infected themselves. These differing observations in the research by Patterson and Knight vs. that of Steinman and Cameron brought forward the first indications that not all DC-HIV interactions were equal. Indeed, across both laboratories, the intricate isolation conditions led to each lab's DCs exhibiting different end-stage phenotypes. For Patterson and Knight, a partially mature DC population that could sustain HIV infection persisted, whilst for Steinman and Cameron, the stringent sorting of blood DCs resulted in the isolation of a purer and more mature DC population. What we now know is that the primary discrepancy in the infection of DCs vs. DCs simply carrying virus is a combination of their maturation state (157, 158) and the source of viral inocula used for infection (159).

Early Observations With Skin-Derived DCs

Shortly following the seminal observations by Steinman and Cameron were observations by Melissa Pope and Steinman of resident dendritic cells that emigrated from skin which could also induce a vigorous cytopathic infection in T cells when exposed to

virus (160). Unlike the early work by Cameron that highlighted the first *in trans* transfer of HIV to T cells, the mechanism of DC-HIV capture and transfer in skin DCs was initially unclear. Follow up studies led by Pope et al. demonstrated that transfer of virus from skin DCs required a low level of infection to ensure efficient viral spread throughout the DC-T cell culture (161). However, like the work that initially dissected mechanisms in blood dendritic cells, it must be noted that emigrating DCs have a phenotype that diverges from their resident immature counterpart. In addition, as we will discuss in more contemporary studies on DC-HIV interactions, the DCs used in these studies would have been comprised of multiple populations of tissue resident DCs. Whilst isolation of emigrating DCs from skin was the method initially used to dissect DC-HIV interactions, follow up studies by Reece et al. (162) and Kawamura et al. (163) would take this closer to an *in vivo* snapshot through the inoculation and infection of Langerhans cells in skin, ultimately proving that transfer of virus from LCs exposed to HIV *in situ* was closely linked to their ability to become infected by the virus (162).

The Introduction of the Monocyte-Derived Dendritic Cell Model

The early observations of DC-HIV interactions were largely constrained by a limited availability of DCs. Often representing <1% of the tissue or blood immune population, isolation of DCs and infection were considered "herculean" tasks. However, an alternative to this limitation was achieved through the identification by Sallusto et al. (77) and Romani et al. (164) in 1994 that a combination of GM-CSF and IL-4 could differentiate abundant $CD14^+$ monocytes into cells that closely resembled DCs found *in vivo*, which could be further matured into a terminally differentiated mature DC using pro-inflammatory cytokines. Whilst many DC "purists" took observations of MDDC-HIV interaction with caution, many studies using this model could recapitulate the results observed using primary DC subsets, namely that MDDCs could be infected (158), could transfer virus once infected, and could also transfer virus independent of infection (158, 165). Furthermore, it was evident in the seminal work by Blauvelt et al., that DCs bound virus in a very different manner to $CD4^+$ T cells (165)—following this work was the re-identification of a HIV gp120-binding CLR (DC-SIGN) expressed at high levels on MDDCs (145) [initially isolated from placental cDNA by Curtis et al. (166)]. Now named DC-SIGN from its description on DCs *in vitro* and ability to facilitate integrin binding during DC-T cell interactions, many of the HIV-DC interactions were initially assigned to this HIV-lectin interaction.

In vivo/Real DC Subsets and the Complexity Beyond the MDDC Model

Shortly after the characterization of the C-Type lectin pathway in MDDCs was the journey into understanding how each DC subset uniquely bound and interacted with HIV. Whilst it was clear that blood dendritic cells could bind and efficiently transfer HIV to $CD4^+$ T cells, it was readily assumed they expressed DC-SIGN. Subsequent studies not only highlighted this was not the case but further demonstrated that each DC subset had its own unique repertoire of binding receptors, some of which would enable transfer of HIV whilst other would mediate their infection (130, 167–169). From here onwards, we will refer to recent contemporary studies that have mapped the diverse landscape of dendritic cells and how they interact with HIV.

Contemporary Understanding of DCs in HIV Infection

Although it has been shown that the majority of HIV binding and uptake in DCs occurs through CLRs (158), this process is highly subset-dependent (not occurring in freshly isolated DCs derived directly from blood) and can be heavily influenced by the maturation state of the DC (130, 157, 158). The same can be said for infection following CD4 and CCR5-dependent entry, which varies across DC subsets and is also influenced by maturation (158). The source and purity of viral inocula also plays a significant role in the outcome of infection in DCs . While many still make the commentary that DCs cannot be infected or that HIV has evolved not to infect DCs, there are several points that should be noted. Firstly, as outlined above, the earliest studies of DC-HIV interactions could observe infection *in vivo*. Secondly, the concept that lentiviral restriction factors such as SAM domain and HD domain-containing protein 1 (SAMHD1) do not enable HIV infection solely in DCs is not correct as lentiviral restriction is also observed across many susceptible HIV targets including T cells and macrophages (170, 171). Finally, maturation of DCs can lead to significant blocks in HIV infection and culture/inoculation conditions that favor maturation of DC subsets will likely not reveal any HIV infection. Indeed, the continuum of DC phenotypes that influence how HIV is captured by DCs, through to if a DC subset can be infected, also modulates its potential to disseminate and transfer virus following exposure to HIV.

Transfer of HIV from DCs to $CD4^+$ T cells appears occurs in two stages, as determined by studies in both *in vitro* MDDCs (158, 172) and *ex vivo* LCs (173). First-phase transfer (within 24 h) relies on transient uptake of virus through pattern recognition receptors (such as Langerin and other CLRs) which either leads to proteolytic degradation of virus in the endosome or immediate transfer across the virological synapse (174, 175) (**Figure 3a**). Alternatively, second-phase transfer occurs over a longer phase (around 96 h) and is mediated by initial CD4/CCR5 mediated neutral fusion at the DC membrane and productive replication of HIV (158, 176, 177) **(Figure 3b)**. *De novo* virus can subsequently be transferred to $CD4^+$ T cells across the virological synapse, which uses adhesion factors such as intercellular adhesion molecular 1 (ICAM-1) to stabilize DC-T cell contacts (178), with infection being established more effectively than direct infection by free virus (156).

The mechanism for uptake of virus in the first-phase is complex—whilst initial studies supported recirculation of virus from endosome networks (179), later studies have revealed the virus for transfer is accessible to the surface (180), but compartmentalized in CD81-positive open membrane invaginations that appear to resemble endosome like compartments (181). Curiously, whilst immature and mature

DCs can both mediate this type of transfer, it is clear that mature DCs can sustain larger reservoirs of virus for first-phase transfer/*trans* infection (157, 158). This *in trans* infection *trans* model has received much attention as it can be applied across many DC subsets and does not rely on the intricacies of studying low level infection of DCs over time. However, two concepts should be emphasized for this form of transfer—firstly, in immature DCs this phenotype is short-lived and independent of the DC subset. For instance, immature Langerhans cells and MDDCs equally bind (and destroy) many incoming virions over periods of ∼24 h (158, 182). Upon maturation, DCs can increase the half-life of bound and trafficked virus, but eventual viral decay and leading to an inability to HIV transfer proceeds (158). Secondly, this process of transfer does not discriminate between strains of HIV that utilize the CCR5 co-receptor or CXCR4 co-receptor for entry (known as R5 and X4 viruses, respectively) whereas whilst it is clear that there is a distinction between them *in vivo* (183). As such, while only a small percentage of DCs become productively infected and can undergo second-phase transfer compared to primary T cells, this still represents a route of transfer as well as a potential mechanism for latent infection of T cells. In addition, productive infection ensures second-phase transfer of virus has a half-life far longer than virus that is simply bound or trafficking through a cell, and in that context provides greater transfer potential. In addition, many studies have observed infected DCs have superior viral transfer capacities than $CD4^+$ T cells and can often efficiently spread infection even in limiting numbers (161, 184).

In vivo: First Contact

Due to their anatomical localization in the outer epidermis of the human anogenital tract, LCs are thought to be the first cells to capture HIV upon sexual transmission (185–187) and therefore their interactions with HIV have been extensively studied. A large amount of this work conducted on LCs has used cells isolated from skin explants rather than anogenital tissue as they are much easier to access and are much larger in size, allowing for significantly higher cell yields and thus enables functional assays to be performed. Early studies have consistently shown LCs to be infected with HIV both *in vivo* (188–190) and *ex vivo* (173, 191–194), suggesting an important role for these cells in transmission. Furthermore Nasr et al. showed uptake and transfer of the virus to $CD4^+$ T cells from *ex vivo* LCs occurred in two phases as described above (173). Furthermore, they went on to show this was mediated by the CLR Langerin and could be efficiently blocked using an anti-Langerin monoclonal antibody or soluble Langerin, highlighting the differences in each specific DC subset and their CLR profiles they use to interact with HIV (173).

LCs have also been investigated in penile tissue (195–200), cervix (196), and vagina (201, 202) as a target cell for HIV infection. It has been shown that within the male genital tract (MGT) there is an increase of LCs found in the glans penis compared to both inner and outer foreskin (195), while other studies have shown increased HIV co-receptor CCR5 expression on LCs in the inner foreskin compared to the outer foreskin (203). Explants on MGT tissue with HIV have shown penetration of the virus to depths where LCs are abundant, particularly within the inner foreskin and uncircumcised penis (197). These findings were consistent with macaque model work performed co-currently, suggesting these explant models are representative of *in vivo* observations. Furthermore, explant and *in vitro* modeling has shown cell-associated virus translocating through inner foreskin keratinocytes can be sampled by LCs which migrate toward the apical epithelium in response to the invading pathogen (199, 200). These LCs quickly internalize HIV and *trans* infect epidermal $CD4^+$ T cells, with increased T cell-LC conjugates following HIV infection confirmed by flow cytometry. Exacerbating this infiltration of HIV via LCs is an increased production of CCL5 (RANTES) by these LCs, driving the migration of $CD4^+$ T cells into the epidermis (198). In *ex vivo* models vagina LCs are able to endocytose HIV (202) and then proceed to *trans* infect $CD4^+$ T cells without showing signs of being productively infected themselves (201).

The recent identification by Pena Cruz et al. of $CD1a^+$ VEDCs, which express high levels of HIV receptors CD4, CCR5, and CXCR4, also require further investigation into what role these cells play in the process of transmission. In their study, only viruses which use the CCR5 co-receptor (known as R5 viruses) and not those which use the CXCR4 co-receptor (known as X4 viruses) were found to replicate efficiently within these cells *ex vivo* (121). However, it was shown that the decreased replication with X4 virus was not due to decreased fusion of the virus to the $CD1a^+$ VEDCs, with levels of R5 and X4 viruses binding and fusing at comparable levels. While both strains showed signs of integration and reverse transcription, R5-enveloped virus had significantly higher integration and reverse transcription levels compared to X4 virus, which was shown to be influenced by the HIV restriction factor SAMHD1. Finally, *in vivo* work showed $CD1a^+$ VEDCs harbored high levels HIV DNA in virologically suppressed women and thus these novel cells may represent a potential latent reservoir for HIV within vaginal tissue.

While a lot of work has been done on LCs and their interactions with HIV, dermal, and *lamina propria* DCs have not been as extensively investigated, particularly within the anorectal tract. However, studies have been able to show intestinal DCs taking up virus and *trans* infecting blood and intestinal $CD4^+$ T cells (204–206). Cavarelli et al. used both an *ex vivo* and *in vitro* model, to show that intestinal DCs (defined as $CD11c^+$ $CD68^-$ to exclude macrophages) migrate toward R5 virus, extending dendrites through the intestinal epithelium to capture R5-HIV (204). This was driven by the R5-envelope itself engaging with cellular CCR5, with no evidence of CCR5-binding chemokines present in these models as a driving force for this migration. Furthermore, these cells were shown to then efficiently *trans* infect target $CD4^+$ T cells. This *trans* infection to $CD4^+$ T cells has also been seen using small intestine explants (206) as well as primary rectal mononuclear cells (205). However, with the ever-changing re-classification of DC subsets, particularly within the human intestine, it is unclear what subset these cells represent and how these findings translate *in vivo*.

Lamina propria myeloid dendritic cells of the vagina (207–210) and cervix (209–211) have also been shown to capture and *trans* infect HIV efficiently, with no significant differences found between different anatomical sites of the female reproductive

tract (FRT) (209, 210). Using vaginal explant models, Shen et al. showed HIV^+ cells that had migrated out of the mucosa after 2 h were of a myeloid DC phenotype expressing CD11c and CD13, while macrophages ($CD11c^-$, $CD13^+$) and lymphocytes ($CD3^+$) were HIV^- (207, 208). Moreover, cervical myeloid dendritic cells ($CD14^+$ $CD11c^+$) have been shown to efficiently take up R5-HIV strain, more so than lymphocytes ($CD4^{+/low}$) and macrophages ($CD14^+$ $CD11c^-$) but do not show signs of productive infection at a later time point, unlike the lymphocyte population (211). Following HIV infection of tissue of the FRT, *lamina propria* DCs show increased secretion of CCL2, CCL3 and CCL4 as well as a moderate increase in IL-8, while there is no difference in secretion of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α (209, 210). Furthermore, these cells show a short-lived increase in secreted antimicrobials including elafin, CCL5 and secretory leukocyte peptidase inhibitor (SLPI) following infection. It must be noted however that the $CD14^+$ $CD11c^+$ cells looked at within the mucosal tissue in these studies may in fact be a subset of macrophage as has been recently identified in the small intestine (136) and therefore will have to be characterized further.

While it is quite well documented that anogenital inflammation is a major risk factor for the sexual transmission of HIV (212–214), it is still not well characterized what DC subsets are present in the human anogenital tract in these conditions and what role these cells may play. While a number of DC subsets have been identified in inflammatory conditions in other tissues, further work needs to be done to confirm these cells reside within the tissues HIV encounters upon sexual transmission, and subsequently how these cells interact with HIV. However, it has been extensively shown that upon inflammation there is a large influx of HIV target cells including DCs as well as $CD4^+$ T cells and macrophages, suggesting that perhaps it is not the occurrence of novel subsets of DCs but rather an increased density of target cells for HIV to interact with.

The Role of pDCs and the Innate Response During HIV Transmission and Infection

Although plasmacytoid DCs are not found constitutively in peripheral tissues, they are recruited to sites of viral exposure and inflammation through engagement of various chemokine receptors, particularly CCR2, CCR5, CCR6, CCR9, CCR10, CXCR1, and CXCR3 (215–221). Within the context of HIV, Shang et al. have demonstrated that CCL3, CCL20, and CXCL8 are produced by cervical epithelium within 24 h of simian immunodeficiency virus (SIV) infection—CCL20 chemotactically recruits $CCR6^+$ pDCs to the underlying endocervical mucosa, with $CCR5^+$, $CCR6^+$, $CXCR1^+$, and $CXCR2^+$ cervical macrophages also attracted through this mucosal signaling axis (222). The cervical macrophages in turn produce CCL3, CCL5, CXCL8, and CXCL10, further recruiting $CCR5^+$, $CXCR1^+$, and $CXCR3^+$ pDCs to the mucosa. As such, a chemotactic "sink" at the site of SIV/HIV exposure leads to the rapid recruitment of pDCs within 1–2 days post infection (222, 223), which then exert a large influence on the course of early HIV infection.

Given their well-documented production of IFN-I in response to HIV (particularly IFN-α1/13, 2, 5, 8, and 14, IFN-β and possibly IFN-ω) (224–229), pDCs have typically been associated with early antiviral responses that limit early viral replication and dissemination. Although HIV completely inhibits the IRF3-mediated IFN-I response in myeloid DCs, macrophages and $CD4^+$ T cells upon uptake or infection (230–235), pDCs constitutively express high levels of IRF7 and upon sensing endosomal HIV ssRNA through TLR7, IFN-I production is rapidly and potently induced (225). Consequently, the IFN response during infection appears to be primarily dictated by pDCs, particularly during acute HIV/SIV infection (223, 236, 237), despite their low frequency in circulation (∼0.001%) (9, 10, 238). The effects of early IFN production on HIV infection appear to be mostly protective and are best observed in rhesus macaque (*Macaca mulatta)* studies where disease outcome can be more easily assessed. Blockade of the IFN-I receptor for 3 days following acute rectal SIV infection accelerated depletion of $CD4^+$ T cells and resulted in an expansion of the viral reservoir due to abrogation of interferon-stimulated gene (ISG) induction, eventually leading to increased and more rapid progression to AIDS (239). Similar results have also been reported upon blockade of IFN-α blockade prior to intravenous SIV infection (240). Meanwhile, exogenous administration of IFN-α2 prior to intrarectal SIV inoculation delayed systemic infection, upregulating the expression of ISGs and necessitating up to four additional challenges for transmission (239). This apparent protection conferred by IFN-I during early infection is also corroborated by a single topical application of IFN-β being sufficient to protect a majority of vaginal simian-human immunodeficiency virus (SHIV)-inoculated macaques from infection (241). Studies of the transmitted/founder (TF) virus that singularly establishes infection also indicate that IFN-resistance is a key trait in most of these viruses, suggesting that IFN-I are amongst the most important selective pressures exerted by the host during transmission (242, 243).

Interestingly, IFN-Is have also been reported to induce T cell activation—topical vaginal IFN-β application led to an increased density of vaginal $HLA\text{-}DR^+$ and $CCR5^+$ $CD4^+$ T cells in rhesus macaques and induced a highly pro-inflammatory state with increased expression of CXCL1/10/11, CCL7/8/23, IL-1β, IL-6, IL-8, TNF-α, and IFN-γ within the female reproductive tract (241). IFN-I produced by pDCs has also been shown to induce CD69 and CD38 expression on peripheral blood $CD4^+$ T cells (244). Altogether, it appears that early IFN-I production exerts an antiviral effect sufficient enough to play an overall protective function despite increasing the susceptibility of local HIV target cells to infection. pDCs are also known to produce IFN-III in response to HIV, which can exert antiviral effects in infected $CD4^+$ T cell (245, 246) how this formally contributes to HIV transmission and early infection is not known.

In addition to IFN-I, pDCs are known to produce other soluble mediators when exposed to HIV inocula including TNF-α, IL-6, IL-13, and IL-12 (238). Both IFN-α and TNF-α have both been shown to mature DCs (247, 248) which may have important consequences for the spread of HIV infection *in trans* by (1) enhancing DC-mediated viral transfer to CD4+ T cells at

initial mucosal sites, (2) accelerating cellular transport of virus to other lymphoid compartments, and (3) increasing the half-life of virions bound and trafficking through DC (158) . In addition, TNF-α is known to upregulate the expression of the transcription factor NF-kB, which is required for HIV proviral transcription in $CD4^+$ T cells (249), and so likely accelerates the lytic HIV lifecycle and $CD4^+$ T cell depletion during infection (250). However, the *in vivo* relevance of these pro-inflammatory effects of pDCs is unclear—during acute vaginal infection in rhesus macaques, SIV-infected $CD4^+$ T cells at the vaginal mucosa appear to predominantly reside in a resting state, typically not expressing CD25 or other T cell activation markers (personal communication with Ashley Haase). Beyond pro-inflammatory cytokines, pDCs also play a key role in recruiting $CD4^+$ T cells to mucosal sites of infection through the production of the inflammatory CCR5-binding chemokines CCL3, CCL4, and CCL5 (222, 223) **(Figure 3b)**. The production of these chemokines leads to an influx of $CCR5^+$ $CD4^+$ T cells to sites of infection and fuels the expansion of infected founder $CD4^+$ populations during acute vaginal SIV infection, thereby allowing foothold infection to be established. Intravaginal application of glycerol monolaurate inhibits secretion of CCL20 and protects against repeated high-dose vaginal SIV challenge, suggesting that pDCs are key cells that underpin the permissive chemotactic and inflammatory milieu during successful transmission events (223). Of note, aside from pDCs and macrophages, other leukocytes such as B cells and neutrophils do not appear to accumulate in SIV-infected cervical tissue or co-localize with clusters of SIV-RNA^+ or $CD4^+$ T cells (222, 223), suggesting $CD4^+$ T cell recruitment is the main chemotactic role pDCs have during infection.

In addition to these multifaceted roles during early infection, pDCs have also drawn interest due to their ability to reactivate virus from latently-infected cells, having been associated with decreases in $CD4^+$ T cell proviral load in suppressed patients upon combined latency-reversal agent and TLR agonist treatment (251–255). IFN-I signaling can lead to the induction of IRF1 (256) which, like NF-κB, promotes transcription of HIV proviral DNA. pDCs have also been reported to prevent the establishment of HIV latency in primary resting $CD4^+$ T cells in an IFN-α-mediated process (257, 258). The deleterious role of pDCs and IFNs during chronic HIV/SIV infection is also well appreciated, where persistent IFN production and ISG expression correlates with higher viral load, hyperimmune activation, decreased CD4+ T cell counts, dysregulated thymopoiesis, and disease progression (239, 259–262).

On a cellular level, HIV has been suggested to retain pDCs in an immature state that chronically produces IFN-Is (263–265), as part of a working model where pDCs become differentially activated into IFN-producing or antigen-presenting cells based on the subcellular compartment pathogens are sensed in (266). Endosomal sensing, such as for HIV, triggers IRF7 signaling and IFN induction leading to retention in an immature "non-DC-like" state whilst engagement in the lysosomes leads to NF-κB-mediated transcription of pro-inflammatory cytokines and maturation into a professional antigen presenting cell (265, 266). How this corresponds with previous reports of (1) HIV transfer to $CD4^+$ T cells, (2) HIV antigen presentation to $CD4^+$ T cells, and (3) HIV-induced increases in CD80, CD83, and CD86 expression by pDCs (247, 248, 267–270) was initially unclear, but may be explained by our new understanding of pDC subsets (P1-P3) and separation from Axl^+ DCs. P1 pDCs may represent the majority of the IFN-producing cells during HIV, responsible for the apparent protective but multifaceted effects of early IFN-I during infection. P3 pDCs and Axl^+ DCs may account for the cDC-like functions of pDCs during infection, namely viral transfer and cross-presentation and production of pro-inflammatory cytokines—upon TLR7 stimulation, Axl^+ DCs are able to produce IL-12 but not pDCs, and it is unclear whether P3 activated pDCs are also capable of this function alongside their potential for T cell stimulation. P2 pDCs may encompass the effects of both P1 and P3 pDCs as a functional intermediate. It is worth noting all three pDC subsets are also able to produce TNF-α following viral stimulation (60), and thus are all likely to play a role in TNF-mediated inflammation during HIV infection.

It is tempting to speculate about the early involvement Axl^+ DCs may have during HIV infection, given they appear to have similar migratory patterns to pDCs (absent in healthy skin but present in lymphoid tissue) (17). $CD11c^{hi}$ Axl+ DCs have a cDC-like gene signature (14), which suggests they may play a similar role to other myeloid DCs in infection, namely efficiently transferring virus to $CD4^+$ T cells and disseminating infection to other physical compartments such as the lymph nodes. In contrast, $CD123^{hi}$ Axl^+ DCs have a gene signature that aligns more closely with pDCs (14)—given they do not produce IFNs in response to HIV, whether they resemble other pDC functions during infection and still possess the potential for viral transfer would be interesting to determine. Whether Axl^+ DCs experience productive HIV infection (and hence can undergo second-phase transfer) like other myeloid DCs is also unknown. The characterisation of the cytokines and chemokines produced by P1-P3 pDCs and Axl^+ DCs in response to HIV and their susceptibility to infection will be critical for unraveling the role of each of these cell types during early and later stages of HIV infection.

CONCLUDING REMARKS

Dendritic cells have most commonly been recognized for their ability to stimulate antigen-specific T cell responses, thereby forming a strong link between innate and adaptive immunity. Like the complexity of this role and other immunomodulatory effects they exert, the repertoire of DCs across different compartments has been difficult to comprehensively capture. However, building on countless years of previous work, we have seen great advances in our conceptualization of the DC spectrum through the emergence of powerful single-cell technologies (one of the most prominent being single-cell RNA sequencing). In peripheral blood, what originally comprised of 3 key DC subsets ($CD141^+$ cDC1, $CD1c^+$ cDC2, and $CD123^+$ pDC) has now been expanded to 6 putative subsets (cDC1, cDC2-A/B, $CD16^+$ DC, Axl^+ DC, and pDC) which can be

distinguished by expression of CD11c, CD16, Clec9a/CADM1, CD1c, CD32b, CD163, Axl, Siglec6, and CD123. Heterogeneity of blood DCs also extends to their developmental relationships, which will require further validation to accurately trace the precursor identities of each fully-differentiated DC. In peripheral tissue, each tissue site contains a discrete collection of DCs, ranging from Langerhans cells and other DC subsets in the outer epidermis of skin and Type II mucosa, to dermal and *lamina propria* cDC1s, cDC2s, and $CD14^+$ cells, as well as their intestinal counterparts defined by CD103 and SIRPα expression.

For a comprehensive understanding of their roles in immunity, it is imperative that we begin to match and dissect these new DC subsets to previous descriptions of their immune functions particularly during disease and infection. In the context of HIV, the relative contributions of Langerhans cells and other epidermal DCs (such as VEDCs) to HIV transmission at the earliest stages must be re-examined, and similarly with pDCs and Axl^+ DCs. Indeed, what are each of their roles in (1) HIV transfer to $CD4^+$ T cells, (2) the secretion of pro-inflammatory and antiviral cytokines, and (3) the recruitment of $CD4^+$ T cells and other HIV targets at the site of infection. By continuously updating our view of DC subsets and development, we can better understand how they influence infection with HIV and other pathogens, and thus precisely modulate their behavior to protect us from disease.

AUTHOR CONTRIBUTIONS

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REVIEW
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Role of Dendritic Cells in Natural Immune Control of HIV-1 Infection

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Dendritic cells (DCs) are professional antigen-presenting cells that link innate and adaptive immunity and are critical for the induction of protective immune responses against pathogens. Proportions of these cells are markedly decreased in the blood of untreated HIV-1-infected individuals, suggesting they might be intrinsically involved in HIV-1 pathogenesis. However, despite several decades of active research, the precise role and contribution of these cells to protective or detrimental host responses against HIV-1 are still remarkably unclear. Recent studies have shown that DCs possess a fine-tuned machinery to recognize HIV-1 replication products through a variety of innate pathogen sensing mechanisms, which may be instrumental for generating both cellular and humoral protective immune responses in persons who naturally control HIV-1 replication. Yet, dysregulated and abnormal activation of DCs might also contribute to sustained inflammation and immune activation accelerating disease progression during chronic progressive infection. Emerging data also suggest that DCs can influence the induction of potent broadly-neutralizing antibodies, and may, for this reason, have to be considered as important components of future HIV-1 vaccination strategies. Apart from their involvement in antiviral host immunity, at least a subgroup of DCs seem intrinsically susceptible to HIV-1 infection and may serve as a viral target cell population. Indeed recent studies suggest that specific DC subpopulations residing in the genital mucosa are preferentially infected by HIV-1 and play an active role in sexual transmission; therefore, DCs may contribute to viral dissemination and possible persistence of the viral reservoirs through either direct or indirect mechanisms. Here, we analyze the distinct and partially opposing roles of DCs during HIV-1 disease pathogenesis, with a focus on implications of DC biology natural immune control and HIV cure research efforts.

Keywords: dendritic cell, HIV-1 controller, IFN, Tfh, bNAb, vaccine

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INTRODUCTION

Dendritic cells (DCs) represent a heterogeneous family of immune cells that link innate and adaptive immunity. The main function of these innate cells is to capture, process, and present antigens to adaptive immune cells and mediate their polarization into effector cells (1). DCs can be subdivided in two main subtypes: plasmacytoid (pDC) and myeloid (mDC) DCs, which specialize in the recognition of different pathogen associated molecular patterns (PAMPs) due to the unique distribution of Pattern Recognition Receptors (PRR), such as toll-like receptors, C-type lectins and intracellular nucleic acid sensors (2–4). As a result, mDCs and pDCs can efficiently induce $CD4^+$ and $CD8^+$ T cell responses against different types of pathogens. In addition, both mDCs

and pDCs are also capable of interacting with Natural Killer (NK) cells, which are particularly relevant during viral infections (5). Therefore, the contribution of different DC subtypes to immune responses against microbial infections seems to be highly complex and be influenced by context- and pathogen-dependent factors.

During HIV-1 infection, several effector components of the innate and adaptive immune system are involved in the host antiviral response, and although these immune responses seem unable to prevent the establishment of the infection, they can influence HIV-1 disease progression. Effective immune control of HIV-1 infection occurs in rare population of HIV-1 infected individuals who are able to spontaneously control HIV-1 replication in the absence of antiretroviral therapy, and to maintain undetectable levels of viral replication as measured by commercial PCR assays. In these individuals, long-lived polyfunctional HIV-1-specific $CD8^+$ T cells have been identified as the main biological correlate of spontaneous immune control of HIV-1 (6–8). However, the contribution of DCs to durable immune control of HIV-1 is still a relatively unexplored area and a matter of active debate. During the last years, new relevant data about DC biology in the context of HIV-1 infection have become available, specifically with regards to DC susceptibility to infection, to DC-mediated immune regulation and to direct host-pathogen interactions between DC and HIV-1. In this review, we have focused on consolidating the most recent advances on DC biology in the context of HIV-1 immunopathology, and on providing a detailed evaluation of the role of DC in HIV-1 immune control.

ANATOMICAL LOCALIZATION AND ACTIVATION OF DCs DURING HIV/SIV INFECTION

DCs are physiologically distributed in mucosal and lymphoid tissues where they capture antigens and present them to T cells, but a small proportion of mDCs and pDCs are also circulating in the blood. mDCs can be identified as lineage marker negative cells that display high surface levels of CD11c and HLA-DR (9) while pDCs are $CD11c^-HLA\text{-}DR^+$ cells characterized by surface expression of the C-type lectin BDCA2, high levels of the alpha chain of the receptor for interleukin-3 (CD123) and the immunoglobulin superfamily receptor immunoglobulin-like transcript 7 (ILT7) (10). Upon HIV-1 infection, the anatomical distribution of DCs is dramatically altered and lower proportions of pDCs and mDCs are present in the blood of infected untreated individuals (11–13). The extent of the depletion of circulating mDC is correlated with rapid disease progression during HIV-1 and SIV infections (14, 15). Interestingly, proportions of circulating pDCs are more profoundly reduced in HIV-1 progressors in contrast to controllers (16); although the exact mechanisms responsible for these differences remain unknown. Despite these discrepancies, circulating pDCs from both controllers and progressors are characterized by upregulated expression of the gut homing integrin $\alpha4\beta7$, suggesting selective trafficking to mucosal intestinal tissue where the majority of HIV-1-infected cells reside (17). Similarly, higher levels of activation in gut resident mDCs and pDCs seem to be associated with changes in gut microbiota and immune homeostasis (18). In addition to migration to the gut, preferential recruitment of pDCs to the lymph nodes also occurs in HIV-1-infected subjects (19).

Besides the changes in anatomical distribution, circulating and tissue-resident DCs display an activated phenotype defined by upregulation of costimulatory molecules in infected individuals (11–13). In fact, higher levels of activation in blood DCs seem to correlate with plasma viremia in progressors (20). In contrast, less pronounced phenotypical signs of immune activation, combined with increased functionality have been described in mDCs from the blood of HIV controllers (21). Interestingly, highly activated mDCs residing in the lymph nodes from HIV-1^+ patients seem to co-express inhibitory costimulatory molecules such as PD-L1 and are still capable of responding to TLR stimulation, in contrast to cells from peripheral blood (19).

A hallmark of circulating pDCs from the blood of HIV-1^+ individuals is the expression of high basal levels of type I interferon (IFN) and IFN-stimulated genes, likely reflecting abnormal immune activation (22). Interestingly, this higher baseline activation of IFN-dependent immune activity seems to make pDCs from progressors refractory to antigenic stimulation (23, 24), and paradoxically reduces their ability to secrete appropriate levels of IFN-α upon PRR stimulation. In contrast, pDCs from controllers maintain IFN-α secretion levels that are comparable to those of healthy individuals. Consistent with these findings, microscopy-based studies indicated differences in the trafficking of intracellular TNF-related apoptosis-inducing ligand (TRAIL) in pDCs from controllers and healthy donors compared to progressors. TRAIL is a molecule known to induce apoptosis of $CD4^+$ T cells through a mechanism regulated by the alarmin High Mobility Group Box 1 (HMGB1) (23, 25). While TRAIL seems to be recycled from the membrane of pDCs in controllers after exposure to HIV-1, pDCs from viremic patients appear to constitutively express TRAIL on the membrane, which may contribute to unspecific induction of cell death in $CD4^+$ T cells and accelerate cell loss and immunodeficiency (26) (**Figure 1**). Overall, these data indicate that pDCs from controllers maintain a functional profile that is similar to healthy persons. In contrast, pDCs from progressors exhibit a hyperactivated state characterized by constitutive TRAIL up-regulation, higher basal levels of IFN-dependent immune responses, and a reduced ability to produce IFN-α in responses to antigen exposure, most likely as a result of generalized immune activation that makes cells refractory to microbial stimulation (**Figure 1**). The normal functional profile of pDCs in controllers therefore could be a consequence, rather than a cause of viral immune control. Notably, the initiation of antiretroviral therapy does not revert the decline in pDC frequency and function observed during progressive infection, suggesting an irreversible defect in pDC physiology in progressors after prolonged exposure to high viremia (27). Interestingly, less pathogenic HIV-2 strains induce lower levels of type I IFN expression in pDCs compared to HIV-1, suggesting that lower levels of pDC activation could be associated with immune control of the infection (28). In addition to pDCs,

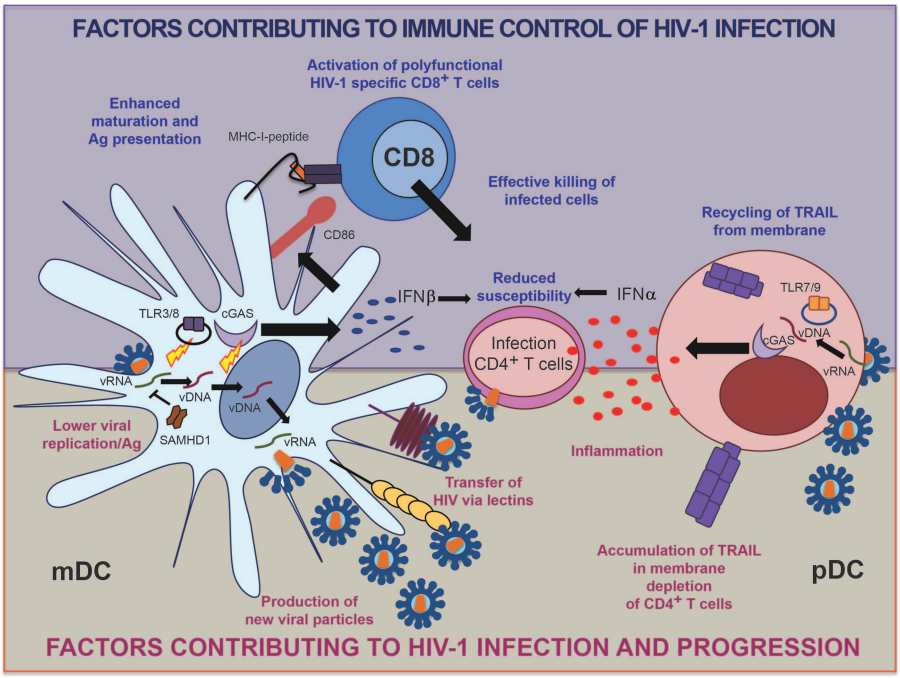

FIGURE 1 | Schematic representation of factors in human mDC and pDC contributing to immune control vs. progression of HIV-1 infection.

recent works in the SIV model have suggested that additional cell types might be responsible for abnormal activation of type I IFN responses at later stages of progressive infection (29). Together, cumulative information from recent studies suggests that DC distribution and function might be critically altered during HIV-1 infection and that preservation of physiological DC distribution and function is associated with immune control of the infection.

DCs AS VEHICLES FOR HIV-1 TRANSMISSION AND DISSEMINATION

During the last few years, several studies have shown that DCs have the ability to transfer HIV-1 particles to target $CD4^+$ T cells and facilitate their infection, in a process known as trans-infection (30). This phenomenon starts with the transference of HIV-1 virions to pockets in the membrane of DCs, where they accumulate and are subsequently actively transferred to T cells through virological synapses (31). The stability of such transmission events depends on the expression of adhesion molecules, such as Intercellular Adhesion Molecule (ICAM) (32) and the actin assembly machinery (33). In order to transfer viruses to $CD4^+$ T cells, DCs require the capture of HIV-1 particles through the lectin Dendritic Cell Intercellular Adhesion Molecule-3-Grabbing Non-integrin (DC-SIGN) (34, 35), and the Sialic acid-binding Immunoglobulin-type Lectin 1 (SIGLEC-1) receptor (36). Recent reports have shown that the ability of DCs to facilitate HIV-1 trans-infection is acquired upon activation with inflammatory molecules associated with poor HIV-1 prognosis, such as IFNα and LPS (37). These stimuli have been shown to induce SIGLEC-1 expression and therefore, enhance the capture and transfer of viral particles. Notably, circulating mDCs but not pDCs facilitate trans-infection of HIV-1 (32). Therefore, increased basal levels of immune activation and high-level viremia might contribute to disease progression through facilitation of viral trans-infection by mDCs. Consistent with a role of mDCs in supporting HIV-1 trans-infection in lymphoid tissues, it was shown that depletion of lymph node-resident mDCs in tissue-suspension cultures reduced the efficiency of HIV-1 infection of $CD4^+$ T cells (38). However, mutations of SIGLEC-1, which naturally occur in small proportions of individuals, did not seem to provide protection from HIV-1 infection or attenuation of HIV-1 disease progression (39), suggesting that classical, SIGLEC-1-independent HIV-1 dissemination within in the host remains the predominant mechanisms fueling viral infection *in vivo*.

DC SUSCEPTIBILITY TO HIV-1 INFECTION AND HOST RESTRICTION FACTORS

Most DCs express the coreceptor CD4 (40), and therefore are in principle susceptible to infection with HIV-1. However, DCs seem to represent a more hostile and restrictive environment for HIV-1 than $CD4^+$ T cells, for reasons that are not completely clear. While initial studies suggested that monocyte derived DCs (MDDCs) are highly resistant to infection with HIV-1 (41), primary mDCs are able to support some levels of HIV-1 replication, at least *in vitro* (42–44).

The main restriction factor that limits HIV-1 replication in MDDCs and mDCs seems to be the cytoplasmic protein SAM domain and HD domain-containing protein 1 (SAMHD1), which is highly expressed in myeloid cells and is able to block HIV-1 replication at the retro-transcription level by depleting endogenous intracellular pools of dNTPs (45), and by directly degrading viral RNA (46). While it is clear that SAMHD1 is a key factor limiting replication of HIV-1 in MDDCs (47) and inhibiting further spread of virions to T cells (48), recent studies demonstrated that MDDCs can actually support productive infection with HIV-1 to a certain degree, despite high levels of expression of this restriction factor (49). The functional ability of SAMHD1 to restrict HIV-1 replication is regulated by phosphorylation mediated by host kinases from the cyclin-dependent kinase family (50). Interestingly, the functionally active, de-phosphorylated form of SAMHD1 is preferentially found in primary DCs isolated *ex vivo* from human blood, which potentially could contribute to a higher resistance to infection (51). However, it is unclear whether restriction of HIV-1 by SAMHD1 in mDCs might truly benefit the host, since restriction of HIV-1 replication via SAMHD1 may impair cytoplasmic viral immune recognition in mDCs and impair their ability to prime HIV-1-specific T cells. On the other hand, interactions of mDCs with T cells induce downregulation of SAMHD1 expression (52), allowing human primary mDCs to be more permissive to infection (44). Importantly, recent data indicate that primary $CD1c^+$ and $CD141^+$ mDC subtypes might differ in their susceptibility to HIV-1 infection. In this regard, expression of the endosomal protein RAB15 prevents fusion of viral particles in $CD141^+$ mDCs and induces a higher level of cell-intrinsic resistance to infection with HIV-1 and HIV-2 compared to $CD1c^+$ mDCs (53). Further proof for the susceptibility of primary mDCs to HIV-1 infection was provided by a recent study identifying a distinct population of $CD1a^+$ mDCs residing in the vaginal mucosa, which supported CCR5-tropic but not CXCR4-tropic HIV-1 replication, in contrast to vaginal Langerhans cells (LC). These data suggest that these vaginal mDCs might play an active role in the selection of transmitted viral variants during heterosexual HIV-1 acquisition (54).

In the context of immune control of HIV-1 infection, recent studies suggest that monocytes and mDCs from HIV-1 controllers restrict early HIV-1 replication steps, specifically at the level of viral integration (44, 55, 56) while restriction of viral reverse transcription is less obvious, possibly due to lower induction of SAMHD1 expression in HIV-1 controllers upon exposure to HIV-1. This specific replicative pattern of HIV-1 may enable enhanced cytoplasmic sensing of accumulated HIV-1 reverse transcripts, which represent the primary substrate for innate immune recognition, and facilitate antigen processing and presentation (44, 56). Interestingly, although SAMHD1 is thought to be an interferon inducible gene, DCs and $CD4^+$ T cells fail to induce its expression in the presence of type I IFNs (57). Therefore, higher permissiveness of mDCs from controllers to viral reverse transcription may represent a key element for supporting cytoplasmic detection of HIV-1 and for inducing potent cell-intrinsic responses that lead to the effective activation of HIV-1-specific T cells (**Figure 1**).

Although SAMHD1 is recognized as a critical host factor limiting HIV-1 replication in myeloid cells, alternative SAMHD1-independent restriction mechanisms might also be playing a role in effective immunological control of HIV-1 replication. Among them, recognition of the HIV-1 capside by cyclophilin A (41, 58) and TRIM5 α (59, 60) or endogenous levels of β-catenin (52), could be actively contributing to block HIV-1 replication in myeloid cells. In addition, some studies suggest that HIV-1 could trigger TLR activation in DCs (61). Indeed, activation of MDDCs through TLR4 and TLR3 resulted in inhibition of HIV-1 replication steps in DC, while simultaneously increasing their ability to prime HIV-specific $CD8^+$ T cells (62). Therefore, TLR-dependent activation of DC could play a relevant role for inducing highly-functional cellular immune responses against HIV-1. Supporting this idea, polymorphisms in the TLR3 gene confer resistance to HIV-1 infection (63). In fact, it was recently suggested that TLR activation could be playing an active role in the detection of HIV-1 by primary $CD141^+$ mDCs (53). Therefore, more studies are required to investigate the mutual interplay between viral restriction in DCs and immune control of HIV-1, and to determine the contribution of myeloid cells to persisting viral reservoirs during suppressive antiretroviral therapy.

INNATE IMMUNE RESPONSES TO HIV-1 IN DCs

DCs are, in principle, capable of inducing secretion of type I IFNs upon recognition of viral nucleic acids, which subsequently leads to transcription of interferon stimulated genes (ISGs) and the upregulation of class II HLA and costimulatory molecules. As a result of such cell-intrinsic, IFN-dependent immune responses, mature DCs become more restrictive for viral replication, while the expression of molecules involved in antigen presentation and co-stimulation is increased. Whether mDCs can induce secretion of type I IFNs in response to HIV-1 is still highly controversial. In MDDCs, HIV-1 seems to be able to induce expression of several IFN-related genes in the absence of actual production of IFN α/β due to the selective activation of IRF-1 mediated signaling instead of inducing phosphorylation of IRF3, which is known to be required for induction of type I IFNs (64). However, the intracellular DNA sensor cGAS is expressed by myeloid cells (65) and is able of producing cGAMP second messengers upon recognition of HIV-1 DNA (66), leading to the activation of the sensor STING and the signal transducer TBK-1, which promote IFN β production (67, 68). Thus, primary DCs are, in principle, able to sense and induce type I IFN upon exposure to cytoplasmic HIV-1 DNA. In fact, activation of cGAS seems to be required for the transcription of IFN β by primary mDCs and MDDCs in the context of HIV-1 and other viral infections (44, 69, 70). This DNA-dependent mechanism of viral sensing leading to type I IFN responses might be more active in human $CD1c^+$ mDCs compared to $CD141^+$ mDCs (53). Interestingly, cGAS triggers TLR9-independent activation of primary pDCs in response to intracellular DNA (71, 72). However, current phenotypic

markers for pDC identify a heterogenous cell population that, in addition to *bona fide* pDC, contains pre-DC precursors of mDCs, which could also be differentially contributing to the observed responses to HIV-1 (73). Such heterogeneity could be the result of different pre-pDC and/or pDCs originated from either lymphoid or myeloid precursors with different functional properties (74–76). Therefore, more studies are required to elucidate the contribution of TLR-independent sensing of HIV-1 in pDCs. Finally, activation of the cGAS pathway by HIV-1 might involve interactions with additional host factors such as the newly identified NONO protein, which apparently is able to bind cGAS and the HIV-1 capsid and facilitate innate sensing of HIV-1 DNA in dendritic cells (77).

Importantly, preserved or enhanced induction of IFN responses has been described in both primary pDCs (23, 24) and mDCs (44) from HIV-1 controllers exposed to HIV-1. A recent single-cell RNAseq study has identified a highly functional population of $CD64^{Hi}CD86^{Hi}$ $PD\text{-}L1^{Hi}$ mDCs characterized by a strong type I IFN signature that is induced more efficiently in HIV-1 controllers than in progressors or healthy individuals in response to HIV-1 (78). The induction of such highly functional mDCs depended on the activation of TBK-1, which acts downstream of several intracellular sensing pathways including cGAS and TLR-3. Therefore, enhanced innate recognition of HIV-1 by both pDCs and mDCs might be a contributing factor to develop effective HIV-1 specific immunity in these individuals (**Figure 1**). However, HIV-1 might have evolved to minimize such mechanisms of viral DNA recognition, since HIV-1 Vif and Vpr are capable of inactivating TBK-1 which is downstream of the cGAS-STING pathway (79). Therefore, additional mechanisms such as alterations in the activation threshold of intracellular sensing pathways might be playing a role in DCs from controllers. In addition to sensing of viral DNA by cGAS, viral immune recognition in DCs could be connected with the RIG-I pathway, which may also contribute to activation of DCs in response to HIV-1 (80). In fact, communication and collaboration between RIG-I and DNA sensing pathways has been reported to amplify innate immune responses against intracellular viral DNA (81, 82). Although no genetic alterations in genes encoding for innate immune sensors for HIV-1 have been found in GWAS studies including large HIV-1-infected populations, a more targeted analysis of innate immune genes may in the future allow to identify immunogenetic polymorphisms in the innate immune system that facilitate innate immune sensing and natural viral control in specific subgroups of HIV-1 controllers.

ANTIGEN PRESENTING CELL FUNCTION OF DCs AND ADAPTIVE IMMUNITY AGAINST HIV-1

Given associations between the polyfunctionality of T cell responses and natural progression of HIV-1 infection (8), several studies have focused on the function of DCs as professional antigen presenting cells (APC) and how these cells are involved in the priming of adaptive immune cells. As mentioned before, both mDCs and pDCs can respond and mature to a certain degree in response to HIV-1, but may become exhausted and hyporesponsive during chronic progressive infection, which might impact their antigen-presenting cell function (83). In the case of pDCs, infection with HIV-1 seems to turn these cells more tolerogenic, and increase their potential to drive polarization of $CD4^+$ T cells into immunosuppressive T regulatory cells (84). On the other hand, although pDCs can activate $CD8^+$ T cells through cross-presentation (85), no studies have yet analyzed the impact of pDCs on the priming of HIV-1-specific cytotoxic $CD8^+$ T cell responses. In contrast, while circulating mDCs from healthy individuals are functionally incapable of efficiently priming T cells *in vitro* after exposure to HIV-1 (86), effective antigen presenting functions of mDCs from HIV-1 elite controllers is associated with enhanced abilities to prime HIV-specific $CD8^+$ T cells in these patients (44, 78). In addition to mDCs, recent *in vitro* studies have shown that MDDCs can acquire HIV-1 antigens from Langerhans cells, become activated and induce cross-presentation to $CD8^+$ T cells (87), suggesting that these cells may also be potentially able to prime protective HIV-1-specific cytotoxic $CD8^+$ T cells. In addition, independent studies have shown that MDDCs can mediate cross-presentation of immuno-dominant HIV-1 peptides and activate HIV-1-specific $CD8^+$ T cells (88). However, MDDC are not a physiological DC subset present and in fact more closely resemble inflammatory DCs (89). Nevertheless, a recent evaluation suggested that primary $CD141^+$ mDCs, obtain HIV-1 antigens from infected $CD1c^+$ mDCs for cross-presentation to $CD8^+$ T cells (53). Interestingly, DCs infected with HIV-1 can also present endogenous viral peptides and mediate activation of HIV-1-specific $CD4^+$ T cells (90). Therefore, mDCs and MDDCs might be involved in the priming of effective HIV-1-specific T cell responses observed in controllers.

Although highly functional HIV-1-specific $CD8^+$ T cell responses were identified as the main correlate of antiviral immune defense (91), the discovery of broadly neutralizing antibodies (bNAbs) against multiple strains of HIV-1 (92, 93), has led to a great interest in understanding their potential contribution to spontaneous immunological control of HIV-1. Recent works have indeed identified a subpopulation of HIV-1 viremic controllers who develop bNAbs in the absence of high levels of viremia or immune activation (94). In previous studies in viremic HIV-1-infected progressors, the induction of bNAbs was associated with the presence of $CXCR5^+$ $PD\text{-}1^+$ T follicular helper cells (Tfh) in the blood (95). Although Tfh cells facilitate B cell maturation and immunoglobulin class switching in lymphoid tissue (96), peripheral $CXCR5^+$ $PD\text{-}1^+$ $CD4^+$ T lymphocytes have been proposed to act as peripheral counterparts of Tfh cells (pTfh) (95, 97) and could serve as a peripheral biomarker of high germinal center Tfh cell activity. Therefore, the priming of Tfh cells by mDC might be important in HIV-1 controllers capable of inducing antibodies with broader neutralizing activity. Supporting this possibility, mDCs from controller neutralizers are more efficient in priming $CD4^+$ T cells into long lived $PD\text{-}1^{Lo}$ Tfh precursors, which can differentiate into functional $PD\text{-}1^{Hi}$ Tfh effector cells upon antigenic stimulation (98). Importantly, higher frequencies of $PD\text{-}1^{Lo}$ Tfh precursors in the blood are

correlated with higher breadth of Ab neutralization in this subset of controllers. Compatible with an indirect role of DCs for influencing humoral immunity through polarization of Tfh, mDCs from controller neutralizers are characterized by high levels of CD40, a molecule previously involved in Tfh cell differentiation (99), and display distinct transcriptional patterns that differ from those present in $CD64^{Hi}$ $PD\text{-}L1^{Hi}$ mDCs from elite controllers with high $CD8^{+}$ T cell priming potential (98). Therefore, these findings might suggest a range of functional specializations of mDCs from controllers that may contribute to immune viral control through different immune mechanisms. To which degree individual DC subpopulations influence other components of the innate and adaptive immune system and contribute to control of HIV-1 is still an open question that requires further study.

CONCLUSIONS

In this review, we have summarized recent advances in understanding DC biology in the context of HIV-1 immune control. While studies have revealed multiple mechanisms by which DCs might contribute to controlling HIV-1 (**Figure 1**), future studies will be necessary to evaluate the complexity of individual DC subsets in promoting beneficial versus detrimental effects during HIV-1 infection. Similarly, our knowledge about the intrinsic ability of pDCs and mDCs to sense and respond to HIV-1 has greatly improved over the last few years, but translating this insight into improved and more specific adjuvants for future preventive and therapeutic HIV-1 vaccines represents a considerable challenge. The development to new humanized animal models that recapitulate human DC biology will likely be critical to identify effective vaccination strategies based on DCs. Together, DCs are emerging as critical players of effective immune responses in HIV-1 and a closer understanding of these cells might contribute to the development of novel effective vaccines or immunotherapies.

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EM-G and XY conceived and wrote the manuscript.

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REVIEW
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How Monocytes Contribute to Increased Risk of Atherosclerosis in Virologically-Suppressed HIV-Positive Individuals Receiving Combination Antiretroviral Therapy

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Combination antiretroviral therapy (ART) is effective at suppressing HIV viremia to achieve persistently undetectable levels in peripheral blood in the majority of individuals with access and ability to maintain adherence to treatment. However, evidence suggests that ART is less effective at eliminating HIV-associated inflammation and innate immune activation. To the extent that residual inflammation and immune activation persist, virologically suppressed people living with HIV (PLWH) may have increased risk of inflammatory co-morbidities, and adjunctive therapies may need to be considered to reduce HIV-related inflammation and fully restore the health of virologically suppressed HIV+ individuals. Cardiovascular disease (CVD) is the single leading cause of death in the developed world and is becoming more important in PLWH with access to ART. Arterial disease due to atherosclerosis, leading to acute myocardial infarction (AMI) and stroke, is a major component of CVD. Atherosclerosis is an inflammatory disease, and epidemiological comparisons of atherosclerosis and AMI show a higher prevalence and suggest a greater risk in PLWH compared to the general population. The reasons for greater prevalence of CVD in PLWH can be broadly grouped into four categories: (a) the higher prevalence of traditional risk factors e.g., smoking and hypertension (b) dyslipidemia (also a traditional risk factor) caused by off-target effects of ART drugs (c) HIV-related inflammation and immune activation and (d) other undefined HIV-related factors. Management strategies aimed at reducing the impact of traditional risk factors in PLWH are similar to those for the general population and their effectiveness is currently being evaluated. Together with improvements in ART regimens and guidelines for treatment, and a greater awareness of its impact on CVD, the HIV-related risk of AMI and stroke is decreasing but remains elevated compared to the general community. Monocytes are key effector cells which initiate the formation of atherosclerotic plaques by migrating into the intima of coronary arteries and accumulating as foam cells full of lipid droplets. This review considers the specific role of monocytes as effector cells in

atherosclerosis which progresses to AMI and stroke, and explores mechanisms by which HIV may promote an atherogenic phenotype and function independent of traditional risk factors. Altered monocyte function may represent a distinct HIV-related factor which increases risk of CVD in PLWH.

Keywords: HIV, monocytes/macrophages, atherosclerosis, foam cells/macrophages, inflammation

INTRODUCTION

For PLWH at the present time, HIV infection is associated with different outcomes depending on access to care and treatment. In resource-constrained settings, those without access to antiretroviral therapy (ART), or with limited access to expensive, alternative ART that is required when first-line therapies fail, experience progressive disease with decreased CD4+ T cell counts ultimately leading to death from AIDS–related diseases. For PLWH who have access to effective antiretroviral regimens, current evidence suggests that life expectancy approaches that of HIV-negative individuals in the general population. Whilst the risk of AIDS and AIDS-related mortality may be low, long term health outcomes for these individuals depend on how soon after infection they are treated, with the best outcomes predicted for those who present early in infection with relatively preserved CD4 counts (1–5). Large cohort studies of persons from the United States, and from North America and Western Europe, have shown that the gap in life expectancy between PLWH and HIV-negative individuals has narrowed as a function of the year in which ART was initiated (2, 6). This is likely due to use of more potent and tolerable ART regimens and adoption of treatment guidelines recommending earlier commencement of ART at diagnosis of HIV.

CARDIOVASCULAR DISEASE IN PLWH

Despite a reduction in AIDS-related mortality, HIV infection remains associated with an increased risk of age-related, inflammatory diseases which cause significant morbidity such as non-AIDS cancers, neurocognitive impairment and cardiovascular disease (CVD). Cardiovascular diseases, or diseases of the heart and vasculature are major causes of morbidity and mortality. They include ischaemic coronary artery disease (chronic angina and AMI), cerebrovascular disease (stroke and transient ischaemic attack), other heart diseases (including arrhythmias, inflammatory heart disease, valvular disease, rheumatic heart disease, and heart failure) and other cardiovascular diseases (peripheral vascular disease, aortic aneurysms, and hypertensive disease). The incidence of many of these conditions is increased in PLWH [for recent reviews see (7, 8)]. The incidence of CVD may be decreased following ART and the impact of ART will depend on the degree to which inflammation and immune activation are decreased following HIV virologic suppression. This review focuses on coronary artery disease (CAD) in well-resourced settings since it is the major cause of mortality in the general population and is an increasing cause of mortality in PLWH as the median age of this population is increasing. Specifically, we look at mechanisms that increase the risk of sub-clinical atherosclerosis which is elevated in these populations and leads to the major causes of death from CVD, i.e., AMI and ischaemic stroke, and focus on the role of activated monocytes as a likely effector contributing to these mechanisms.

ELEVATED RISK OF CAD IN VIROLOGICALLY SUPPRESSED PLWH

In virologically suppressed individuals on stable and effective ART, CAD has become one of the leading causes of death (9). Early hospital record-based studies that compared the rate of AMI in large cohorts of HIV-positive and HIV-negative individuals presenting at the same US hospitals between 1996 and 2004, showed that the relative risk of AMI was 1.75-fold higher after adjusting for age, gender (*sic*), race, diabetes, hypertension, and dyslipidemia (10). Similarly, a study of US veterans enrolled since 2003 and followed-up until 2009, confirmed that the incidence of AMI was higher [HR = 1.48 after adjustment for Framingham risk score (FRS), co-morbidities and substance abuse] in HIV-positive veterans matched demographically to HIV-negative veterans (11). In both of these studies, participants were not restricted to those virologically suppressed by ART, and information on the proportion of successful virological suppression were not presented. Similar results were obtained in analyses of incidence of AMI in the French Hospital Database on HIV (FHDH-ANRS CO4) cohort (12) where standardized mortality ratios were 1.4 [95% confidence: 1.3–1.6] and 2.7 [1.8–3.9] compared to the general population for men and women, respectively. More recent studies have reported that the incidence is also higher when restricted to type 1 events, i.e., those associated with atherosclerotic plaque rupture or thrombosis, which are relevant as inflammatory co-morbidities in PLWH who are virologically suppressed on ART (13).

The higher rate of AMI reported in the above studies contrasts with a gradual decline of incidence in individuals enrolled via the Kaiser-Permanente health plan (California, USA) as a function of when HIV patients started ART. In those individuals who commenced ART between 2010 and 2011, the rate of AMI decreased to the point where the incidence was not statistically different from that in HIV-negative controls (14) and also no difference was observed in rates of AMI in PLWH who commenced ART at CD4 cell counts of $\geq$500/μL (15). Other studies report a reduction in AMI incidence over time as well;

Masia et al reported a decrease in standardized incidence rates in PLWH relative to the general population in Spain when the intervals between 2006–2009 and 2010–2014 were compared (16). The observed reduction in AMI risk may be, in part, due to prescription of ART regimens with lower associations with CVD, adoption of guidelines for earlier initiation of ART, and increased awareness of traditional risk factors for CVD in PLWH. The more frequent interactions of PLWH within healthcare settings, and the consequent impact on CVD risk monitoring and treatment, is difficult to correct for in cohort analyses, and may be an important confounder in comparing CVD risk in HIV-positive and HIV-negative populations. Also, we have limited long term data on CVD risk in virologically-suppressed PLWH and the HIV-related CVD risk in virologically suppressed PLWH on ART for long periods of time remains to be established.

WHY IS CVD RISK ELEVATED IN VIROLOGICALLY SUPPRESSED HIV-POSITIVE INDIVIDUALS?

There are several potential and recognized factors that may elevate risk of CVD in PLWH.

Traditional Risk Factors

A higher prevalence of traditional risk factors for CVD such as smoking (17–19), diabetes (20, 21), dyslipidemia (including hypercholesterolemia, triglyceridemia, low HDL cholesterol and abnormal fat distribution) and hypertension (22, 23) is found in HIV-positive populations in well-resourced settings. As many of these factors are modifiable, reduction in CVD risk attributable to traditional risk factors is as effective in PLWH as strategies and treatments for the general population (24). Recently, Althoff and colleagues have used data from the North American AIDS Cohort Collaboration on Research and Design to estimate the contribution of population-attributable risk factors for AMI, in addition to other non-AIDS comorbidities, and concluded that considerable reduction in risk could be obtained by reducing total cholesterol levels and hypertension in PLWH (25).

Antiretroviral Drugs

The risk of AMI associated with ART use was robustly reported in the Data collection on adverse events of ARV Drugs (D.A.D) study (26) in which duration of ART was associated with an adjusted relative risk of AMI of 1.26 [1.12–1.41] per year. Specific drugs, or classes of drug, have also been associated with CV events; recent, but not cumulative use, of abacavir (27) and continued use of protease inhibitors (26, 28) including some recently-introduced combinations (29). The study by Ryom and colleagues using data from the D:A:D cohort, showed that cumulative use of the boosted protease inhibitor combination darunavir/ritonavir was associated with a 60% increase in baseline risk of CVD (using a composite endpoint that included AMI and stroke, and also sudden cardiac deaths and invasive cardiovascular procedures) over 5 years of use. In contrast, use of atazanavir/ritonavir was not associated with an increase in risk. These findings were not explained when analyses were controlled for effects of dyslipidemia or a protective effect of hyperbilirubinemia. Deleterious effects of antiretroviral drugs contribute to traditional CVD risk factors including increased circulating levels of cholesterol and triglycerides (26), visceral adiposity (30), reduced high-density lipoprotein-associated cholesterol (HDL_c) levels (31), hypertension (32, 33) and metabolic syndrome (34–36) shown by studies linking the prevalence of these syndromes with years of ART (33). The hypothesized mechanism for the effect of current use of abacavir on AMI is via increased platelet reactivity, which reverses when abacavir is ceased (37, 38). Antiretroviral drugs probably mediate much of their effect on hypertension via their effects on other components of the metabolic syndrome such as diabetes, changes in HDL and low-density lipoprotein (LDL) levels and alterations in fat distribution which are themselves associated with hypertension; for a more complete discussion see (23, 33). Risks associated with ART can be ameliorated by avoidance of abacavir and specific protease inhibitors. Another factor is the effect that weight gain following ART initiation with various PI-based, integrase inhibitor-based and earlier non-thymidine analog-based regimens may have on CAD risk (39, 40). For further information, the reader is referred to a recent comprehensive review (41).

HIV-Related Chronic Inflammation

Atherosclerosis is an inflammatory disease (42). A recent meta-analysis has confirmed an association of plasma markers of inflammation with CVD in PLWH (43) particularly interleukin 6 (IL-6), D-dimer and high-sensitivity C-reactive protein (hs-CRP), which are the most extensively studied biomarkers. However, in individual studies measuring various outcomes related to CVD, biomarkers of myeloid activation such as sCD14 and sCD163 have been more closely associated [(44–46) and discussed further below].

Monocytes/macrophages possess a full complement of pattern recognition receptors that promote inflammation by stimulating production of high levels of pro-inflammatory factors. Measuring their activation status, most conveniently via soluble plasma markers or cell surface markers on monocytes, is therefore a valuable approach to assessing inflammation and innate immune activation in individuals. Using cross sectional studies we reported that virologically suppressed PLWH have higher levels of circulating plasma biomarkers of inflammation and of myeloid activation compared to age-matched HIV-negative individuals, but similar to levels found in much older individuals (47, 48) which is consistent with findings from other laboratories (45, 49). We further estimated that the increase in immune activation was equivalent to an additional 2–4 years of aging in virologically suppressed individuals (50) and reasoned that this increased the risk of age-related inflammatory co-morbidities by a commensurate rate. We measured phenotypic markers on blood monocytes and reported their continued alteration in virologically suppressed individuals (47), however plasma biomarkers of monocyte/macrophage activation such as CXCL-10 correlate with monocyte subset and phenotypic alterations

(51) and are a more convenient and robust measure in clinical studies.

The significance with respect to CAD of the extent to which immune activation and inflammation persist in PLWH on effective ART is evident from studies showing that measures of myeloid activation and inflammation are associated with non-calcified plaque (52, 53), coronary artery calcium (54, 55), carotid intima-media thickness (46) and predict cardiovascular events and death (56–59). Expression of tissue factor on monocytes, important for initiating platelet activation, is increased by TLR ligands such as LPS elevated in settings of HIV infection (60), although not at the concentrations found in the plasma of PLWH (61), and by thrombin (61). Schechter and colleagues further showed that tissue factor expression was restricted to CCR2+ classical monocytes (a discussion of human monocyte subsets is given below) although others have implicated CD16+ monocytes (62). While a discussion of late events in atherosclerosis and mechanisms of plaque rupture are outside the scope of this review, it is important to note that in a primate model of HIV, administration of an inhibitor of tissue factor activity on monocytes, Ixolaris, decreased immune activation (61). The association of monocyte activation markers and soluble myeloid activation biomarkers with incidence of CAD and with cardiovascular events is strong circumstantial evidence for a mechanistic role of activated monocytes in these processes. However, the mechanisms by which monocytes may contribute to CAD are best identified by functional comparisons of these cells present in PLWH and in well-matched HIV-negative individuals.

HIV-Specific Mechanisms

HIV produces pathogenic factors such as the Negative Regulatory Factor, Nef, that are potential mediators of HIV-related morbidities. HIV-infected foam cell macrophages have been detected in coronary arteries obtained post-mortem from HIV-positive individuals, and Nef has been shown to stimulate their formation by inhibiting reverse cholesterol efflux from macrophages (63), suggesting a direct mechanistic link. The significance of these observations is discussed in more detail below.

HIV-ASSOCIATED ATHEROSCLEROSIS

AMI follows thickening of the arterial wall by atherosclerotic plaque formation and eventual occlusion of coronary arteries. Ischaemic strokes result when unstable atherosclerotic plaques rupture and lodge in the brain, while peripheral artery disease shares similar initial events to CAD of plaque formation and narrowing of arteries at other sites. Both are significant co-morbidities elevated in PLWH. Atherosclerotic plaques identified in virologically suppressed PLWH are more likely to be non-calcified plaques considered to be unstable and high risk (64, 65).

As atherosclerosis underlies coronary artery diseases it is important to understand whether it is more prevalent in virologically suppressed HIV-positive individuals and how HIV impacts its development. Early studies reported increased pre-clinical atherosclerosis assessed by surrogate measures such as carotid artery intima media thickness (cIMT) in PLWH compared to HIV-negative controls (66, 67). More recently, Leon et al. reported that in a well-controlled virologically suppressed HIV-positive population with a low cardiovascular risk, as measured using the FRS, of <10%, 21% had sub-clinical atherosclerosis determined by carotid artery ultrasonography (68). In this group of patients, who had median FRS of 1% and all with an FRS<8%, traditional risk factors did not account for the high prevalence of sub-clinical atherosclerosis, but the authors found high IL-6 levels (>6.6 pg/mL) resulted in an odds ratio of 9 for sub-clinical atherosclerosis. These findings suggest an involvement of inflammation in heightened CVD risk in these subjects rather than the traditional risk factors accounted for by FRS (age, smoking, dyslipidemia, total and HDL cholesterol, systolic blood pressure).

An important factor underlying increased immune activation in PLWH is that most are cytomegalovirus (CMV) seropositive, and have an impaired ability to control CMV reactivation. CMV infection in PLWH has been associated with expansion of a unique population of adaptive natural killer cells which serve as a sensitive marker for the presence of CMV infection (69, 70). Using small cross-sectional studies of 93 PLWH and 37 healthy, HIV-negative controls, it has been shown that sub-clinical atherosclerosis (as measured by cIMT) is correlated with CD8 T cell responses to CMV antigen (pp65) (66). CMV infection in PLWH is also associated with expansion of CD8+ T cells expressing fractalkine receptor (CX3CR1; involved in endothelial homing and adhesion) and the PAR-1 receptor which can be activated by thrombin, although the impact of these cells on clinical atherosclerosis is unknown (71). Other studies have found correlations between CMV antibody (IgG) levels and carotid artery stiffness in HIV-positive women, although association of IgG with the prevalence of cardiovascular lesions was restricted to those who achieved virologic suppression (72). More recently, in a small cross-sectional study of 105 PLWH matched to 105 healthy, age- sex- and smoking-matched controls, a correlation was observed between CMV IgG levels and cIMT (73).

There are few well-controlled studies comparing the relative prevalence of atherosclerosis in well-managed virologically suppressed PLWH to appropriate HIV-negative controls. One such study found that the prevalence of coronary plaque as measured by Coronary Computed Tomography Angiography (CCTA) was higher in 78 HIV-positive men (of whom 95% were currently receiving cART and 81% were virologically suppressed) compared to 32 HIV-negative men recruited from the same clinics in Boston (59 vs. 34%, $p = 0.02$) (74). A similar larger study from the Multicentre AIDS Cohort Study confirmed these findings with a 1.25-fold higher prevalence of plaque after adjustment for cardiovascular risk factors (75). In contrast, studies from the Swiss HIV cohort have reported little or no increase in prevalence of high risk, non-calcified plaque and lower coronary atherosclerosis involvement than HIV-negative persons with a similar FRS (76). A smaller study demonstrated that HIV-positive women are more likely to have non-calcified plaque compared to HIV-negative women, after controlling for known cardiovascular risk factors, although prevalence of

calcified plaque was not increased (52). The presence of non-calcified plaque is associated with higher levels of the macrophage activation marker sCD163 and with CVD in HIV-positive individuals (52, 53). A meta-analysis of 9 studies involving 1,229 HIV-positive individuals and 1,029 HIV-negative controls, concluded there was an increased prevalence of non-calcified plaque in PLWH as detected by CT (OR = 3.26 [1.30–8.18]) (77). Larger studies are required to measure the relative prevalence of non-calcified plaque in PLWH who commence ART with well-preserved CD4 counts.

ACCUMULATION OF ACTIVATED MACROPHAGES IN THE ASCENDING AORTIC ARCH IS ASSOCIATED WITH CVD IN HIV-POSITIVE INDIVIDUALS

[^{18}F] FDG-PET is a technique which uses positron emission tomography to image uptake of [^{18}F] 2-deoxyglucose into cells and tissues. There is evidence [for a summary, refer to papers listed in (44)], and it is assumed, that the main cell type that accumulates [^{18}F] 2-deoxyglucose is the activated monocyte-derived macrophage, and that therefore the intensity of radioactivity detected by PET reflects accumulation and activation of monocytes to the activated endothelium. More specific macrophage tracers are being developed which leverage the fact that macrophages and dendritic cells express CD206, the mannose fucose-receptor: these include ^{99m}Tc-diethylenetriaminepentaacetic acid—mannosyldextran (Tilmanocept or LymphoSeek^{TM}) (78, 79) and radiolabeled anti-MFR monoclonal antibodies (80) but they have yet to be used extensively in studies of CVD risk in HIV.

In a proof-of-concept study, a greater uptake of [^{18}F] FDG occurred in both the carotid arteries and the aorta of 9 PLWH compared to 5 HIV-negative participants (81). Similarly, in 27 virologically suppressed HIV-positive individuals without evidence of cardiovascular disease and age-, sex-, and CVD risk-matched to 27-HIV-negative individuals, the HIV-positive participants had greater accumulation of ^{18}F radiolabel in the ascending aortic arch at levels comparable to those measured in 27 HIV-negative individuals with cardiovascular disease (44). These results provide evidence that HIV infection, even with successful virologic suppression, is associated with similar levels of arterial inflammation as found in individuals with pre-existing CVD. Follow-up studies from the same group showed that the arterial inflammation in a similar group of 41 HIV-positive patients was associated with higher numbers of high-risk coronary plaque (82).

The accumulation of [^{18}F] FDG is evidence for activated macrophages accumulating in the arteries of the heart, and its association with plaque with low attenuation and positive remodeling emphasizes the clinical significance of this accumulation, but it does not provide a mechanism by which this occurs. In HIV-positive participants, higher accumulation of FDG was moderately associated with elevated plasma levels of sCD163, but not D-dimer or hs-CRP (44). This is consistent with a specific link with activated macrophages but not with generalized inflammation or thrombotic disease. Unfortunately, plasma markers of endothelial activation were not investigated. Taken together these studies suggest that atherosclerosis is elevated in PLWH receiving effective ART due to a greater accumulation of monocyte-derived macrophages in coronary arteries leading to formation of non-calcified, high risk plaques. Significantly, it has been reported that whereas treatment of PLWH with atorvastatin improved lipid profiles and reduced the prevalence of non-calcified plaque in these individuals after 12 months, there was no reduction in the accumulation of FDG measured by [^{18}F] FDG-PET (83). This suggests that accumulation of activated monocyte-derived macrophages in the heart is caused by factors other than dyslipidemia and that it may represent an independent risk factor for CVD in PLWH. This may be specifically associated with myeloid cell activation. It is therefore important to understand how effective therapies used to reduce hypercholesterolemia are at reducing inflammation and monocyte activation that may contribute to CVD in PLWH. Initial reports from the SATURN-HIV trial showed that whereas treatment with the statin rosuvastatin was effective at reducing LDLc levels in HIV patients after 24 weeks, it was not effective in reducing inflammatory markers studied including well-established markers such as IL-6, hsCRP, and soluble TNF receptor isoforms (84). In contrast, lipoprotein-associated phospholipase A2 [an enzyme produced by myeloid cells and an independent predictor of CVD (85)] was significantly decreased in the statin arm compared to the placebo arm of the trial. Follow up studies of the same cohort indicated greater reduction in immune activation as assessed by various biomarkers: after 48 weeks, treatment with rosuvastatin reduced levels of the inflammatory marker IP-10 (interferon-γ-inducible protein-10 i.e., CXCL10) and the myeloid activation marker sCD14, as well as the proportion of non-classical monocytes expressing tissue factor (86). However, even after 48 weeks treatment with rosuvastatin, inflammatory markers IL-6, hsCRP, D-dimer and soluble TNF receptors were not reduced in this study (*ibid*). These data underscore the importance of monocyte activation in the mechanism leading to increased atherosclerosis and emphasizes the necessity of examining the functional properties of monocytes in these individuals to understand how their altered behavior might favor atherosclerotic plaque formation.

INITIATION OF ATHEROSCLEROTIC PLAQUE FORMATION

Atherosclerotic plaques tend to form in arteries at sites of perturbed blood flow such as near bifurcations in arteries. The initiation of atherosclerotic plaque formation is accelerated by the presence of an activated endothelium at these sites. In individuals with traditional risk factors such as high levels of triglycerides, total cholesterol, or LDL_c in plasma, lipid accumulates in the intimal layer beneath the endothelium where it is oxidized by reactive oxygen intermediates produced by activated endothelial cells and macrophages. The action of oxidative pathways derived from endothelial cells and macrophages produce different oxidized species in LDL particles

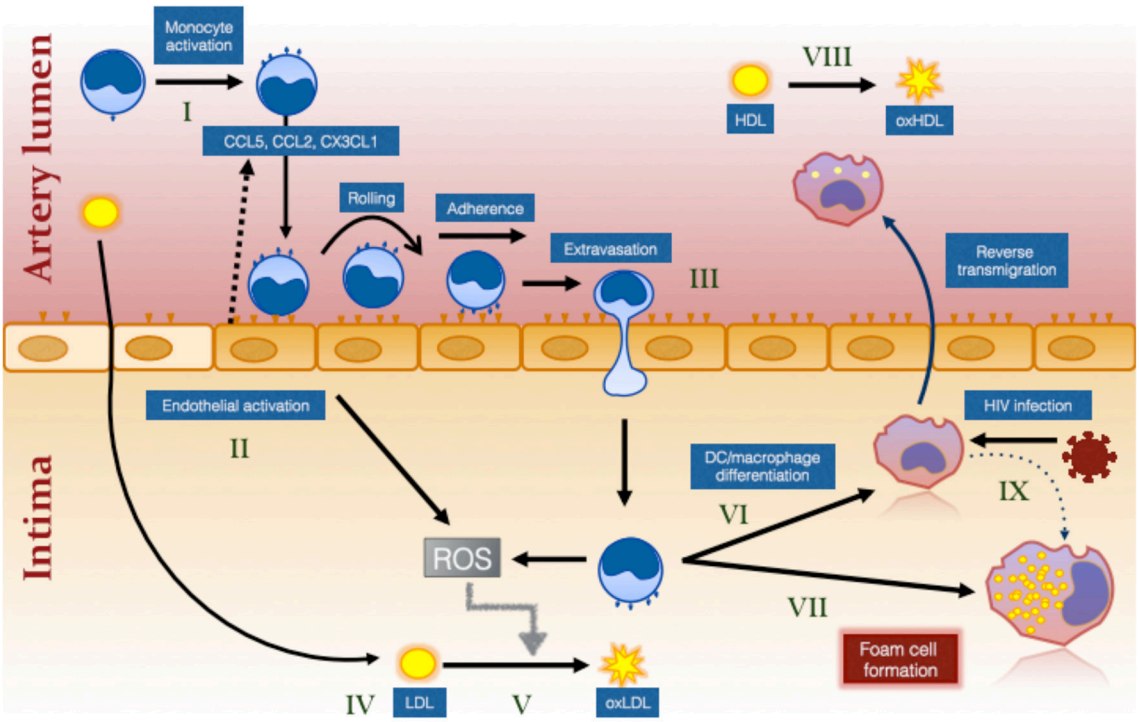

FIGURE 1 | The effect of HIV infection on monocyte- and inflammation-mediated mechanisms of foam cell formation. HIV infection causes systemic monocyte activation (I) due to factors unresolved by ART including residual HIV viremia, CMV reactivation, elevated bacterial ligands and oxidative stress. Gut bacterial ligands (eg., lipopolysaccharide; LPS) activate classical and intermediate monocytes via CD14/TLR4 receptor while viral ligands activate non-classical monocytes via TLR7/8 (144). Unresolved inflammation also activates the endothelium (II) which secrete chemokines to attract different monocyte subsets (not illustrated) via specific chemokine receptors, CCR2, CCR5, and CX3CR1 (99). Intermediate monocytes in particular exhibit increased pro-atherogenic properties (127). Activation of the endothelium and of monocytes results in greater monocyte adherence, rolling, firm adhesion and extravasation, the last via either paracellular, or transcellular mechanisms (III) [reviewed in (145)]. Cholesterol accumulates in the intima due in part to ART-induced dyslipidemia and to increased traditional risk factors in PLWH (IV). This is oxidized by the activated myeloid and endothelial cells which produce reactive oxygen intermediates (ROS) (V). Monocytes that have migrated into the intima ingest LDL and oxLDL via LDL receptor and CD36/SR-A1/II (131), respectively and either mature into monocyte–derived dendritic cells (VI) and reverse migrate out of the intima or mature into immobile foam cells (VII). Changes in monocytes, including decreased ABCA1 expression (126), favors pathway (VII) over pathway (VI). Oxidation of HDL (VIII) that also occurs in virologically suppressed PLWH impairs the protective function of this lipoprotein and further increases the risk of atherosclerotic plaque formation. Direct infection of macrophages in the intima also induces an atherogenic phenotype promoting foam cell formation (IX).

(87, 88) but both lipid species have been found in atherosclerotic plaque-derived lipids, suggesting that both endothelial cells and macrophages participate in this pathway (89). Oxidized LDL (oxLDL) is a known risk factor for CVD which has been shown in one study of HIV-positive individuals (91% of whom received ART) (90) and in a second study (of patients with varying degrees of HIV viremia) (91) to be elevated in plasma, and is associated with subclinical atherosclerosis in this population (92, 93).

THE ROLE OF MONOCYTE SUBSETS IN ATHEROSCLEROTIC PLAQUE FORMATION

Monocytes initiate the process of atherosclerotic plaque formation following their transmigration from blood into the intima of arteries at sites of activated endothelium and cholesterol deposition ("fatty streaks") (**Figure 1**). Here they mature into foam cells which are macrophages containing large numbers of lipid droplets (94). They also have important inflammatory roles in atherosclerotic plaque progression and rupture. The present review focuses on their role in atherosclerotic plaque initiation.

Monocytes are currently classified into subsets based on the expression of CD14 which is the co-receptor for Toll-like receptor 4 (a pattern recognition receptor that recognizes lipopolysaccharide derived from gram-negative bacterial cell walls) and CD16 (the intermediate affinity IgG receptor, FcγRIII). The majority of monocytes in circulation are $CD14^{hi}$CD16- classical monocytes which represent ∼90% of the total in healthy individuals, while non-classical or patrolling monocytes ($CD14^{dim}$CD16+) and intermediate monocytes ($CD14^{hi}$CD16+) are minor subsets which each represent ∼5% of

circulating monocytes. It is likely that monocytes in circulation represent a continuum of classical monocytes newly emerging from the bone marrow and gradually maturing into intermediate then non-classical monocytes (95) or re-emerging from tissue sites such as the spleen (96, 97).

Monocyte subsets have different functional properties and must therefore be considered separately with respect to their roles in inflammation and atherosclerosis. Recruitment of monocytes to arteries at sites of inflammation is directed by chemokines released by activated endothelial cells, but the multiplicity of chemokines and their receptors makes it difficult to determine the role of individual chemokines. Mouse models have been particularly valuable in this context: using the ApoE knockout/western-type diet model of atherosclerosis, early experiments revealed the importance of the chemokine receptor CCR2 in atherogenesis (98) and subsequently that the murine equivalent of the classical monocyte subset ($Ly6C^{hi}$ CCR2+ monocytes) migrates in response to the chemokine CCL2 (99, 100). It was reported by Tacke et al that classical monocytes also migrate into atherosclerotic plaques in response to the fractalkine receptor, CX3CR1 which is unexpected since they do not express high levels of this receptor, and furthermore that non-classical monocytes ($Ly6C^{lo}$CX3CR1+) mainly migrate into atherosclerotic lesions in response to CCR5 but not CX3CR1 even though the latter is highly expressed in this subset (99). The recruited monocytes were shown in these studies to differentiate into lesional macrophages and therefore to potentially participate in disease progression. Neither study examined the migration of intermediate monocytes which are not a well-defined subset in mice. Support for the role of CCR5 in monocyte recruitment into atherosclerotic plaques has been obtained using the ApoE knockout mouse model of atherosclerosis, where it was shown that monocyte recruitment into plaques, and plaque progression, was reduced with treatment using the CCR5 antagonist Maraviroc, which is of relevance to PLWH who may be treated with ART regimens containing this drug (101). Following AMI, monocytes are also recruited into atherosclerotic plaques with distinct kinetics to co-ordinate left ventricular repair. In this setting, classical monocytes are recruited initially in response to CCR2 ligands, where they phagocytose necrotic debris and orchestrate pro-inflammatory responses (102). Non-classical monocytes are recruited later and participate in tissue repair, angiogenesis and extracellular matrix deposition, although in this study their migration was reported to be dependent on fractalkine receptor (*ibid*).

There are no extensive data on how HIV infection, especially in settings of virologic suppression with ART, affect chemokine receptor expression on individual monocytes to modulate monocyte recruitment to atherosclerotic lesions. It is reported that HIV-infection alters proportions of CCR2- and CX3CR1-expressing monocytes in circulation, but this may be due to alterations in the distribution of monocyte subsets. In a cohort of virologically suppressed HIV-positive individuals with low cardiovascular risk, we have previously demonstrated an association between the proportion of CX3CR1+ CD16+ monocytes and cIMT, supporting a potential link between monocyte recruitment and atherosclerosis (103). We, and others, have reported that intermediate and non-classical CD16+ monocytes, are expanded in viremic PLWH (104–108) but no difference in subset proportions is seen in PLWH who are virologically suppressed (109). Recently, using a well-controlled longitudinal cohort of PLWH commencing ART, we showed that intermediate and non-classical monocyte proportions decreased rapidly on commencement of ART, reaching control levels within ∼6 months of therapy (108). Taken together, these data suggest that when assessing the roles of individual subsets in CVD risk and progression, the impact of HIV infection and of ART on monocyte subsets must be considered.

Recruitment of monocytes to sites of atherosclerotic plaque formation is favored by increased expression of adhesion receptors such as ICAM-1 on activated endothelial cells (110). Systemic inflammation may also activate circulating monocytes to increase expression of adhesion receptors such as CD11b/CD18 and CD11d/CD18 (111) which recognize their cognate ligands such as ICAM-1 and VCAM-1 expressed on activated endothelium (112). Following attachment, monocytes transmigrate across the endothelium and enter the intima where they ingest deposited lipids via scavenger receptors including CD36 and scavenger receptor A1 and AII, the major receptors for oxLDL expressed on macrophages. Monocytes can reverse transmigrate and transfer lipid to acceptor molecules such as HDL, a process thought to maintain the health of the artery and regress atherosclerotic plaque development (113, 114) or they can differentiate into foam cells, which are large, immobile cells (115) characterized by lipid accumulation in lipid droplets and lysosomes, and are among the earliest pathogenic feature of atherosclerotic plaque formation. Lipids derived from oxLDL accumulate in lysosomes and in this intracellular compartment, unlike lipids found in lipid droplets, do not readily participate in cholesterol efflux pathways (116). Lysosomal foam cell lipids persist as a stable store, as data from the White Carneau Pigeon model of atherosclerosis indicates, after a change in diet (117). Thus, the differentiation/maturation pathway of the monocyte, which is dictated in part by the type of lipid ingested, may determine the initiation of atherosclerotic plaque development.

While models of atherosclerotic plaque development rightly emphasize the role of lipid accumulation in the artery wall as the initial stimulus activating the endothelium, attracting monocytes and acting as a substrate for foam cell formation, it is also clear that inflammatory mechanisms intersect this mechanism by independently activating monocytes and endothelial cells and oxidizing lipids by promoting oxidative stress pathways (118) (See **Figure 1**). However, as discussed above, PLWH may have elevated risk of atherosclerosis independent of traditional risk factors such as elevated total cholesterol and LDL_c, and this increased risk may in part be accounted for by their increased levels of inflammation and monocyte activation. It is important to realize that foam cell formation by monocyte-derived macrophages may occur in response to toll-like receptor ligands including HIV ssDNA (119), rather than lipoprotein particles [reviewed in (118)] which is of relevance to HIV infection where there is evidence for elevated levels of circulating endotoxin as well as other TLR ligands derived from microbial translocation even in

virologically suppressed individuals (47, 120, 121). Finally, as discussed further below, foam cells may be induced following alterations in macrophage cholesterol metabolism and efflux and metabolic changes in circulating monocytes may predispose them to differentiate into foam cells following transendothelial migration.

HOW DOES HIV INFECTION INFLUENCE MONOCYTES TO PROMOTE CVD?

The association of plasma biomarkers of myeloid activation with CVD in HIV-positive individuals, especially where other markers of inflammation are not strongly associated (44, 53), suggest that activated monocytes or macrophages play a direct role in promoting atherosclerosis. However, while plasma levels of molecules released from activated myeloid cells such as sCD163 may be useful prognostic biomarkers, they do not provide information about the mechanistic link between CVD and particular myeloid cell types and their functions. For this, functional studies of cells obtained from PLWH are required.

Macrophages containing the major HIV antigen (p24 capsid protein) have been detected in coronary arteries present in autopsy material obtained from patients receiving ART (63). These cells were adjacent to the lipid core of the plaque and had morphological characteristics of foam cells. Using an *in vitro* infection model, Bukrinsky and colleagues showed that HIV infection of monocyte-derived macrophages impaired reverse cholesterol transport and down modulated the cholesterol transporter ATP Binding Cassette family member A type 1 (ABCA1) via a mechanism requiring the HIV accessory protein, Nef (122). This molecule is the major transporter through which cholesterol is effluxed from macrophages to acceptor molecules like apolipoprotein A1 in HDL particles. It was further shown that injecting Nef protein into mice to achieve circulating levels reported in viremic HIV+ individuals, exacerbated dyslipidemia and development of plaque in the high fat diet ApoE$^{-/-}$ mouse model of atherosclerosis. This work suggests a plausible mechanism for promoting atherosclerosis in PLWH, but its significance to their CVD risk depends on the prevalence of HIV-infected macrophages in coronary arteries and on the concentration of Nef circulating in virologically suppressed individuals, for which there is currently a paucity of information. It would be of interest to use modern techniques such as DNA- and RNAscope to evaluate HIV infection in resected coronary arteries and to accurately determine circulating levels of Nef protein in a current cohort of PLWH.

Given the persistence of monocyte activation in ART-treated PLWH and its association with CVD, we reasoned that monocytes present in PLWH may have pro-atherogenic functional properties induced via bystander mechanisms dependent on HIV infection, even if these cells are not infected with the virus. To address this, we used a static model of atherosclerotic plaque development in which a type 1 collagen matrix is overlaid with a monolayer of primary human endothelial cells (HUVEC) and monocytes freshly isolated from study subjects are added (123, 124). In this model, monocytes transmigrate across the HUVEC monolayer to enter the collagen matrix, and the fate of the monocytes is followed over the subsequent 48 h. This model has been used extensively to determine the reverse transmigration properties of monocytes and the endothelial cell-expressed molecules such as PECAM-1 governing extravasation (113, 125), but had not previously been used to compare monocyte behavior from clinical cohorts. Using this model, we have shown that monocytes isolated from virologically suppressed PLWH have a higher propensity to mature into foam cells than monocytes from age-matched HIV-negative individuals, and that plasma from these individuals contains factors that promote this functional phenotype (126). In this study, monocytes from PLWH also exhibited impaired reverse transmigration out of the collagen and across the HUVEC monolayer, which is possibly linked to their greater propensity to mature into immobile foam cells. These functional defects were accompanied with impaired cholesterol efflux and reduced ABCA1 expression at the mRNA level. We are currently examining monocytes isolated from a larger group of virologically suppressed PLWH with low/medium FRS, to determine whether we can detect the same monocyte atherogenic phenotype and, if so, whether interventions to reduce CV risk will improve their functional properties (Angelovich, Hearps, Trevillyan, Hoy, and Jaworowski unpublished).

Experiments we have conducted using this *in vitro* model have shown that the intermediate monocyte subset has the greatest propensity of the three monocyte subsets to differentiate into foam cells (127) and expresses the highest levels of intracellular TNF and IL-6, both in the steady state and in response to LPS (128). Thus, to the extent that the proportion of this monocyte subset is elevated in PLWH, this may exacerbate the pro-atherogenic properties of monocytes in these individuals.

It is interesting that monocytes isolated from virologically suppressed PLWH possess similar functional defects in cholesterol transport as the HIV-infected monocyte-derived macrophages described by Bukrinsky and colleagues, although there is no evidence to date that Nef is the circulating factor causing this functional change in the subjects we studied. Monocytes from relatively old but healthy HIV-negative individuals have similar properties (127) strengthening the view that there are similarities in monocyte inflammatory behavior induced by HIV and by healthy aging, and suggesting that mechanisms apart from Nef contribute to this phenotype.

The model we use to study monocyte atherogenicity *in vitro* involves activation of the endothelial monolayer with TNF, simulating an inflammatory milieu *in vivo*. TNF can decrease expression of ABCA1 (129) and via this mechanism promote foam cell formation, but in the above experiments it was removed from the culture medium before monocytes were added. Furthermore, ABCA1 expression was decreased in freshly isolated monocytes from HIV-positive subjects. The factors that reduce ABCA1 expression in monocytes

from PLWH *in vivo* remain to be established. As a static model, this system does not provide reliable information about the adhesion and transmigration properties of monocytes *ex vivo*, only their behavior post migration. Measurements conducted under shear flow conditions will need to be made to address whether monocytes from virologically suppressed PLWH have abnormal endothelial adhesion and transmigration properties.

ABNORMAL LIPOPROTEINS IN PLWH MAY INFLUENCE MONOCYTE BEHAVIOR

It is important to consider that the pro-atherogenic phenotype of monocytes from virologically suppressed HIV-positive individuals revealed using the atherosclerotic plaque model is evident in the absence of added exogenous lipid or lipoprotein particles. However, we have used the same model to show that transmigrated monocytes can be induced to differentiate into foam cells by the addition of known atherogenic lipids such as oxLDL (124) and by LPS (127) into the culture medium. This may, in part, explain the pro-atherogenic properties of plasma from these individuals. LDL is oxidized (89) and taken up by macrophages via scavenger receptor A and CD36 (130–132) and since their expression is not regulated in a cholesterol-dependent manner, this promotes foam cell formation.

HDL is normally a protective lipoprotein with respect to CVD since its major protein component, apolipoprotein A1, functions as a cholesterol acceptor from cells. However, HDL in plasma of PLWH exhibits an increased level of oxidation, and the levels of oxidized HDL (oxHDL) correlate with markers of systemic inflammation such as IL-6 and hsCRP (133). We have isolated HDL particles from plasma obtained from a limited number of virologically suppressed PLWH, incorporated the isolated particles into the atherosclerotic plaque model and showed that they promote foam cell formation by monocytes from healthy, HIV-negative individuals. This behavior was associated with defective redox properties of the HDL particles (134). Functional defects in HDL particles isolated from PLWH have been reported by others, specifically a decreased level of the enzymes Paraoxonase (PON) 1 and 3 and a decreased level of PON redox activity (135). PON enzymes have important anti-inflammatory properties: in the context of atherosclerosis they inhibit the oxidation of LDL particles (136–138) and reduce monocyte-attracting chemokine expression by the endothelium (139, 140). PON also inhibits cholesterol biosynthesis (141) and stimulates cholesterol efflux from macrophages (89), properties that reduce the propensity of macrophages to develop into foam cells. While human macrophages do not express PON1 or PON 3 these effects can be mediated by PON delivered via HDL particles (*ibid*).

The behavior of monocytes isolated from virologically suppressed PLWH and of monocytes from healthy subjects in response to HDL particles from PLWH are consistent with the observation of lower PON1 and 3 levels in these particles. As PON can be displaced from HDL particles by inflammatory proteins such as serum amyloid A in circumstances such as acute infection (142, 143) it will be critical to understand which factors promote loss of PON1 and 3 in chronic HIV infection.

CONCLUSIONS

While the relative risk of death from AMI has decreased significantly for PLWH and has approached that for comparable members of the general community, CVD will become an increasing cause of morbidity and mortality as the HIV-positive population ages. It is still unclear to what extent the risk of atherosclerosis is elevated in patients who commence ART at high CD4 counts, especially as individuals may be treated with ART for many decades, which is longer than follow up studies conducted to date. Monocytes are principal effectors that initiate atherosclerotic plaque formation at sites of endothelial activation and damage. The "decision" of monocytes to differentiate into foam cells rather than extravasate from the intima influences the chance that initiation and progression of atherosclerotic plaques occurs. Functional studies using freshly isolated monocytes from virologically suppressed HIV-positive individuals are consistent with a phenotype that promotes atherosclerosis and therefore suggest that therapies designed to target this phenotype will prove beneficial. Future studies should assess the impact of therapies that reduce inflammation including statins and agents that target pathogen-induced monocyte/macrophage activation to reduce the tendency of monocytes to migrate across activated endothelia and differentiate into foam cells. A better understanding of the transcriptional changes in monocytes in PLWH, especially those involved in cholesterol/lipid metabolism and accumulation, will inform pathways that need to be targeted to prevent this. Emerging technologies such as improvements to [^{18}F] FDG-PET might be useful in assessing how successful interventions are at reducing the accumulation of activated macrophages in the heart. PLWH should be monitored for CV risk, encouraged to reduce modifiable risk factors and treated appropriately. The use of novel biomarkers such as oxLDL and oxHDL to improve risk prediction in the setting of HIV infection should also be considered.

AUTHOR CONTRIBUTIONS

AJ conceived and wrote the manuscript. AH, JH, and TA contributed to the manuscript. All authors reviewed and approved the final manuscript.

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REVIEW
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The HIV Reservoir in Monocytes and Macrophages

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In people living with HIV (PLWH) who are failing or unable to access combination antiretroviral therapy (cART), monocytes and macrophages are important drivers of pathogenesis and progression to AIDS. The relevance of the monocyte/macrophage reservoir in PLWH receiving cART is debatable as *in vivo* evidence for infected cells is limited and suggests the reservoir is small. Macrophages were assumed to have a moderate life span and lack self-renewing potential, but recent discoveries challenge this dogma and suggest a potentially important role of these cells as long-lived HIV reservoirs. This, combined with new HIV infection animal models, has led to a resurgence of interest in monocyte/macrophage reservoirs. Infection of non-human primates with myeloid-tropic SIV implicates monocyte/macrophage activation and infection in the brain with neurocognitive disorders, and infection of myeloid-only humanized mouse models are consistent with the potential of the monocyte/macrophage reservoir to sustain infection and be a source of rebound viremia following cART cessation. An increased resistance to HIV-induced cytopathic effects and a reduced susceptibility to some antiretroviral drugs implies macrophages may be relevant to residual replication under cART and to rebound viremia. With a reappraisal of monocyte circulation dynamics, and the development of techniques to differentiate between self-renewing tissue-resident, and monocyte-derived macrophages in different tissues, a new framework exists to contextualize and evaluate the significance and relevance of the monocyte/macrophage HIV reservoir. In this review, we discuss recent developments in monocyte and macrophage biology and appraise current and emerging techniques to quantify the reservoir. We discuss how this knowledge influences our evaluation of the myeloid HIV reservoir, the implications for HIV pathogenesis in both viremic and virologically-suppressed PLWH and the need to address the myeloid reservoir in future treatment and cure strategies.

Keywords: HIV, monocytes/macrophages, reservoir, DNAscope, animal models

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INTRODUCTION

Whilst CD4+ T cells are the primary targets of HIV, myeloid cells also express the HIV primary receptor CD4 and the chemokine co-receptor CCR5, and are also infected *in vivo* by R5-tropic and dual tropic strains of HIV. Monocytes and macrophages are significant mediators of inflammation, and dysregulation of their inflammatory functions either by direct or bystander

mechanisms during HIV infection is a key driver of comorbidities with an inflammatory etiology in PLWH. The significance of macrophage infection in viremic individuals is well established: HIV species within individuals become increasingly macrophage-tropic with disease progression (1) and by late stage infection, CD4 T cells are depleted and infected macrophages are a principal reservoir driving viremia (2, 3). Moreover, monocyte and macrophage infection is linked to HIV pathologies including the development of HIV-associated dementia (HAD) by promoting inflammation and production of neurotoxins, and by impaired immunoprotective functions leading to thriving opportunistic infections (4). Currently, the role and relevance of monocytes and macrophages during virologically-suppressed HIV infection remains poorly defined, and the persistence, extent and relevance of a monocyte/macrophage HIV reservoir is not clearly understood. With effective cART, the extent of monocyte/macrophage activation and dysfunction is substantially reduced as compared to untreated PLWH, but is not completely ameliorated (5, 6) and contributes to comorbidities including milder HIV-associated neurocognitive disorders (HAND) (7), cardiovascular disease (8, 9), early immune aging (10, 11) and also all-cause mortality (12) [reviewed by (13)]. In this context, the relative contributions of direct infection of monocytes/macrophages vs. bystander effects of persistent, chronic inflammation remain unclear, but the low frequency of monocyte/macrophage infection, particularly during cART, implies the latter is more relevant. However, the contribution of HIV infected monocytes/macrophages to comorbid disease development and the persistence of the HIV reservoir in the setting of long-term, effective virologic suppression is not well understood and needs to be addressed. In current scenarios of controlled HIV infection with successful cART, many questions remain including the extent to which monocyte/macrophage reservoirs persist, how long lived are HIV-infected macrophages, does it include latently infected cells, is it an important source of cryptic viremia in sanctuary tissue sites such as the brain and other tissues and can it contribute to rebound viremia following cART cessation? These questions will need to be addressed to inform research into HIV cure strategies. This review will focus on the detection and measurement of the monocyte/macrophage reservoir and recent advancements in the field of monocyte and macrophage ontogeny and circulation dynamics which affect the way in which the myeloid reservoir should be evaluated.

MONOCYTE/MACROPHAGE BIOLOGY

A basic understanding of the origins and functions of monocytes and macrophages forms the foundation for understanding and targeting the myeloid HIV reservoir. Recent discoveries have challenged early dogma that macrophage populations are terminally differentiated cells, sustained through continual replenishment by bone-marrow derived monocytes. Long-lived tissue resident macrophage populations, which are derived from yolk sac-progenitors and fetal liver-derived monocytes, have been described and shown to be capable of self-renewal, independently of circulating monocytes (14). The discovery of this new macrophage niche represents a paradigm shift in the field of macrophage ontogeny, which needs to be reflected in how monocytes and macrophages are evaluated in the context of HIV infection.

Monocyte Subtypes and Circulation Dynamics

Monocytes are derived from granulocyte/monocyte progenitors in the bone marrow and enter circulation under the influence of the chemokine CCL2 via the CCR2 receptor (15). The monocyte lineage is derived from pluripotent hematopoietic stem cells which progressively differentiate into common CD34+ myeloid progenitors, granulo-monocyte progenitors and committed monocyte progenitors within the bone marrow (16). These cells express CD4 and the coreceptor CCR5, albeit at very low levels, and there is inconsistent data regarding their susceptibility to HIV infection *in vitro* (17–20). Further *in vivo* evidence suggests that a limited CD34+ myeloid progenitor HIV reservoir exists in some individuals (21, 22), although this has not been found in other studies (23, 24). Importantly, the bone marrow is a secondary lymphoid organ to which T cell homing is increased in PLWH (25), and can thus be a site of infection of CD34+ progenitors and monocytes. Following differentiation, these infected CD34+ cells may be involved in trafficking of virus to tissue compartments including the brain (see **Figure 1**), but the extent to which this occurs *in vivo* is not known. The clinical relevance of the HIV-infected CD34+ progenitor cell reservoir is difficult to assess as there is very limited information regarding its prevalence and persistence in HIV+ individuals on current cART regimens with long term virological suppression.

In circulation, monocytes circulate through blood and lymph with a half-life of ∼71 h (26) before migrating into tissues and differentiating into macrophages. Under inflammatory conditions, monocyte turnover is increased and specific monocyte-derived macrophages (MDM) populations are expanded at inflammatory sites. Human monocytes are subdivided into 3 subsets based on surface expression of CD14 and CD16: classical (CD14++/CD16-), pro-inflammatory intermediate (CD14++/CD16+) and "patrolling" non-classical monocytes (CD14+/CD16++) monocytes, which each represent about 90, 5, and 5% of total circulating monocytes, respectively, in healthy individuals (27). Classical monocytes appear to be the first subset to appear in peripheral blood, followed by intermediate and non-classical monocytes (28), with evidence suggesting that populations transition sequentially from classical monocytes to non-classical monocytes via intermediate monocytes (29). To understand how reservoirs are established in different monocyte subsets, a knowledge of monocyte ontogeny, monocyte subset dynamics and migration behavior is required.

Recent modeling of human monocyte circulation dynamics by Tak et al. suggests <10% of classical monocytes mature into circulating intermediate monocytes, and 82–89% of these subsequently mature into circulating non-classical monocytes (30). Interestingly, their data indicated intermediate monocytes spend an average of 1.6 days outside circulation before

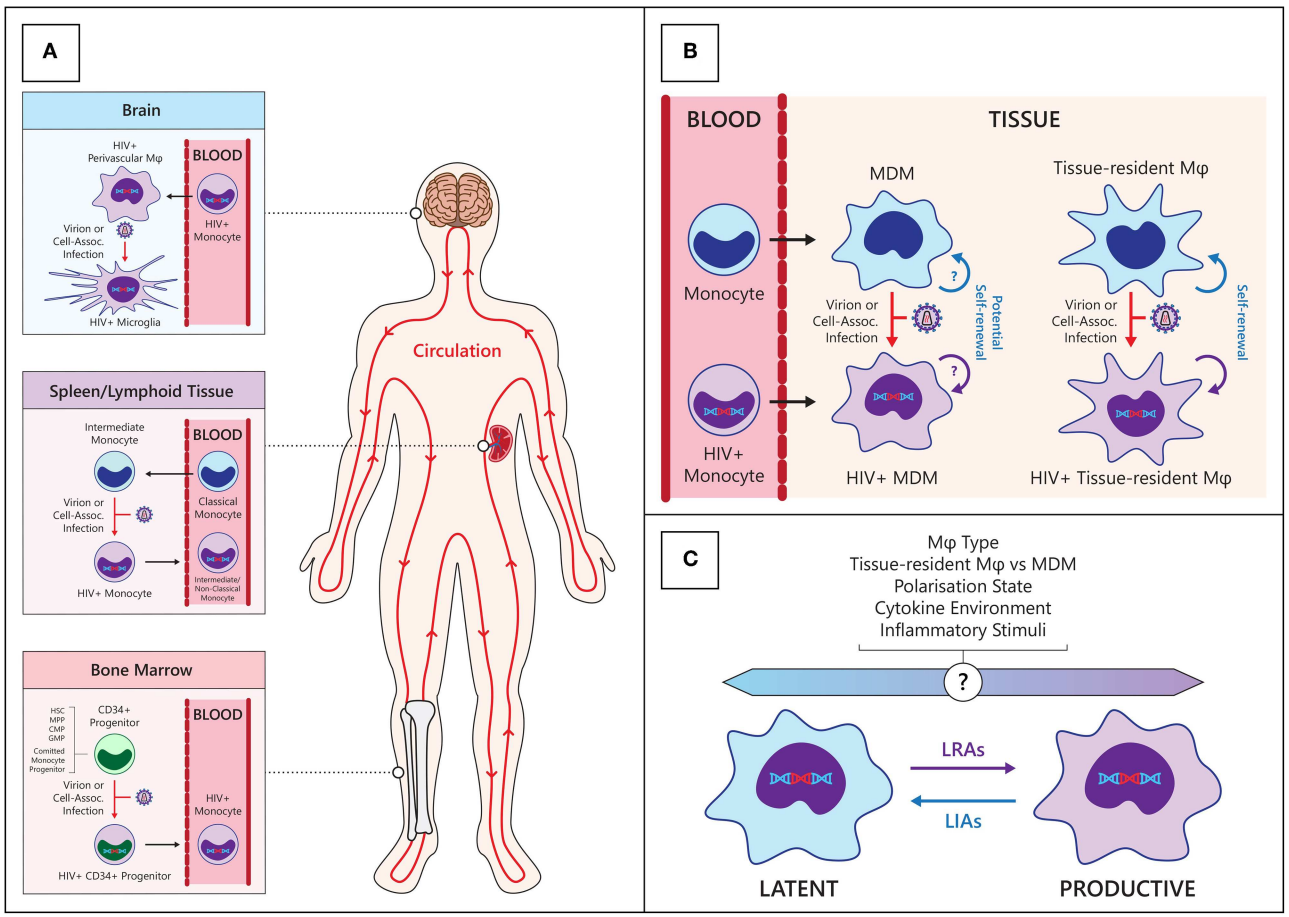

FIGURE 1 | Establishment and maintenance of the HIV myeloid reservoir. **(A)** CD34+ progenitors—hematopoietic stem cells (HSC), multipotent progenitor (MPP), committed myeloid progenitors (CMP), granulo-monocyte progenitors (GMP), and committed monocyte progenitors—in the bone marrow may become infected with HIV then migrate and differentiate into monocytes in circulation (Bottom panel). Classical monocytes differentiate into intermediate monocytes and migrate into tissues such as the spleen, where they may become infected with HIV and re-enter the circulation (center panel). Circulating HIV-infected monocytes can enter anatomical sanctuary sites such as the brain, differentiate into macrophages (ϕ) and thus seed tissue reservoirs (Top panel). **(B)** HIV macrophage reservoirs in tissues can be maintained by infiltrating infected monocytes, de novo infection of monocyte-derived macrophages (MDM) within tissues, and by homeostatic self-renewal of infected tissue-resident macrophages. **(C)** Various endogenous and exogenous factors may influence the state of HIV infection within macrophages. LRAs, latency reversing agents; LIAs, latency inducing agents.

re-entering circulation as non-classical monocytes, suggesting intermediate monocytes may be infected with HIV in tissues during this trafficking. Discrete pools of non-circulating, mature monocytes have been described in bone marrow (31), spleen (32), and patrolling blood vessel adherent monocytes (33), which are distinct from MDM in tissues (30). This has implications for where monocytes/macrophages can be infected with HIV and how they may subsequently traffic virus to other regions in the body (**Figure 1**).

The trafficking of individual monocyte subsets is influenced by their distinct chemokine receptor expression profiles and has implications for their ability to seed tissue reservoirs. Classical monocytes express high levels of CCR2 and migrate out of the bone marrow and into sites of infection and inflammation in response to CCL2, whereas non-classical monocytes express very low levels of CCR2 and high levels of the fractalkine receptor CX3CR1, allowing blood-vessel wall patrolling functions (34–36). Intermediate monocytes express intermediate levels of CCR2 (37) and are efficiently recruited to lymph nodes following immune activation/inflammation (38). It has been reported that the more mature CD16+ monocytes are preferentially infected with HIV (39), which is consistent with their higher level of CCR5 expression as compared to classical monocytes. This observation is hard to reconcile with a model in which the HIV reservoir in monocytes is established in precursor cells in the bone marrow and HIV-infected monocytes progress through their maturation pathway in peripheral circulation. The preferential infection of CD16+ subsets may also be due to their heightened ability to undergo extravasation from blood into tissues such as lymph nodes, where they are more likely to encounter productively infected cells. This has clinical implications, as HIV+ intermediate monocytes preferentially

migrate across the blood brain barrier and contribute to HAND (40). This represents a potential mechanism for trafficking of virus between tissue reservoirs during HIV infection, especially as the intermediate monocyte subset is expanded in inflammatory states including viremic HIV infection (41, 42).

Macrophage Polarization and Heterogeneity

Given their range of functions in tissue homeostasis and protection against infection, macrophages are highly sensitive to changes in the local cytokine environment and exhibit extensive heterogeneity [recently reviewed in (43)]. In response to environmental stimuli, macrophages can be primed toward more pro-inflammatory, anti-inflammatory or tissue remodeling responses. Macrophages can be polarized *in vitro* to inflammatory M1 and anti-inflammatory M2 macrophages using a range of cytokines and maturation factors (44). While the *in vivo* relevance of these experimentally induced states has been debated, M1 and M2 macrophages may be considered as representing extremes of macrophage polarization, with macrophage populations *in vivo* either falling on a spectrum between them or exhibiting overlapping M1-like and M2-like phenotypes (45). *In vivo,* splenic macrophages can be polarized (46) and display similar phenotypes to those generated in *in vitro* systems. Thus, macrophage polarization is a useful tool to highlight the different responses which may be elicited in different cytokine environments.

Macrophage polarization is relevant in HIV infection where acute and chronic inflammation, characterized by different macrophage polarization states, are present at different stages of disease (47). Disease progression is associated with a shift in the cytokine environment from a type-1 inflammatory environment to a type-2 immunosuppressive environment (48–50) and is posited to drive the shift from HIV infection-driven M1 polarization of macrophages (51) toward an M2 polarization state (47, 52). The effects of M1 and M2 polarization *in vivo* are varied, with each stimulating pathways that both benefit and inhibit host defenses, as discussed extensively (52). Although the implications for HIV infection and pathogenesis are subject to debate, it is clear that macrophage polarization is an influential factor with respect to establishment of HIV infection and pathogenesis (**Figure 1**). *In vitro* macrophage infection studies have shown that relative to unpolarized macrophages, both M1 and M2 macrophages have impaired function during chronic and acute HIV infection (53) and are refractory to HIV infection (54–58), albeit through different mechanisms. Extensive work by Graziano et al. who have investigated *in vitro* macrophage plasticity, demonstrates that further stimulation of polarized macrophages with the same or opposing cytokines can modulate HIV restriction (58). This emphasizes the intricate link between flux in the cytokine environment and HIV macrophage reservoir dynamics. Changes in macrophage polarization and functions may therefore act as a mechanism for controlling latency and may contribute to stochastic reactivation and viremic "blips" often seen in patients receiving cART, but this remains to be investigated *in vivo.* Moreover, macrophage polarization can induce differential expression of drug efflux transporters, perhaps contributing to sub therapeutic antiretroviral concentrations (59). Recently, Ganor et al. investigated the polarization states of *ex vivo* urethral macrophages from HIV-, and virologically suppressed HIV+ individuals and reported an intermediate polarization state (Mi) which expresses both M1 (IL-1R) and M2 (CD206) markers. Mi-polarized macrophages are enriched in HIV+ individuals and preferentially infected by HIV (60). Thus, whilst *in vitro* macrophage polarization is a useful and relevant tool to investigate how macrophage heterogeneity and plasticity may influence HIV infection, more *ex vivo* analyses are clearly required to fully understand infection dynamics in this complex cell type.

Macrophage Self-Renewal

Our understanding of macrophage heterogeneity has expanded with the discovery of self-renewing tissue resident macrophages which originate from embryonic yolk sac and fetal liver precursors rather than from circulating monocytes. Experiments in mice have provided evidence for self-renewing populations of myeloid cells including epidermal Langerhans cells (61) and microglia (62) which were maintained via host-derived local expansion, independently of donor-derived circulating monocytes. Murine fate-mapping (63) and lineage tracing experiments have since revealed 3 waves of macrophages sourced from embryonic yolk sac progenitors, fetal liver progenitors and hematopoietic stem cells [reviewed in (64)]. The extent to which tissue macrophage populations are maintained by tissue resident (TR) macrophage expansion or MDM is unique to different tissues, with microglial and Langerhans cell populations largely self-renewing, while gut macrophage populations are largely monocyte-derived (65, 66). Trauma or inflammation can affect these dynamics with greater monocyte infiltration to bolster local macrophage populations (67); however, multiple studies have shown inflammation-recruited MDM populations to be relatively transient (68, 69). Under homeostatic conditions, macrophage half-lives vary between tissues from <6 days for dermal CD14+ MDM (70) to ∼2 months for alveolar macrophages (71), but Réu et al. have also suggested that the average age of microglial cells is 4.2 years whilst individual microglial cells could potentially be decades old (72). Bone-marrow derived macrophages can give rise to long-lived self-renewing heart (73) and lung (74) macrophages in humans, suggesting some MDM have potential to form a stable, self-renewing, tissue resident population in different tissues (**Figure 1**). To the extent that these cells are infected with HIV, this has significant implications for the maintenance of long-lived macrophage HIV reservoirs comparable in duration to those found in memory T cells, and their persistence in settings of treatment with cART. Moreover, while these MDM and tissue resident macrophages can be induced toward similar functions by local stimuli, they are discrete populations with different responses and functions, even within the same tissue. Currently, it is not known which macrophage populations are preferentially infected by HIV with respect to tissue type, ontogeny, and polarization legacy, and current literature has largely been restricted to whole macrophage populations within different tissues. Given

the highly heterogeneous nature of human macrophages, future studies into HIV macrophage reservoirs will need to consider different macrophage populations.

TECHNIQUES TO STUDY THE MYELOID HIV RESERVOIR

Sensitive and specific techniques are needed to measure the scope of the HIV reservoir which persists after cART given the low frequency of infected cells. Current techniques used primarily in T cells to detect HIV infection are subject to limitations [reviewed in (75, 76)] and a combination of techniques will likely be required to comprehensively and specifically map the myeloid HIV reservoir. Detection of HIV-infected cells is often performed using qPCR for HIV DNA, qRT-PCR for HIV RNA, and quantitative viral outgrowth assays (qVOA) using isolated cells. Some HIV DNA qPCR assays are not specific for integrated HIV DNA, or do not distinguish between replication competent and defective proviruses, thus overestimating the amount of latent, activatable viral genomes. Heiner et al. estimate only 5% of proviruses within T cells are intact, and potentially replication competent (77) and there is no information on this proportion in myeloid cells. The qVOA specifically detects replication competent viruses, and quantifies the inducible, replication competent HIV reservoir; however, this technique can underestimate the HIV reservoir as it depends on the production and release of HIV capsid protein (p24) from productively-infected cells and may not detect latently-infected cells. Indeed, Ho et al. estimate that the qVOA measures only 1% of the HIV infected cell population due to inefficient induction of productive infection from quiescent, latently-infected cells (78). It also requires large numbers of cells which can be very difficult to acquire, particularly from tissue samples, and is thus less feasible for use with cells such as macrophages. These qPCR and qVOA HIV detection assays are also indicators of infection in bulk tissue samples and are unable to identify the specific cellular source of HIV. The use of these techniques to detect the HIV monocyte/macrophage reservoir is thus vulnerable to T cell contamination during sample preparation as HIV-infected myeloid cells are a relatively low frequency target in patients receiving cART.

Recently, more specific and sensitive techniques such as DNAScope and RNAScope, and single cell assays, have overcome some technical limitations to studying the monocyte/macrophage reservoir. DNA and RNA Scope are improved *in situ* hybridization technologies which couple highly selective probe sets with extensive amplification, allowing for detection of both HIV DNA and RNA with single cell resolution coupled with immunohistochemistry with appropriate lineage markers to identify infected cells. This technology has been used in tissue sections obtained from animal models of HIV infection (see below) to study the impact of cART on reservoirs in specific cell types using tissues not readily available from human subjects. Moreover, viral DNA and RNA can be detected simultaneously using Scope technology allowing detection of potentially latent HIV DNA+RNA- cells (79). Ko et al. have also used DNAScope and immunohistochemistry to detect HIV DNA in CD68 or CD206 expressing macrophages and microglia, but not in astrocytes, in human brain tissue in the setting of suppressive cART (80). These data demonstrate that this technology may be used to quantify and identify specific cell populations harboring integrated HIV DNA and actively transcribing HIV RNA, or to demonstrate potential latently-infected cells, all in the context of native tissue microenvironments (81). Other single cell techniques including laser capture microscopy to isolate nuclei or whole cells from fixed tissue samples, single cell transcriptomics, and the Full-Length Individual Proviral Sequencing assay, which identifies near full length intact proviral sequences (77), although time-consuming and laborious, complement existing detection assays to offer new insights into specific cells of the HIV reservoir. These methods are especially useful for the study of cells in tissue samples whose behavior with respect to viral production often correlates poorly with peripheral blood compartments (82, 83). Given the low frequency of latently infected cells and the small size of the myeloid reservoir, these emerging techniques which allow for the interrogation of single cells, in context of cellular subtype and tissue microenvironment, address technical weaknesses inherent on older detection technologies and are ideal for contemporary studies measuring the myeloid reservoir.

MONOCYTE/MACROPHAGE HIV RESERVOIRS IN HIV+ INDIVIDUALS

The extent and relevance of the monocyte and macrophage HIV reservoir in humans has been a subject of debate. The drastic reduction in reservoir size with successful cART, limited access to relevant tissue samples and inherent weaknesses in detection techniques has failed to convincingly demonstrate the presence of a replication-competent monocyte/macrophage HIV reservoir in virologically-suppressed individuals. Nevertheless, data showing the presence of HIV DNA in myeloid cells in virologically suppressed individuals, and the persistence of myeloid cell associated comorbidities, argue against the dismissal of this important reservoir.

Monocytes and Macrophages During Untreated HIV Infection

Macrophage reservoirs are seeded within the first few days (84) and are sustained throughout HIV infection, including during the asymptomatic stage of disease during which it can still drive pathogenesis. Jambo et al. detected HIV in alveolar macrophages present in chronically infected, cART-naïve HIV+ individuals using a fluorescence *in situ* hybridization-based flow cytometry assay, specifically gating on CD206 positive macrophages and excluding CD3+ T cells (85). Detection of an infected macrophage population was associated with impaired phagocytic activity, although this may not be restricted to infected cells as both direct (86, 87) and bystander mechanisms (88) of macrophage phagocytic inhibition have been described. Central nervous system (CNS) infiltration by HIV occurs as early as 8 days post infection (89), and within 1 year, structural changes

in the brain are detectable (90). Increased glial cell activation and neuronal injury is observed in HIV infected, cART-naïve individuals (91) and HIV has been detected in microglia in post mortem samples of brain from asymptomatic HIV+ individuals (92). Yakasai et al. detected symptomatic HAND in 40% of cART-naïve HIV+ individuals (93) and brain injury has been associated with HIV DNA in peripheral blood monocytes (94, 95). These data suggest an ongoing contribution of infected monocytes and macrophages to disease pathology over the course of HIV infection, including during asymptomatic, chronic infection (96).

The Monocyte Reservoir During cART-Mediated Virological Suppression

In individuals receiving cART, the extent of *in vivo* infection of monocytes and macrophages is more contentious, and evidence is limited. Monocytes have host-cell restriction mechanisms which limit HIV infection, including the restriction factors SAMHD1 and APOBEC3, and cellular microRNAs (97–102); however, several groups have detected HIV in blood monocytes in PLWH with defined cART status including virologically suppressed individuals (**Table 1**) (39, 103–116). In addition to the studies summarized in **Table 1**, other groups have detected HIV in monocytes of suppressed individuals (117–121) but have not been included in **Table 1** due to insufficient information to determine the virological status of participants. Most of the above studies detected HIV in monocytes to varying extents in 30–100% of donors tested; however integrated DNA and replication competence were assessed in only a small number of studies (103–106). Many studies utilized patient study groups with mixed virologic histories and variable treatment effectiveness, and were limited by small sizes, which may explain the variation in results. Moreover, not all studies determined the degree of contamination of monocyte preparations with T cells. This is especially relevant in context of older monocyte isolation techniques which do not achieve the extremely high purity expected of FACS sorting. Nevertheless, studies satisfying the criteria discussed above have detected HIV DNA in monocytes isolated from long-term virologically suppressed individuals, with highly purified monocyte isolates (106–108, 114) (**Table 1**). PCR-based detection of HIV DNA is highly sensitive; however, HIV DNA was not detected in all donors, and detection rates varied, between studies suggesting the extent of the persisting monocyte reservoir under cART is highly variable and/or very small. Indeed, Spivak et al. were only able to detect HIV DNA in monocytes from 2 of 13 patients on cART (112) and Almodóvar et al. were unable to detect HIV DNA in monocytes from any of the 14 donors with virologic suppression they studied (109). Moreover, while Cattin et al. were able to detect HIV DNA in monocytes isolated from 4 of 10 HIV+ individuals receiving cART, none of these individuals had detectable levels of integrated HIV DNA (116). Difficulties in consistently detecting HIV infection of monocytes has engendered skepticism, resulting in a data set which, while suggestive of the persistence of a reservoir, has yet to demonstrate replication competent virus in monocytes, with solid evidence of no T cell contamination or phagocytosis. The technical advances in detection methodologies described above such as Scope technology will be required to convincingly demonstrate the existence and relevance of the monocyte/macrophage reservoir in the cART era.

The Macrophage Reservoir Persists During cART-Mediated Virological Suppression

Macrophages are more permissive to HIV infection than monocytes and may be productively infected *in vivo*. Studies of individuals on effective cART are limited but have detected HIV DNA, RNA and even HIV Capsid p24 protein in tissue macrophages using multiple techniques (**Table 2**) (60, 80, 114, 116, 122–131). Zalar et al. detected HIV DNA in macrophages present in duodenal tissue from gut biopsies from 9 of 20 virologically suppressed HIV+ individuals, and reported CD68+ macrophages expressing p24, suggesting productive infection (122). Moreover, Ganor et al. have demonstrated replication competent HIV in urethral macrophages and measured total and integrated HIV DNA, HIV RNA and p24 in all virologically suppressed donors tested (60). Further studies have shown HIV DNA and RNA within CD68+ macrophages in brain tissue of cART-treated virologically-suppressed individuals via DNA and RNAScope (80, 127, 130), and Lamers et al. suggested that this virus is actively replicating (132), providing strong evidence for a local macrophage reservoir, which may contribute to the development of HAND.

HIV DNA and RNA has also been detected in alveolar macrophages obtained from bronchoalveolar lavage (BAL) of virologically suppressed individuals (126) but the methodology used was not able to distinguish infected macrophages from cells that had ingested infected T cells, nor rule out T cell contamination during sampling. Nevertheless, Cribbs et al. showed significant impairment of alveolar phagocytic function in donors with detectable as compared to undetectable proviral HIV DNA in BAL cells (126), consistent with trends found in cART-naïve HIV+ individuals. As the degree of macrophage infection is small, this functional impairment is likely due to bystander effects of persistent inflammation and/or other infected cell populations with the tissue, however, unintegrated HIV DNA and incomplete or even productive infection of macrophages may also contribute to this impairment. HIV DNA has also been detected in BAL macrophages from virologically suppressed individuals in some (133) but not other studies (129). Damouche et al. were also unable to detect HIV DNA in CD14+CD206+ adipose tissue macrophages from any of 3 virologically suppressed donors tested (134). These contrasting observations may be expected given the small size of the HIV macrophage reservoir, especially given that many of these studies are limited to very small cohorts. Nevertheless, inconsistency between studies does highlight the variability between PLWH with respect to factors related to both HIV clinical history and host immunity, and emphasize the need for further studies to better characterize macrophage reservoirs in different contexts and tissues. Taken together, these data suggest that tissue macrophage reservoirs persist under suppressive cART in some PLWH and can be associated with impaired or dysregulated macrophage function. Few studies have assessed replication competence of the HIV detected within

TABLE 1 | Evidence for the infection and persistence of HIV within monocytes.

Reference	*n*=	Isolation technique (purity)	Virological suppression	Years on cART	Detection methodology and number positive	Replication competence	T cell specific contamination
Lambotte et al. (103)	5	Microbead (>96% purity)	Yes for all	1–2	HIV DNA detected by PCR. 5/5	N.D.	<2% CD3+ cells detected
					HIV RNA detected by PCR. 5/5	HIV RNA detected in supernatant	Not specifically assessed
Calcaterra et al. (104)	11	Plate adherence and complement-induced CD3 lysis (>94% purity)	$n = 7$ Yes $n = 4$ No	1–3	Total and integrated HIV DNA detected by PCR. Total: 9/11, Integrated: 3/11	N.D.	Not detected
					Soluble HIV p24: 0/11	Not detected	N.D.
Sonza et al. (105)	10	Plate adherence ± microbead CD3 depletion (purity not determined)	Yes for all	N/A	Integrated HIV DNA and 2LTR circles detected by PCR. Integrated: 10/10, 2LTR: 4/5	N.D.	≤0.1% contain TCR mRNA
					Multiply spliced (ms) and virion associated RNA detected by PCR. msRNA: 4/5, Virus: 5/10	HIV RNA detected (supernatant and cell lysates)	
Zhu et al. (106)	7	Microbead and FACS sorting (98.3–100% purity)	Yes for all	2–4	HIV DNA and RNA detected by PCR. DNA:7/7, RNA: 7/7	HIV msRNA detected	N.D.
Garbuglia et al. (107)	18	Microbead (>99% purity)	Yes for all	>2	HIV DNA detected by modified HIV-1 Amplicor assay. 16/18	N.D.	N.D.
Delobel et al. (108)	3	Microbead and FACS sorting (>99% purity)	Yes for all	7–12	HIV DNA detected by PCR. 3/3	N.D.	N.D.
Almodóvar et al. (109)	14	Microbead (95–99% purity)	$n = 12$ Yes $n = 2$ No	N/A	HIV DNA detected by PCR. 0/14	N.D.	≤1.1% CD3+ cells detected
Ellery et al. (39)	17	Microbead and plate adherence, or FACS sorting (>95% purity)	$n = 5$ Yes $n = 12$ No	N/A	HIV DNA detected by PCR. 16/17	N.D.	≤0.01% contained TCR mRNA
Gibellini et al. (110)	34	Microbead (96–99.5% purity)	Yes for all	2–3	HIV DNA detected by PCR. 12/34	N.D.	N.D.
Valcour et al. (111)	27	Microbead (purity Not Determined)	$n = 26$ Yes $n = 1$ No	1	HIV DNA detected by PCR. 8/27	N.D.	N.D.
Spivak et al. (112)	13	Microbead (purity not determined)	Yes for all	N/A	HIV DNA detected by PCR. LTR: 2/13, Nef: 1/10	N.D.	1.5% CD3+ cells detected
Ndhlovu et al. (113)	12	Magnetic Separation (purity not determined)	Yes for all	2–24	HIV DNA detected by PCR. 12/12	N.D.	N.D.
Hansen et al. (114)	6	FACS sorting (>98% purity)	Yes for all	9–22	HIV DNA detected by ddPCR. 6/6	N.D.	N.D.
Pasquereau et al. (115)	31	Plate adherence (purity not determined)	Yes for all	1–27	HIV DNA detected by PCR. Detection rate not stated.	N.D.	N.D.
Cattin et al. (116)	15	Microbead and FACS sorting, or FACS only (>99% purity)	Yes for all	0.3–16	HIV DNA detected by PCR Total: 4/10, Integrated: 0/10	Not detected by qVOA 0/3	N.D.

N/A, not available; N.D, not determined; ms, multiply spliced; ddPCR, droplet digital PCR; TCR, T cell receptor; qVOA, quantitative viral outgrowth assay.

TABLE 2 | Evidence for infection and persistence of HIV in macrophages.

Reference	*n* =	Virological suppression	Years on cART	Tissue	Detection methodology and number positive	Replication competence	T cell specific contamination
Zalar et al. (122)	30	*n* = 20 Yes *n* = 10 No	>5	Duodenum	HIV p24 detected in CD64+ and CD68+ in mucosal monocytes and macrophages by flow cytometry. Suppressed: 9/20, Viremic: 6/10	N.D.	CD3+ and CD4+ cells excluded
Deleage et al. (123)	9	*n* = 7 Yes *n* = 2 No	N/A	Seminal vesicles	HIV RNA and p24 detected in CD163+ macrophages in sectioned tissue via *in situ* hybridization and IHC. Detection rate not stated.	N.D.	CD3 stained but not specifically discussed
Josefsson et al. (124)	8	Yes for all	4–18	GALT	HIV DNA detected in CD3-CD4+ FACS sorted myeloid cells by PCR. GALT: 4/8	N.D.	TCR mRNA detected (not quantified)
Yukl et al. (125)	7	Yes for all	N/A	Rectum	HIV DNA and RNA detected in CD13+CD45+CD3- FACS sorted myeloid cells by qPCR. DNA: 7/7, RNA: 1/7	N.D.	<0.05% CD4+ cells detected
Cribbs et al. (126)	23	*n* = 18 Yes *n* = 5 No	N/A	Lung	HIV DNA and RNA detected in alveolar macrophages by PCR. DNA: 16/23, RNA: 8/23	N.D.	N.D.
Lamers et al. (127)	1	Yes for all	N/A	Brain	HIV DNA and RNA detected in CD163+CD68+ macrophages in sectioned tissue via RNAScope and IHC. 1/1	N.D.	N.D.
Rose et al. (128)	1	Yes for all	N/A	Cerebellum, lymph node	HIV DNA and RNA detected in CD163+CD68+ macrophages in sectioned tissue via RNAScope and IHC. 1/1	N.D.	N.D.
Hansen et al. (114)	1	Yes for all	0.5	Lung	HIV DNA and RNA detected in alveolar macrophages (>95% purity) by ddPCR. DNA: 1/1, RNA: 0/1	N.D.	N.D.
Di Napoli et al. (129)	8	*n* = 6 Yes *n* = 2 No	3	Lung	HIV DNA detected in alveolar macrophages by qPCR. 1/8	N.D.	0.3 copies of TCR DNA per myeloid cell
Tso et al. (130)	2	*n* = 1 Yes *n* = 1 No	1–9	Brain	Detection of HIV DNA and RNA in CD68+ macrophages in sectioned tissue via RNA/DNAScope and IHC. Virologically suppressed: 1/1 DNA; RNA not detected. Viremic: 1/1 DNA, 1/1 RNA.	N.D	CD4 staining: no colocalization.
Kandathil et al. (131)	8	Yes for all	1–12	Liver	HIV DNA and RNA detected in macrophages via qPCR and liver macrophage VOA. DNA: 6/8, RNA: 1/8	Viral outgrowth detected	CD3 mRNA undetectable
Ko et al. (80)	16	Yes for all	N/A	Brain	HIV DNA and RNA detected in CD68+ microglia and CD206+ perivascular macrophages by RNA/DNAScope and IHC. DNA: 16/16, RNA: 6/16	N.D.	N.D.
Ganor et al. (60)	20	Yes for all	3–22 (*n* = 16) N/A (*n* = 4)	Urethra	Integrated HIV DNA detected in FACS sorted CD68+ macrophages via PCR. 3/3	N.D.	Not specifically assessed.
					Integrated HIV DNA, RNA and p24 detected in macrophages in sectioned tissue via FISH and IHC. DNA: 3/3, RNA: 3/3, p24: 6/6	N.D.	No colocalization of CD3 or CD4
					HIV replication from macrophage-rich urethral tissue detected by modified qVOA. 3/3	Viral outgrowth detected.	Tissue contained 25–30% CD4+ T cells
Cattin et al. (116)	8		6–18 (*n* = 7)	Colon	HIV DNA and HIV replication from FACS sorted myeloid cells detected by PCR and qVOA. DNA: 1/8, qVOA: 0/8	Viral outgrowth not detected.	CD3+ cells excluded

N/A, Not Available; N.D, not determined; GALT, gut associated lymphoid tissue; ddPCR, droplet digital PCR; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; TCR, T cell receptor; qVOA, quantitative viral outgrowth assay.

macrophages; Kandathil et al. has observed viral outgrowth from liver macrophages in only 1 of 8 donors (131) whilst Ganor et al. demonstrated replication competent DNA in urethral macrophages from all 3 donors assessed (60). These data suggest that replication competent DNA can persist during suppressive cART, but more work is required to determine the extent of the replication competent reservoir, and in which macrophage populations it can exist.

MONOCYTE/MACROPHAGE INVOLVEMENT IN ANIMAL MODELS OF HIV INFECTION

Non-human Primate SIV Infection Models

Given the difficulties in obtaining tissue resident cells from humans, animal models such as the SIV-infected macaque non-human primate (NHP) model has been invaluable for studying HIV replication dynamics in macrophages. Similar to HIV infection in humans, SIV targets macrophages in addition to CD4+ T cells (135, 136) and exhibits a similar distribution within blood and tissues. Rhesus macaques are often used in reservoir and cure studies as infection is associated with sustained viral loads, progressive CD4+ T cell depletion and chronic immune activation (137), and viremia can be controlled but not eliminated by cART, leading to viral rebound if cART is interrupted (138). These similarities to HIV disease have made the SIV-macaque model extremely useful for studying the early establishment of HIV infection and reservoir dynamics in the setting of virologic suppression with cART due to access to repetitive blood sampling and ready access to appropriate tissue samples. This model has been used to demonstrate the persistence of the HIV monocyte/macrophage reservoir under cART, using modified macrophage-qVOAs to detect replication competent virus in myeloid cells from blood, brain, BAL, lungs and spleen of virologically suppressed subjects (139–141). DNA and RNAScope technology has also been leveraged to specifically detect SIV infected macrophages *in situ* and distinguish genuine macrophage infection from phagocytosis of infected T cells (79). Using DNAScope, Di Napoli et al. observed SIV DNA in splenic macrophages of macaques receiving cART for at least 5 months with undetectable viral load; however, they were unable to detect replication-competent virus from this reservoir and were also unable to detect SIV DNA in BAL macrophages (129). This may explain inconsistent detection of myeloid-associated HIV in HIV+ individuals on cART and suggests the myeloid reservoir is likely not only small and stochastic, but may also be influenced by viral and host response factors that we are yet to fully understand.

The SIV macaque model has also been used to study disease pathologies such as SIV encephalitis (SIVE). Several SIVE NHP models have been developed [reviewed in (142)], and are often derived via infection of macaques with neurotropic- and macrophage-tropic SIV strains, complemented in some studies with T cell depletion (142). These infection models lead to a higher incidence of SIVE and more rapid onset of pathogenesis compared to the slow progression of HAND observed in HIV+ individuals on suppressive cART, but they have been used to emphasize the association of SIVE with macrophage-tropic viruses, increased monocyte turnover (143, 144) and infiltration and accumulation of inflammatory macrophages into the brain (145, 146). Currently, SIVE models combined with cART are in development which may better reflect the persistence of the milder HAND observed in HIV+ individuals on suppressive cART and represent a promising new avenue for investigating the specific contribution of infected and bystander monocytes and macrophages to neuropathology.

Monocyte/macrophage targeted therapeutics have also been evaluated in the SIV model as an approach to minimize neurodegenerative disorders and cardiovascular disease. Campbell et al. investigated the effect of anti-α4 integrin blocking antibody (natalizumab), which prevents monocyte and lymphocyte trafficking into the brain and gut in SIV infected macaques. Early administration during acute infection blocked CNS infection and macrophage accumulation, and administration of the antibody during chronic infection (after established neuronal damage and macrophage accumulation) led to stabilization of neuronal injury (147), which strongly supports the critical role of infected monocytes in seeding SIV infection within the brain and the importance of myeloid cell trafficking and accumulation in the development of neurodegenerative disorders during SIV infection. Moreover, natalizumab blocks monocyte/macrophage trafficking to heart tissues which was associated with decreased cardiac fibrosis, inflammation, and cardiomyocyte degeneration (148). These findings are consistent with the observation of HIV-infected macrophages in atherosclerotic plaques (149). The correlation of monocyte and macrophage trafficking with neurodegenerative disorders and cardiovascular disease suggests the potential for pharmacological treatment of these persistent comorbidities found in HIV+ individuals on cART.

Mouse Models of HIV Infection

Humanized mouse models have also allowed targeted studies of the monocyte/macrophage reservoir alone, or in combination with T cells, and its potential contribution in the context of cART suppression or disease states like HIV-associated encephalitis (150). Arainga et al. have demonstrated that mice transplanted with human hematopoietic stem cells (huHSC) can sustain HIV infection and respond well to cART with substantial reductions in detectable viral DNA and RNA, but similar to the response of humans, cART does not eliminate HIV reservoirs (151). Humanized BLT mice are readily infected with HIV and were validated to have detectable HIV DNA and RNA in both T cells and macrophages in the absence of cART and reduced and undetectable reservoirs, respectively, in the presence of cART (152). Honeycutt et al. have developed a humanized myeloid-only mouse (MoM) by transplanting hematopoietic stem cells into NOD/SCID mice which are unable to support human lymphocyte development. Using this novel model, they were able to show that the monocyte/macrophage reservoir can sustain infection independently of CD4+ T cells (152) and that viremia is undetectable in cART treated mice compared to cART-naïve mice (153). Upon discontinuation of cART, 3 of 9 mice examined had detectable viral rebound within 7 weeks

post-treatment interruption. The presence of rebound viremia correlated with a higher viral load prior to cART initiation (153). These data demonstrate that the HIV monocyte/macrophage reservoir potentially remains a source of reactivatable virus even in a setting of suppressive cART. This MoM study is limited by a short antiretroviral treatment duration (5 weeks) and follow-up period after cART interruption (7 weeks). Also, it may not recapitulate conditions found *in vivo* in humans since human macrophage turnover in this model was estimated to be 1.05 days, which is far shorter than the estimated half-lives of normal tissue and MDM, and T cell interactions may be necessary for the persistence of the HIV macrophage reservoir. Nevertheless, data from these models are consistent with persistence and relevance of this reservoir, and are noteworthy due to limited *in vivo* data from people living with HIV.

In the MoM model, HIV infected mice have a greater accumulation of human macrophages in the brain compared to non-infected mice. HIV infection of human macrophages resident in the brain of these mice was demonstrated by immunohistochemistry for HIV capsid protein p24 and by detection of HIV RNA using qRT-PCR on RNA from isolated macrophages (152). This underscores the role of HIV-infected myeloid cell infiltration and accumulation to establish HIV reservoirs within the brain. This model has not yet been used to evaluate monocyte and macrophage infection in the context of the CNS and neuropathology but would be a highly relevant and interesting avenue for future studies.

Other mouse models have been used to evaluate the contribution of the monocyte/macrophage reservoir to HIV associated pathologies, specifically HIVE. In early studies, intracranial injection of HIV-infected human macrophages or microglia into SCID mice were used to produce a SCID-HIVE model (154) which effectively recapitulates some of the neuropathology of HIV encephalitis in humans (astrogliosis, multinuclear giant cells, and monocyte migration), and does so on an accelerated timeline compatible with the short lifespans of mice. This methodology, however, results in unavoidable confounding factors like trauma at the injection site and xenoreactivity. Attempts at using transgenic mice and humanized mouse models have had limited success in mimicking neuropathology [reviewed in greater depth in (155) and (156)]. Humanized mouse models are attractive as a model for HAND as infection in the brain can be established via a systemic route.

Mouse models have also been developed to measure HIV/SIV replication from infected human and NHP cell samples. Mice are not naturally susceptible to HIV infection; however, they can be engineered to host *in vivo* modified qVOA systems allowing for long-term viral outgrowth detection to better capture reactivation of the latent reservoir. These systems involve the xenografting of cells from HIV+ individuals into immune-modified mice and detecting virus in plasma following an incubation period to detect low frequency reactivation of latent proviruses. Two types of mouse-based qVOAs, reviewed by Schmitt and Akkina (157), have been developed: the mVOA which uses immunodeficient NOD *scid gamma* NSG mice, and the huVOA, which uses humanized Hu-HSC or BLT mice which have a reconstituted human immune system through injection of human hemopoietic stem cells (and in BLT mice, implantation of fetal liver and thymic tissue) into irradiated NSG mice. The mVOA, developed by Metcalf Pate et al. involves injecting immunodeficient NSG mice with large numbers of PBMC or CD4+ T cells, combined with antibody mediated CD8 depletion and, in some cases, CD3 activation (158). This mVOA technique has successfully detected reactivation of latent virus in PBMC derived from an elite controller with a negative *in vitro* qVOA result (158) and from other negative qVOA samples (159), but is limited by variable engraftment rates and rapid onset of graft vs. host (GvH) responses. The use of humanized mice overcomes GvH and provides a larger range of humanized target cells. HuVOAs have been validated and demonstrated to be more sensitive than qVOA (160). Both the mVOA and HuVOA are useful, ultrasensitive systems for detection of low frequencies of HIV-infected cells but have not yet been used to detect HIV in purified monocytes. These techniques may be used to evaluate future cure strategies and attempts.

Conclusions derived from the use of these mouse models are constrained by the short natural lifespan of mice which precludes studies of age-related inflammatory comorbidities found in humans with long-term cART suppression. Nevertheless, development of humanized mice susceptible to HIV infection are a valuable resource that can be used for accelerated models of disease, for studying HIV persistence and infection dynamics, and detection of low frequency infection. These mouse models are thus useful tools given the scarcity and barriers to access of human samples and their capacity to recapitulate aspects of disease pathology consistent with those found in PLWH.

RELEVANCE OF THE MONOCYTE/MACROPHAGE RESERVOIR FOR HIV CURE STRATEGIES

The myeloid HIV reservoir may be clinically relevant as it is potentially long-lived, relatively resistant to the cytopathic effects of HIV infection (161) and resistant to CTL-mediated killing (162). Moreover, being widely dispersed throughout the body, they can inhabit sanctuary sites and tissue reservoirs such as the brain and lymph tissue which may experience reduced penetrance of antiretroviral drugs (163–165). Macrophages are also intrinsically resistant to some antiretroviral drugs [reviewed in (166)]; protease inhibitors saquinavir and ritonavir showed ∼2–10 fold lower activity in chronically-infected macrophages compared to chronically-infected lymphocytes (167). Moreover, *in vitro* cultures treated with antiretrovirals demonstrated that concentrations of nucleoside analogs are 5–140 fold lower in macrophages than in lymphocytes, and their antiviral activity was significantly decreased when combined with M-CSF stimulation (an M2 polarizing factor) (168). These characteristics, combined with the persistence of HIV within monocytes and macrophages, and the observation of a non-T cell source of rebound viremia in some PLWH (169), suggest the existence of a replication competent, and clinically relevant monocyte/macrophage HIV reservoir.

Current cure research is focused on T cells, the primary HIV reservoir. Cure strategies are varied and include CRISPR based gene therapy, vaccines to boost anti-HIV immune response,

broadly neutralizing antibody immunotherapy approaches to cure, and the "shock and kill" strategy, in which the latent reservoir is targeted by reversing latency under continued suppressive cART and triggering cell death (170). This strategy is still in development and has seen limited success (171–178). If monocytes/macrophages are considered a legitimate reservoir which persists under cART, the effect of latency reversing interventions should be investigated in these cells. The observation that latency reversing agents (LRAs) can reactivate latently infected macrophages in the CNS leading to increased immune activation and inflammatory responses in a SIV model (179) demonstrate the potential risks of LRAs potentiating macrophage-mediated pathologies such as HAND. Moreover, CTL mediated killing activity against HIV-infected macrophages is not only ineffective but leads to increased inflammation (162). Thus, strategies to target the macrophage reservoir and minimize unintended activation may need to be investigated in parallel with T-cell centric cure strategies.

CONCLUSIONS

The development of new animal models and HIV detection techniques coupled with greater understanding of the scope of monocyte and macrophage biology has significant implications on how HIV myeloid reservoir research is appraised. The circulation of monocytes and trafficking through tissues, including through lymph nodes which are tissue reservoirs of HIV, represents a new avenue by which monocytes can become infected and contribute to HIV persistence. Moreover, the existence of long-lived macrophage populations which can maintain a HIV reservoir via homeostatic cell division is analogous to memory T cell reservoirs and indicates the potential for tissue macrophages to be a viable HIV reservoir. With this greater understanding of how a myeloid reservoir could be seeded and maintained, coupled with mouse studies indicating that the myeloid reservoir can sustain infection and serve as the source of rebound viremia independently of T cell infection, there has been a resurgence in interest in the myeloid reservoir as a legitimate barrier to HIV cure. Current data suggests that the monocyte/macrophage reservoir is very small in PLWH and in non-human primates with cART suppression and is likely to be a minor contributor to viral rebound compared to the latent T cell reservoir. Nevertheless, the persistent monocyte/macrophage reservoir may be relevant in HIV+ individuals on cART through contribution to pathologies but also as a potential viral source which should not be ignored in attempts to cure or treat HIV.

AUTHOR CONTRIBUTIONS

MW wrote the article. AJ and AH conceived the topic and contributed to the manuscript.

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Corrigendum: The HIV Reservoir in Monocytes and Macrophages

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In the original article, there was an error. The individual who generated artwork for Figure 1, was not duly acknowledged.

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"The authors kindly thank Mr. Hans Kek for generating the artwork for Figure 1. We gratefully acknowledge the contribution to this work of the Victorian Operational Infrastructure Support Program received by the Burnet Institute."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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MINI REVIEW
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Negative and Positive Selection Pressure During Sexual Transmission of Transmitted Founder HIV-1

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Sexual transmission of HIV-1 consists of processes that exert either positive or negative selection pressure on the virus. The sum of these selection pressures lead to the transmission of only one specific HIV-1 strain, termed the transmitted founder virus. Different dendritic cell subsets are abundantly present at mucosal sites and, interestingly, these DC subsets exert opposite pressure on viral selection during sexual transmission. In this review we describe receptors and cellular compartments in DCs that are involved in HIV-1 communication leading to either viral restriction by the host or further dissemination to establish a long-lived reservoir. We discuss the current understanding of host antiretroviral restriction factors against HIV-1 and specifically against the HIV-1 transmitted founder virus. We will also discuss potential clinical implications for exploiting these intrinsic restriction factors in developing novel therapeutic targets. A better understanding of these processes might help in developing strategies against HIV-1 infections by targeting dendritic cells.

Keywords: dendritic cell, langerhans cell, transmitted founder HIV-1, IFITM, Type I IFN, Trim5a, viral restriction and dissemination

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INTRODUCTION

The number of new HIV-1 infections globally continues to decline. From a peak of 3.4 million new infection a year in 1996 to 1.8 million in 2017. The intervention of early combination antiretroviral therapy (cART) is clinically beneficial to patients and very effective in preventing HIV-1 transmission (1–3). The introduction of pre-exposure prophylaxis (PrEP) will further interfere with HIV-1 transmission (4–6). However, currently there is no curative treatment or vaccine to prevent HIV-1 infection. Uncovering the mechanisms underlying viral transmission and pathogenesis is crucial to develop methods to prevent HIV-1 transmission. Sexual transmission of HIV-1 results most commonly from virus exposure at mucosal surfaces (7, 8). The identification of transmitted founder (TF) viruses emphasizes the existence of selection pressure mechanisms that lead to the transmission of only specific HIV-1 strains (9). Host factors influence whether virus exposure leads to productive infection. These may include the physical barrier of the mucosa (10), the amount of available target cells (11), altered mucosal microbiota (12, 13), and immune activation by genital inflammation established by other sexual transmitted infection

(14–18). Also, genital fluids are known to contain proteins that enhance viral infection, like semen-derived enhancer of virus infectivity (SEVI) and complement (**Figure 1**) (19, 20). The transmission risk is associated with the specific within-host barriers, which creates a selection bias with an advantage for viruses with higher between-host transmission potential (21–24). Important cells that exert opposing selection pressures are the different dendritic cell (DC) subsets localized in the mucosal tissues.

VIRUS-HOST INTERACTIONS AT MUCOSAL SITES

DC subsets play an important role in transmission of viruses such as HIV-1 across mucosal tissues (14, 25). The cellular plasma membrane is the first interaction of HIV-1 with its host and an important step in viral transmission and pathogenesis. HIV-1 spreads either as cell-free virus particles or via cell-cell transmission. While cell-free virus allows spread of virions in more distant tissues, cell-cell transmission is much more efficient and it helps the virus to overcome physical and immunological barriers (26). *In vitro* studies illustrate that cART and broadly neutralizing antibodies (bnAb) do not neutralize HIV-1 cell-cell transmission as potent as cell-free spread (27–29), which underscores the importance of understanding the mode of viral transmission for drug design.

The compartments where fusion of HIV-1 with the host cell occurs differs and is dependent on the cell type and mode of viral transmission. For CD4 T cells, HIV-1 fuses at the plasma membrane using the CD4 receptor and a co-receptor such as CCR5 and CXCR4 (30–32). For DCs viral fusion occurs at the plasma membrane (33) or after internalization via clathrin mediated endocytosis (34, 35). Internalization minimizes the exposure of viral epitopes at the cell surface, thereby reducing the efficacy of inhibitors targeting these epitopes (35). In contrast, endocytosis does not always lead to productive infection. When HIV-1 is endocytosed in multivesicular bodies (MVBs) the virus can be recycled back toward the plasma membrane for transfer to uninfected cells (36, 37). However, internalization can also lead to autophagic destruction in specific cells (38). Indeed, different DC subsets have distinct roles in HIV-1 dissemination because of the differences in handling the virus. Mucosal langerhans cells (LCs) capture and internalize HIV-1 leading to degradation, thereby preventing HIV-1 dissemination (38, 39), whereas DCs play a key role in transmitting the virus to target CD4 T cells.

DENDRITIC CELLS FACILITATE HIV-1 DISSEMINATION UPON SEXUAL CONTACT

DCs patrol the submucosal tissues to capture invading pathogens for antigen presentation to T cells in the lymph nodes, thereby facilitating HIV-1 transmission (**Figure 1**) (40). DCs facilitate viral transmission to T cells either by HIV-1 fusion and productive infection of the DC, leading to viral transmission to permissive cells or by capture and internalization of HIV-1 into MVBs and transmission independent of DC infection (36, 37, 41). Besides their role in HIV-1 dissemination they are also important in triggering an innate immune response upon viral exposure. DCs express relatively low levels of the CCR5 and CXCR4 co-receptor and CD4 entry receptor, which could account for the lower levels of infection seen in DCs (42). DCs express many attachment molecules that mediate virus internalization and transfer. Indeed, the C-type lectin receptor (CLR) DC-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN) is thought to play an important role in HIV-1 binding and internalization via endocytosis into clathrin coated pits (41, 43, 44). After internalization the virions can stay infectious for many days and can be transmitted to CD4-positive T cells (45). In this way DCs serve as virus reservoirs to mediate *trans*-infection of CD4-positive T cells, thereby facilitating spread of HIV-1 to the lymph nodes (45, 46). DC-SIGN is highly expressed on *in vitro*-generated monocyte-derived DCs (moDCs), at mucosal sites on CD14-positve dermal DCs (dDCs) and on sub epithelial-based vaginal myeloid DCs (47–49). For DCs that do not express DC-SIGN (50), different attachment receptors like Siglec-1 (CD169) have been identified to transfer HIV-1 (51, 52). Also external factors can promote *trans*-infection, like semen fluid, which contains fragments of prostatic acid phosphatase forming amyloid aggregates termed SEVI that promote viral attachment to DCs thereby increasing *trans*-infection of CD4-positive T cells by HIV-1 (19, 53). There are different processes described by which *trans*-infection occurs. One process is internalization via clathrin-mediated endocytosis (41, 43, 44). Antigen bound to DC-SIGN in mature DCs localizes in compartments with a neutral pH close to the cell surface, which could represent early endosomes (43). In contrast, in immature DCs DC-SIGN co-localizes with late endosomes or lysosomes (41). A different *trans*-infection route is dependent on invagination. For mature DCs, HIV-1 is internalized in a CD81 tetraspanin rich compartment, which is distinct from endocytic vesicles but adjacent to the plasma membrane (36, 54). This allows protected transfer of virions by DCs and delivery to target cells in the lymph node. Interestingly, more recently it has been shown that a process called micropinocytosis is involved in transfer of HIV-1 from immature DCs to CD4-positive T cells. Dynamin 2 (DNM2), a gene involved in organelle and membrane trafficking limits HIV-1 endocytosis and maintains virions on the surface of DCs for efficient transfer (55). Once in the lymph node HIV-1 can be transmitted from DCs to T cells via different mechanisms. DC-SIGN-bound HIV-1 facilitates optimal contact with CD4 and CXCR4/CCR5 co-receptors on T cells, enhancing viral transfer (56), HIV-1 is transferred via virological synapses which is formed by direct contact between DCs and T cells (57–59) or DCs transfer HIV-1 to T cells via exosomes (60, 61). Interestingly, exosomes derived from HIV-1 infected cells contain HIV-1 viral genome (62) and are able to establish productive infection in target cells (61, 63). All these mechanisms contribute to viral escape and promote further dissemination in the newly infected host.

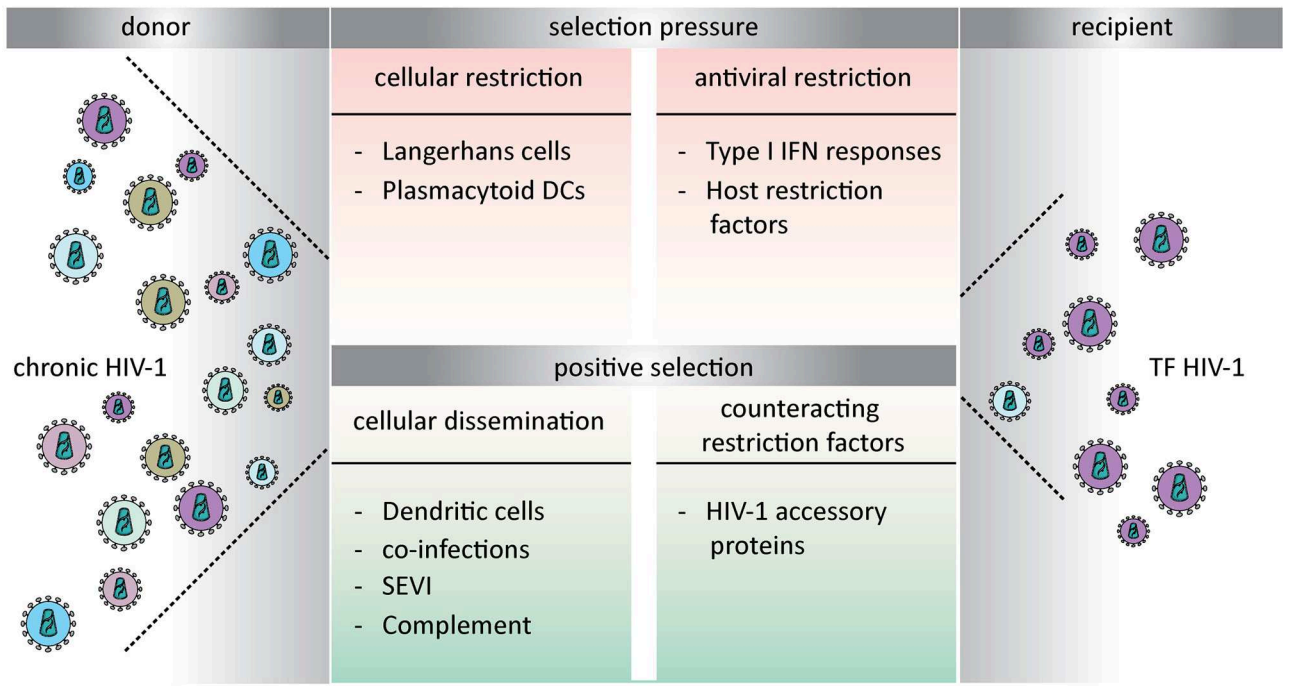

FIGURE 1 | Opposing selection pressures during transmitted founder HIV-1 transmission at mucosal surfaces. The within-host evolution creates a selection bias that leads to either viral restriction or establishes a new infection. Transmitted founder viruses are able to escape host restriction and are therefore responsible for establishing productive infection. TF HIV-1, transmitted founder HIV-1; DCs, dendritic cells; IFN, Interferon; SEVI, semen-derived enhancer of virus infectivity.

LANGERHANS CELLS ARE EITHER PROTECTIVE AGAINST OR PROMOTE HIV-1 TRANSMISSION

LCs a subset of DCs are abundantly present at epithelia of vagina, foreskin and within the anal tissues (64, 65) and under normal conditions are therefore the first immune cells to encounter HIV-1 during sexual transmission (38, 39). Langerin (CD207) is a CLR expressed almost exclusively by LCs and is important for antigen capture and internalization, which induces Birbeck granules formation and routing of antigen into organelles (66). In contrast to DC-SIGN, langerin has a role in antiviral protection as immature LCs do not become infected by HIV-1 but capture HIV-1 via langerin, leading to TRIM5α-mediated degradation of HIV-1 and thereby preventing HIV-1 dissemination (**Figure 1**) (38, 39). LCs from inner foreskin explant cultures and vaginal explant are not productively infected by HIV-1 but several studies suggest that these cells support *trans*-infection of CD4-positive T cells (64, 67). Since the restrictive nature of LCs is dependent on the activation state and can be saturated, the amount of virus and isolation method could explain differences observed in restriction and infection (68). Taken together, the outcome of these studies suggest that immature LCs generally seem to be more restrictive to HIV-1 infection, whereas activation of LCs allows *cis*-infection and subsequent transmission of HIV-1 to T cells (14, 64). Inflammatory stimuli like TNFα, Pam3CSK4 or Interleukin-7 increase HIV-1 transmission by increasing HIV-1 replication or capture (14, 69). Also, viral coinfections, such as HSV-2, breach the protective function of LCs by abrogating langerin function, which increases HIV-1 susceptibility (15, 65). This implies that activation of LCs by inflammation or genital co-infection alters the protective function of LCs, mediating HIV-1 transmission (**Figure 1**), which might be associated with lower expression of langerin on activated LCs as langerin has anti-viral properties (39).

INTERFERON PRESSURE AT MUCOSAL SITES

Viral infections sensed by pattern recognition receptors (PRRs) lead to the activation of signaling cascades that results in the release of interferons (IFNs). Upregulation of type I IFN production is one of the earliest innate responses observed in HIV-1 infection. Production of type I IFNs during viral infections promotes an antiviral environment by an autocrine feedback loop triggering the IFN receptor and subsequently inducing cellular expression of IFN-stimulated genes (ISGs) within the infected cells but also in bystander cells (70). Several studies have shown that HIV-1 is able to escape intrinsic IFN-β response triggering by limiting replication of viral DNA (71) or actively

blocking virus sensing by PRRs, which contributes to efficient HIV-1 replication (72). Besides cellular IFN responses upon viral infection, plasmacytoid dendritic cells (pDCs) secrete a second wave of type I IFNs in response to viruses or tissue damage (73, 74). pDCs develop in the bone marrow and circulate in the blood. Macaque studies have shown that upon SIV exposure, pDCs are recruited to the mucosal sites of virus transmission, become activated and start producing high levels of type I IFNs (75). The outcome of this high IFN response by pDCs has conflicting functions in antiviral defense. Some of the induced ISGs act as host restriction factors to prevent HIV-1 infection and dissemination. In contrast, during acute infections, IFN produced by pDCs results in maturation of bystander myeloid DCs that play a crucial role in transporting the virus to secondary lymphoid organs thereby promote transmission (**Figure 1**) (74). It has been shown that DCs upregulate the interferon-inducible receptor Siglec-1 which is able to transfer HIV-1 to T cells (76). Also, studies show that ISGs are upregulated during chronic infection (77, 78). The persistent activation of pDCs during chronic infection may contribute to immune activation and inflammation, which is associated with AIDS disease progression (79, 80). These consequences of high IFN production promote viral dissemination. Earlier studies suggested that IFN-α responses in mucosa of non-human primates could enhance infection and the IFN-α induction did not protect animals from SIV infection (75, 81). However, IFN production may also create an antiviral environment. Recently an elegant study showed that early type I IFN responses in macaques prevent SIV infection and slow disease progression (82). Moreover, in uninfected but high exposed individuals, higher IFN-α levels have shown to be protective against infection (83). Also, the induction of an effective early antiviral immune response at mucosal sites creates selective pressure for viruses that are resistant to type I IFN (84).

TRANSMITTED FOUNDER VIRUSES ARE RESPONSIBLE FOR INITIAL HIV-1 INFECTION

In 60–80% of mucosal infection, a single specific HIV-1 variant, the TF virus, establishes productive clinical infection (**Figure 1**) (9, 85, 86). To be able to cross intact mucosal barriers TF viruses have specific properties that provide an advantage to establish new infections more efficiently (87, 88). TF virus strains are relatively resistant to IFN compared to viruses isolated later in infection (84, 88–90), suggesting adaptations in HIV-1 evolution to escape host restriction. TF viruses replicate and spread more efficient in CD4 T cells in the presence of IFN-α than chronic viruses (84, 88). This suggest that IFN resistance of TF viruses is specifically important during initial infection as type I IFNs are produced at lower levels during systemic infection when chronic viruses replicate. Also, initial HIV-1 infection occurs predominantly with R5 HIV-1 strains (31, 91) and TF viruses have the chemokine receptor 5 (CCR5) tropism (9, 92). TF viruses incorporate more envelope glycoprotein (Env) per particle compared to chronic HIV-1 viruses, which is associated with enhanced infection of target CD4 T cells (88). Furthermore, it has been shown that TF viruses bind more efficiently to DCs than their chronic counterparts giving TF viruses a potential selection advantage in transmission to a new host (88). Phenotypic analyses of TF viruses show an enhanced resistance to fusion inhibitors, masking of CCR5 co-receptor binding sites, and more neutralizing antibodies compared to chronic HIV-1 strains (9, 93). Since TF viruses need to establish infection they might have specific capabilities to infect immune cells such as DCs an LCs. Moreover, certain TF virus strains might infect immature LCs more efficient compared to their chronic counterparts, which could indicate that TF viruses might have an intrinsic capacity to escape LC restriction (**Figure 1**). These findings underscore the importance of LCs as initial targets for sexual transmission of HIV-1 and understanding these phenotypic properties of TF viruses is essential for vaccine design. Especially in the era of PrEP, transmitted drug resistance could be of concerns as it could select for higher virulent TF viruses (94).

HOST ANTIRETROVIRAL RESTRICTION FACTORS AGAINST HIV-1

Host restriction factors play an important role in suppressing retroviral replication and dissemination (**Figure 1**). Many restriction factors that target HIV-1 are induced by type I IFN. Well-known HIV-1 restriction factors in DCs are IFITM (Interferon-induced transmembrane proteins), TRIM5α (E3-ubiquitin ligase tri-partite-containing motif 5a) (38) SAMHD1 (SAM- and HD domain-containing protein 1) (95), APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) (96), Mx2 (Myxovirus resistance 2), and bone marrow stromal antigen 2 (BST2 or Tetherin) (97). Because of the potent antiviral potential of IFN many viruses have developed mechanisms to promote their survival. HIV-1 although sensitive to type I IFNs, is able to antagonize host restriction factors that inhibit virus entry to facilitate viral dissemination (**Figure 1**) (98). HIV-1 accessory proteins well known to counteract important restriction factors are: viral protein R (Vpr) viral infectivity factor (Vif) which antagonizes APOBEC3 proteins, negative regulatory factor (Nef) and viral protein unique (Vpu) antagonizing BST2 (99, 100).

IFITMs are small membrane-associated cellular factors that inhibit the replication of HIV-1 at the entry step (101). IFITMs do not block the internalization of viruses but inhibit fusion of the virus with the host cell. Whether HIV-1 is sensitive to IFITM restriction is determined by the subcellular localization of the IFITMs and HIV-1 co-receptor usage (102). TF viruses are more resistant to the antiviral activity of IFITMs. The ability by TF viruses to evade IFITM restriction is due to its relative resistance to IFN. Interestingly, IFITM restriction contributes to the increased IFN sensitivity of chronic HIV-1 viruses (102).

TRIM5α targets incoming retroviral capsid before integration to block infection. TRIM5α expression levels and polymorphisms have been associated with the clinical course of HIV-1 infection

in cohort studies underscoring the antiviral effect of TRIM5α (103–105). Unique about TRIM5α is that it can restrict diverse retroviruses in a species-specific manner. Rhesus TRIM5α (rhTRIM5α) strongly restricts HIV-1, whereas human TRIM5α has been thought to have poor restriction activity against HIV-1 (106). More recently some primary isolates of HIV-1 have been found to be more sensitive to human TRIM5α restriction than lab strains (107, 108). So restriction of TRIM5α on replication may vary according to the virus. The functional capacity of TRIM5α also depends on the localization of the restriction factor in the cell. It has been suggested that non-human primate DCs lack efficient TRIM5α mediated retroviral restriction because TRIM5α is unable to restrict incoming viruses because it is absent from the cytoplasm (109). TRIM5α localization to the nucleus triggers induction of type I IFN during infection (109). Notably, recent data show that TRIM5α restriction might be cell specific. Immature LCs protect against HIV-1 infection by inducing langerin-mediated autophagic degradation of captured HIV-1 (38). The LC specific restriction factor TRIM5α is dependent on the CLR function. HIV-1 binding to Langerin routes HIV-1 into the TRIM5α mediated restriction pathway which targets virions for degradation and thereby prevents infection of LCs. Taken together, the outcome of these studies support a role for human TRIM5α in HIV-1 transmission and pathogenesis *in vivo*.

SAMHD1 is highly expressed in myeloid cells like DCs and macrophages (95). SAMHD1 also targets the early phase of viral infection as it inhibits reverse transcription by depleting the pool of cellular dNTPs (95, 110, 111). HIV-2 viral protein X (Vpx) is able to counteract SAMHD1 restriction. Degrading SAMHD1 by treating DCs with SIV-Vpx leads to infection and maturation of DCs promoting viral dissemination (95). Whether HIV-1 infection leads to DC maturation is unclear as it has been shown that interfering with SAMHD1 restriction increases infection of DCs but not DC maturation (112). Furthermore, higher infection observed with SAMHD1 depletion correlates with a stronger suppression of maturation, suggesting that HIV-1 might actively suppress PRR sensing (112). HIV-1 complement opsonization bypasses SAMHD1 restriction in DCs by enhancing SAMHD1 phosphorylation, which results in DC infection (113).

Upon HIV-1 infection APOBEC3 is encapsulated into budding virions. In newly infected cells during reverse transcription of the viral RNA, APOBEC3G triggers G-to-A hypermutations leading to the production of defective proteins and non-functional virus particles which results in a strong inhibition of HIV-1 replication (96). Interestingly, exosomes can transfer host restriction factors such as APOBEC3 from cell to cell and thereby inhibit HIV-1 infection (114). Vif antagonizes APOBEC3 proteins by inducing the recruitment of proteins leading to polyubiquitylation and proteasomal degradation of APOBEC3, thereby preventing incorporation of APOBEC3 into virions (115, 116).

BST2 or Tetherin prevents the release mature Env virions by anchoring virions to the plasma membrane of infected cells (117, 118). The retention of viral particles at the plasma membrane leads to endocytic uptake and the accumulation of these virions in endosomes which may result in viral degradation and thereby inhibit the spread of newly formed virions (119). Similarly, Vpu interacts with tetherin, preventing tetherin trafficking to the cell surface, promoting ubiquitination and subsequent targeting to late endosomes and degradation in lysosomes (118, 120). This prevents incorporation of tetherin into virions thereby enhancing viral budding and release.

Accessory proteins positively contribute to transmission by allowing HIV-1 to escape host restriction. The continuous adaptation of HIV-1 to the antiviral activity of host restriction factors emphasizes their importance in controlling HIV-1 infection and viral transmission.

UNDERSTANDING HOST-VIRUS INTERACTIONS FOR SPECIFIC INTERVENTIONS

Mucosal DCs are among the first immune cells to encounter HIV-1 upon sexual contact. Therefore, receptors expressed or host antiviral factors induced by DCs or LCs could be used in immunotherapeutic strategies to prevent HIV-1 transmission. Langerin binds to glycan ligands for pathogen capture and internalization. A recent study identified chemical compounds with a high binding affinity to langerin (121). Interestingly, these compounds were found to modulate cellular signaling and to suppress inflammation (121, 122). Also, it has been shown that rhTRIM5α is very potent in HIV-1 restriction. Interestingly, human TRIM5α restriction is specific for LCs and is dependent on HIV-1 binding to langerin. Therefore, targeting langerin, host restriction factors like TRIM5α and other ISGs that contribute significantly to viral control could be interesting candidates for therapeutic applications (125). A better understanding of the specific properties of TF viruses, which will relate to different selection biases during transmission, will allow us to identify the specific selection mechanisms and thereby providing novel strategies to counteract the transmission of these TFs (24). The majority of TF viruses are of R5 tropisms and use CCR5 co-receptor for their initial infection, which makes CCR5 an interesting candidate for blocking early transmission. The higher incorporation of Env per particle may increase the sensitivity to neutralization by antibodies.

CONCLUDING REMARKS

At mucosal sites DC subsets patrol the microenvironment and are therefore the first cells to interact with HIV-1 after exposure. If the virus carries specific properties and interacts with DCs or LCs determines the fate of the virus which can result in either routing of the virus for degradation or further dissemination. Strategies to counteract suppression mechanisms by HIV-1 leading to HIV-1 sensing and induction of type I IFN responses upon viral infection can be a powerful strategy to restrict viral dissemination. The induction

of host factors and the ability of HIV-1 to counteract viral restriction shows the intricate interplay between HIV-1 and host. Further understanding of the specific within-host barriers provides new insights important for developing novel therapeutic approaches at the site of initial infection. Understanding the specific properties of TF viruses that create advantages to promote between-host transmission may contribute to the development of immunotherapeutic strategies to combat HIV-1 dissemination.

AUTHOR CONTRIBUTIONS

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REVIEW
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Illuminating the Role of Vpr in HIV Infection of Myeloid Cells

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Vpr is a 14 kDa accessory protein conserved amongst extant primate lentiviruses that is required for virus replication *in vivo*. Although many functions have been attributed to Vpr, its primary role, and the function under selective pressure *in vivo*, remains elusive. The minimal importance of Vpr in infection of activated CD4+ T cells *in vitro* suggests that its major importance lies in overcoming restriction to virus replication in non-cycling myeloid cell populations, such as macrophages and dendritic cells. HIV-1 replication is attenuated in the absence of Vpr in myeloid cells such as monocyte-derived dendritic cells (MDDCs) and macrophages, and is correlated with the ability of Vpr to overcome a post-integration transcriptional defect in these cells. Intriguingly, recent identification of the human hub silencing (HUSH) complex as a target for $DCAF^{CRL4}$-mediated degradation by numerous ancestral SIV Vpr alleles, and the Vpr paralog Vpx, signifies the potential function of HIV-1 Vpr to alter yet-to-be identified chromatin remodeling complexes and prevent host-mediated transcriptional repression of both invading viral genomes and pro-inflammatory responses. Myeloid cells constitute an important bridge between innate and adaptive immune responses to invading pathogens. Here, we seek to illustrate the numerous means by which Vpr manipulates the myeloid cellular environment and facilitates virus replication, myeloid cell-dependent HIV transmission, and systemic virus dissemination.

Keywords: HIV, Vpr, myeloid, DDR, DCAF, VprBP, ubiquitin ligase

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INTRODUCTION

Sexual transmission is the predominant means by which HIV is acquired ("Global AIDS Update" 2016). While the exact cell type targeted by HIV in the genital mucosa remains a matter of debate (1–3), various subsets of dendritic cells (DCs) and macrophages are present at high concentrations within the genital mucosa, and therefore may be early targets of HIV (4–6). Infection of DCs and macrophages is particularly important as they are uniquely poised to transmit HIV with high efficiency to CD4+ T cells during antigen presentation within the secondary lymphoid organs (5, 7, 8). As professional antigen presenting cells, DCs and macrophages have a unique cellular architecture to initiate and sustain robust interactions with CD4+ T cells. Virological synapse formation between DCs /macrophages and CD4+ T cells ensures directed delivery of HIV to its most permissive host: activated CD4+ T cells (9). In order for mucosal transmission and establishment of productive infection, HIV not only has to navigate tissue barriers (3), but also a number of cell-intrinsic immune defenses, or restriction factors, such as APOBEC3G (a cytidine deaminase that dramatically increases genome mutations), tetherin (which prevents HIV viral budding and enforces a positive type I IFN loop upon suppression of viral budding), and SAMHD1 (a dNTPase that limits dNTP levels within the cytoplasm to hinder reverse transcription), that

prevent primate lentiviral infection of DCs, macrophages and resting T cells (10–13). However, primate lentiviruses have evolved to counteract these restriction factors by encoding accessory proteins that selectively inhibit their anti-viral function. Although postulated to act in this capacity, the function of Vpr has yet to be fully understood.

Vpr, or viral protein R, is a 14 kDa protein, encoded by all extant primate lentiviruses. Vpr is actively packaged into virions through its interaction with the p^6 region of Gag (14, 15), and as such has roles in both pre- and post-integration steps of the viral life cycle. Although originally described as an accessory protein, and thus dispensable for virus replication *in vitro*, it has since been shown to play an important role in the infection of macrophages and dendritic cells (16–19). Importantly, Vpr function is necessary for viral pathogenesis *in vivo*. In 1993, six rhesus macaques were infected with pathogenic $SIV_{mac}239$ isolate either containing or lacking Vpr (20). Within 16 weeks, wild type Vpr sequences were isolated from three of the five animals infected with Vpr null virus. Moreover, the remaining two animals infected with Vpr null virus displayed delayed pathogenesis, and reversion to Vpr-expressing virus by 66 weeks (20, 21). These studies validated retrospective work that found reversions of an internal Vpr stop codon to an open reading frame in an accidentally infected laboratory worker and experimentally infected chimpanzees (22, 23). Together, these studies were seminal in igniting research into the role of Vpr in the pathogenesis of HIV. Vpr is best known to induce an Ataxia-Telangiectasia and Rad3-related (ATR) dependent-DNA damage response, or DDR (24). Vpr-mediated DDR activation results in a G_2 to M cell cycle arrest in cycling cells, which is perplexing as the cell populations whose infections are seemingly most reliant on the presence of Vpr are terminally differentiated and thus not susceptible to cell cycle arrest. The ability to induce a G_2 to M cell cycle arrest is thought to be advantageous for viral transcription as the HIV LTR has been shown to be most active during this phase of the cell cycle (23, 25). What's more, the Vpr residues that confer G_2 to M cell cycle arrest capabilities are under positive selection *in vivo* and are thus the most well-studied in the field (26). It remains to be determined if induction of DDR by Vpr, an evolutionarily conserved function amongst all primate lentiviral Vpr proteins (27–29), is necessary for establishment of virus replication in metabolically quiescent immune target cells, such as monocytes, macrophages and DCs.

Vpr/Vpx and the Importance of Co-opting the Ubiquitin-Ligase $DCAF^{CRL4}$ in Myeloid Cells

Vpx, or Viral protein x, arose following the duplication of Vpr post-diversion of the primate lentiviral lineages that gave rise to HIV-1 and HIV-2 (30, 31). Vpx has a well-characterized role in degrading the retroviral restriction factor SAMHD1 (10, 11). In terminally differentiated or non-cycling cells, SAMHD1 reduces the concentration of dNTPs within the cytoplasm, thereby drastically limiting reverse transcription (32–35). Vpx bridges SAMHD1 to the E3-ubiquitin ligase CUL4A-DDB1 DCAF ($DCAF^{CRL4}$) leading to its polyubiquitination and proteasomal degradation (10, 11, 36). There has been much interest in identifying the restriction factor(s) targeted by Vpr as it similarly co-opts the $DCAF^{CRL4}$ complex to ubiquitinate target host proteins. Unlike Vpx however, infection of myeloid cells by either HIV-1 or HIV-2 still occurs in the absence of Vpr, albeit with significantly different outcomes (19, 24, 37–41). It is likely that the replication advantage conferred by Vpr lies in its ability to induce a DDR, though the mechanisms by which Vpr-induced DDR facilitates enhanced virus replication and spread *in vivo* are still to be determined. The multitude of DDR proteins associated with the Vpr-$DCAF^{CRL4}$ complex (24, 37–39, 41), suggests that Vpr by co-opting a host protein complex involved in multiple cellular pathways, has managed to maximize its impact at the interface of virus and host to promote HIV spread.

Vpr Residues Involved in $DCAF^{CRL4}$ Engagement

An NMR structure of HIV-1 Vpr provides insight into how it interacts with multiple proteins. Both N and C-termini are unstructured (nucleotides 1–16, and 77–96, respectively) and flank three α-helices from nucleotides 17–33, 38–50, and 56–77 (42). The HIV-2 Vpr, as well as the closely related Vpr alleles from SIV_{smm} and SIV_{mac}, are predicted to be structurally homologous to that of HIV-1. Whilst the unstructured C- and N-terminal domains facilitate interactions with host targets, the $DCAF^{CRL4}$- binding domain is isolated to the third α-helix (42–45). The HIV-1 Vpr mutants Q65R and H71R for example, and corresponding residues in HIV-2/SIV_{mac} Vpr alleles fall within this region and fully abrogate Vpr-$DCAF^{CRL4}$ interactions. These mutations prevent Vpr-mediated transcriptional enhancement in MDDCs (19), decrease degradation of multiple DNA damage response proteins (46–50), and prevent G_2/M cell cycle arrest in CD4+ T cells (51). Furthermore, ablation of Vpr-$DCAF^{CRL4}$ interaction, as occurs with a VprQ65R mutation (albeit not with the VprQ77R mutation), has been associated with long-term non-progression *in vivo* (52). Investigations into the $DCAF^{CRL4}$-mediated enhancement of infection in myeloid cells use these select mutations to infer mechanisms of action and are thus worthy of mention.

$DCAF^{CRL4}$-DEPENDENT ROLES OF Vpr IN MYELOID CELLS

DNA Damage Response Proteins

Human Uracil DNA Glycosylase

Monocyte-derived macrophages (MDMs) have a high ratio of dUTP/TTP in their cytoplasm that can lead to the mis-incorporation of uracil in the reverse transcribed genome. The ratio of dUTP/TTP in macrophages was found to be as high as 60 (53, 54). Human uracil DNA glycosylase (hUNG) excises mis-incorporated UTP and recruits additional repair enzymes to the site of genome mutation. Thus, HIV-1 Vpr-mediated $DCAF^{CRL4}$-dependent ubiquitination and proteasomal degradation of hUNG was hypothesized to restrict virus replication through either degradation of uracilated viral DNA prior to integration or via transcriptional interference of the uracilated provirus

(53, 55, 56). However, due to the intrinsically low levels of hUNG in MDMs (56), the utility of uracil-dependent restriction of HIV-1 in MDMs is limited. Furthermore, infection of MDDCs with HIV-1 expressing hUNG-binding deficient VprW54R mutant does not result in transcriptional attenuation nor deficiency in viral spread (19). Thus, the significance of hUNG degradation by the Vpr-DCAF^{CRL4} complex (39, 57) remains unclear.

SLX4-SLX/MUS81-EME1

The importance of Vpr in the Holliday junction repair pathway has been of great interest as it promised to provide insight into the role of Vpr at the viral integration step. Original reports suggested that HIV-1 Vpr-DCAF^{CRL4}-mediated ubiquitination of MUS81 which, in the presence of phosphorylated EME1 and kinase-active PLK1, prematurely activates the quaternary endonuclease complex SLX4com (47). This activation was shown to precede G_2/M cell cycle arrest and result in the formation of FANCD2 foci as a result of activation of the Fanconi anemia pathway (47). Notably, virion-associated Vpr-mediated activation of SLX4com was shown to prevent type I IFN production which is of great importance due to the myriad of interferon stimulated genes (ISGs) that modulate myeloid cell function and determine the dissemination efficiency of virus through the host (47). However, subsequent studies have only shown a Vpr-DCAF^{CRL4} dependent degradation of the SLX4com subunits MUS81-EME1 (50, 58–60) and have not addressed whether the active SLX4com suppressed innate immune detection of HIV-1 in myeloid cells. Additionally, interaction of SLX4com with Vpr is not conserved amongst all primate lentiviral Vpr alleles (59). Together, published findings so far, suggest that the Vpr-mediated activation of SLX4com does not have a conserved role in suppressing innate immune detection of primate lentiviruses in myeloid cells.

Helicase-Like Transcription Factor (HLTF)

Helicase like transcription factor, or HLTF, is a target of Vpr-mediated DCAF^{CRL4} degradation (46, 48). Like UNG2 and SLX4-SLX1/MUS81-EME1, HLTF is involved in DNA damage repair. Specifically, HLTF is critical to the remodeling and repair of stalled replication forks (61). Although HLTF is degraded in macrophages in a Vpr- DCAF^{CRL4} dependent manner, it is unclear whether HLTF antagonizes viral replication in myeloid cells.

Exonuclease 1

Exonuclease 1, or Exo1, is a Rad2/XPG 5′ to 3′ exonuclease involved in numerous DNA repair processes that ensures genome stability throughout the cell cycle (62). Exo1 has recently been identified as a substrate for Vpr-DCAF^{CRL4} polyubiquitination and proteasomal degradation in CD4+ T cells (49). The authors speculate that Exo1 antagonism prevents deleterious processing of reverse transcription- and viral integration-intermediates, and thereby attribute Exo1 restriction to virion-associated Vpr rather than its *de novo* synthesized partner (49). As of yet, Exo1 has not been shown to play a role in promoting HIV-1 infection of macrophages or dendritic cells.

While these published studies highlight the numerous interactions of Vpr with diverse DDR proteins, contribution of these interactions to viral pathogenesis have remained unclear. Although understudied in the case of HIV-1 infection, there is a robust literature tying innate immune signaling and DDR (63, 64). It should be noted that manipulation of the DDR is not unique to HIV. Rather, it is a shared pathogenic strategy used extensively at the interface of hosts with both bacteria and viruses that can promote pathogen replication and pathogenesis (65). Kaposi sarcoma herpesvirus, for example, encodes a protein (Latency-Associated Nuclear Antigen or LANA) that sequesters Rad50, Mre11, and NBS1, all members of the DDR signaling activator MRN complex to prevent cytoplasmic sensing of viral DNA and innate immune activation (66). Another example of virus subversion of DDR pathway includes murine γ-herpesvirus which specifically encodes orf36 whose role is to induce an ATM-dependent DDR and H2AX phosphorylation (67). In the absence of orf36 or ATM activation, virus replication is attenuated, pointing toward a role for the DDR in facilitating virus replication (67). Overall, it is evident that Vpr uses DCAF^{CRL4} to induce a DDR, with potentially divergent outcomes in different cell populations. What remains unclear is how activation of the DDR and interaction of Vpr with DNA repair proteins allows viral evasion of immune detection in myeloid cells. Since the kinetics of reverse transcription of HIV-1 in myeloid cells is relatively slow, it is tempting to speculate that manipulation of diverse DDR pathways is a conserved strategy by primate lentiviral Vpr alleles to overcome premature host repair of viral reverse transcription intermediates (63), though definitive evidence for this hypothesis has been lacking. Rather, it is likely that activation of DDR promotes multiple discrete stages of the virus life cycle. For example, Vpr can induce DDR through both the ATM and ATR pathways (24, 68). Unresolved ATM activity can lead to activation of NF-κB (69) and increased production of inflammatory cytokines, such as IL-6, both of which can result in enhanced viral gene expression and macrophage-dependent HIV-1 transmission to CD4+ T cells (70).

Vpr Functions in Transcriptional De-repression

Transcriptional Enhancement

Previous work by our group has shown a post-integration defect in monocyte-derived dendritic cells (MDDCs), infected with Vpr deficient HIV-1 (19). Infections in the absence of virion associated Vpr were characterized by low proviral transcription despite similar levels of integration, and reduced infection of CD4+ T cells in co-cultures (19). This defect is dependent on Vpr binding to DCAF^{CRL4} as it is fully abrogated upon infection with Vpr mutants (Q65R or H71R) lacking DCAF^{CRL4} interactions. It should be noted that numerous viruses besides HIV-1, most notably Hepatitis B virus, can also manipulate the E3 ubiquitin ligase DCAF^{CRL4} to enhance transcription (71). While, the mechanism of HIV-1 Vpr-mediated transcriptional enhancement remains unclear, previous research has shown Vpr-mediated degradation of HDACs (38) and members of the NuRD chromatin remodeling complex (72) which may globally

enhance transcription. Furthermore, DCAF^{CRL4} also has a well-known role in the degradation of a transcriptional repressor, ATF3, which is necessary to correct UV-damage (73). This explanation is not satisfactory given the cell type dependency of the transcriptional enhancement. Whether a MDDC-specific repressor/activator is degraded or sequestered remains unknown and warrants further investigation.

TET2

Members of the TET DNA dioxygenase family have recently been shown to be degraded in a Vpr-DCAF^{CRL4} dependent manner (70). In myeloid cells TET2 is naturally monoubiquitinated. TET2 N-terminal monoubiquitylation allows for efficient binding to chromatin and subsequent recruitment of chromatin remodeling machinery and transcription factors. However, in the presence of Vpr, TET2 is rapidly polyubiquitinated at a site independent of its natural monoubiquitylation site and undergoes DCAF^{CRL4}- dependent proteasomal degradation (70). This is relevant in myeloid cells as TET2 is an upstream suppressor of IL-6 expression. TET2 recruits HDAC1 and HDAC2 to the IL-6 promotor thereby repressing IL-6 transcription. Importantly, in monocyte-derived macrophages and the monocytic cell line THP-1, the lack of Vpr-mediated degradation of TET2 was associated with reduced viral particle release and slower spread of HIV-1 infection. Upon TET2 knockout, the differences in infection between Vpr-competent and Vpr-deficient viruses was lost. Since IL-6 has long been recognized as a transcriptional enhancer of HIV in monocytes (70, 74–76), these findings are further suggestive of a direct link between Vpr, TET2 degradation, and persistent IL-6 production, which might result in enhanced efficiency of viral spread from myeloid cells to CD4+ T cells.

Epigenetic Regulation of Provirus

Until recently, it was not known whether there were host-intrinsic mechanisms to restrict retroviral replication following integration. However, recent investigations have identified a novel method of cell-intrinsic restriction: that of deposition of transcriptionally suppressive methylation marks at proviral LTRs. Following reverse transcription and integration, the LTR of proviruses within heterochromatin are methylated through the sequential recruitment of HP1 and the methyltransferase Suv39H1 (77) Tri-methylated H3K9 recruits the HUSH (HUman Silencing Hub) complex of which there are three components; TASOR, MPP8, and periphilin (78). Although the HUSH complex does not harbor methyltransferase activity itself, HUSH recruits the methylase SETDB1 which induces further H3K9me3 methylation of the provirus. Notably, shRNA-mediated knockdown of each HUSH complex protein rescues endogenous and exogenous retroviral gene expression, thereby signifying the importance of its quaternary assembly for transcriptional repression (78). Interestingly, TASOR is targeted by the SIVmac/HIV-2 lineage Vpx for DCAF^{CRL4}-mediated polyubiquitination and proteasomal degradation, thereby increasing the transcriptional activity of proviruses that would otherwise be suppressed (78–80). While HUSH complex can repress transcription from integrated HIV-1 LTR (78, 80, 81), surprisingly, HIV-1 Vpr does not target the HUSH complex proteins for degradation (79). However, multiple Vpr alleles from ancestral primate lentiviruses to HIV-1, including alleles derived from SIV_{AGM}, $SIV_{MUS2,}$ and $SIV_{SAB,}$ have been shown to prevent HUSH-mediated silencing (79, 80). These studies mark the beginning of investigations into Vpx/Vpr antagonism of antiviral host proteins at the proviral DNA level. Although HUSH-mediated transcriptional silencing is not a myeloid specific anti-viral mechanism, the HUSH complex and its associated facilitators are active in myeloid lineages. Recent studies in the literature provide evidence for epigenetic control of proinflammatory cytokine responses in macrophages (82, 83). For instance, the histone methyltransferases, SETDB1 and Smyd2, potently suppress TLR4- mediated induction of IL-6 and TNFα production, and mice with macrophage-specific SetDB1 deficiency are hyper-responsive to endotoxin challenge (82). Whilst antagonism of HUSH complex has not been attributed to HIV-1 Vpr, transcriptional silencing of the HIV-1 LTR in MDDCs in the absence of Vpr (19) suggests the existence of additional mechanisms of myeloid cell-intrinsic transcriptional repression that are targeted by HIV-1 Vpr.

Dicer and miRNA Processing

Modulation of the RNA interference (RNAi) and microRNA (miRNA) pathways is an integral means by which pathogens usurp host functions to their advantage (84, 85). MicroRNAs in particular have long been known to play a role in HIV replication in multiple cell populations. For instance, miR-29a, has been implicated in the suppression of HIV mRNA levels through its binding to the 3′-UTR of HIV RNA and subsequent attachment to P body proteins and RISC complexes (86). Dicer is required for processing pre-miRNA substrates to reveal a double-stranded miRNA complex, which then binds to the RISC complex and represses target mRNA expression, either via translation inhibition or via mRNA degradation (87). Recent studies have also demonstrated the ability of miRNAs to negatively regulate proinflammatory responses in macrophages by restricting chromatin remodeling and enforcing transcriptional silencing of promoters of select inflammatory genes (88). Interestingly, Dicer has been identified in complex with Vpr-DCAF^{CRL4} prior to its degradation and depletion of Dicer within infected MDMs has been shown to increase viral replication via unknown mechanisms (89). We posit that Vpr-Dicer dependent modulation of select miRNA expression might contribute to the de-repression of inflammatory responses. It should be noted that Vpr-mediated Dicer depletion has also been shown in CD4+ T cells and as such, is not a myeloid-specific antagonist of innate restriction (89). The role of Dicer degradation has yet to be fully understood, particularly as research into the function of miRNA and RNAi in HIV pathogenesis is increasing (90).

DCAF^{CRL4}-INDEPENDENT ROLES OF Vpr IN MYELOID CELLS

Envelope Trafficking

Myeloid cells often populate mucosal tissues and as such are poised to disseminate HIV from the periphery to sites harboring abundant activated CD4+ T cells. Macrophages and

dendritic cells are capable of transferring virus via cis or trans infection. Both methods facilitate the concentration of virions to the infectious synapse, and in doing so greatly increase the probability of CD4+ T cell infection (9, 91, 92). Two studies have investigated the role of Vpr in the concentration and delivery of virus at the virological synapse. Both of these studies show that in the absence of Vpr, Env-positive virions are trafficked to the lysosome for degradation, thereby reducing the efficiency of macrophage to CD4+ T cell virus spread at low multiplicity of infection (93, 94). In contrast, our investigations into whether Vpr facilitates evasion of this Env-dependent reduction in virus release in other cells types, such as MDDCs, have not yielded similar results (19), suggesting that the effect of Vpr on Env expression might be restricted to specific cell types and not universally observed.

Type I IFN and Pro-Inflammatory Responses

There is mounting evidence to suggest that Vpr modulates the immune response of myeloid cells to favor viral replication and dissemination throughout the host. Early studies suggested a possible defect in the activation of MDMs and MDDCs upon treatment with recombinant Vpr (95). This defect was characterized by low CD33 surface expression, poor CD80/86 upregulation, and impaired antigen presentation to activated CD4+ T cells (95). In contrast to studies utilizing exogenous addition of recombinant Vpr, there has been a preponderance of research investigating the role of Vpr in the context of a viral infection. For instance, *de novo* expression of Vpr in productively infected MDDCs induced pro-inflammatory cytokine (TNF-α, IL-6 and IL-8) production (70, 96, 97). Previous work by our group has shown enhanced proviral transcription in MDDCs in the presence of Vpr (19). Other studies have similarly shown a role for Vpr in proviral transcription. Liu et al. showed that Vpr alters the availability of the NF- κB p50-p65 heterodimer and AP1 (98), both of which are necessary for the initiation of HIV transcription from the 5′ LTR (99, 100) and expression of pro-inflammatory cytokines. In this study, Vpr was shown to facilitate the polyubiquitination and subsequent phosphorylation (activation) of TAK1, an upstream regulator of NF-κB and AP1 (98). Interruption of TAK1 phosphorylation, and thus inhibiting its activation, significantly reduced proviral transcription (98).

Both our study (19) and the work showing Vpr-mediated modification of TAK1 (98)are important in light of the recent identification of a novel viral detection pathway: one in which host sensing of *de novo* expressed intron-containing HIV-1 RNA

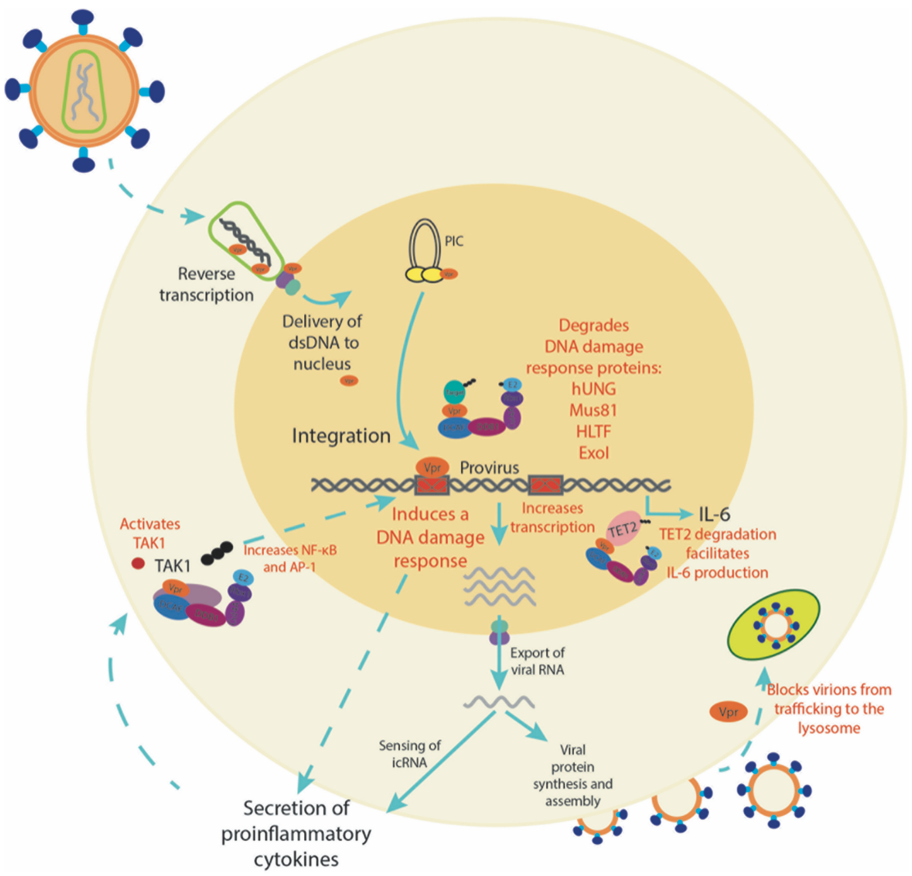

FIGURE 1 | The role of Vpr in the infection of myeloid cells. A summary of the multiple functions Vpr plays in myeloid cells, from enhancing transcription and inducing a DDR to the secretion of pro-inflammatory cytokines. In red are processes in which Vpr has been directly investigated.

(HIV icRNA) in MDMs and MDDCs results in ISG expression and proinflammatory chemokine and cytokine production, including IP-10 and IL-15 (101). IP-10 is an inflammatory chemokine and is a ligand for the receptor CXCR3 (102), while IL-15 is a γ-chain cytokine critical for the proliferation and homeostasis of T cells (103). CXCR3 is expressed on activated CD4+ T cells, and thus secretion of IP-10 from productively infected myeloid cells may result in additional recruitment of virus-susceptible cells to sites of viral replication in the peripheral tissues. Furthermore, IL-15 exposure can result in SAMHD1 phosphorylation and inactivation of its dNTPase activity, thus alleviating restrictions to viral replication in quiescent CD4+ T cells (104). Interestingly, a TAK1 inhibitor reduced production of IP-10 from HIV-1 infected macrophages (101). Thus, in this model, Vpr-dependent enhanced proviral transcription potentially increases the pool of viral icRNAs subject to cell-intrinsic innate immune sensing, resulting in the establishment of a pro-inflammatory state and enhanced virus dissemination.

It should be noted that an increase in viral transcription in myeloid cells is a double-edged sword for HIV in that there is also induction of ISG expression and establishment of a putative anti-viral state. While the nucleic acid sensing mechanism responsible for detection of HIV icRNA is yet to be determined, Vpr can block phosphorylation and nuclear translocation of interferon regulatory factor 3 (IRF3) via inducing ubiquitination and proteosomal degradation of IRF3, though this has only been shown in CD4+ T cell lines (105). Additionally, Vpr has been shown to bind to TBK1 in myeloid cells and prevent its phosphorylation, thereby preventing induction of type I IFN production (106). Thus, we posit that Vpr might function to promote NF-κB-dependent pro-inflammatory responses while contributing to the suppression of induction of anti-viral host defenses. Furthermore, induction of ISGs such as CD169 in MDMs and MDDCs upon host sensing of HIV icRNA in myeloid cells (101) might further tip the balance toward enhanced virus dissemination as opposed to virus restriction. For instance, induced CD169 expression on HIV-infected macrophages and dendritic cells can facilitate cell-to-cell transmission of CD4+ T cells across infectious synapses (101, 107, 108). Together, these studies point to the role of Vpr as a protein that carefully navigates multiple viral sensing systems to induce recruitment of additional cellular targets of virus, whilst evading antiviral immunity.

CONCLUSION

It is clear that Vpr plays an important role in the infection of myeloid cells (see **Figure 1**). A number of tissue-resident macrophages, such as microglia, kupfer cells, alveolar, intestinal, testicular and vaginal macrophages harbor proviral DNA (109–113), and tissue-resident macrophages are estimated to compromise up to 4% of infected cells *in vivo* (114), and importantly, can remain persistently infected with HIV-1 even in the presence of cART (109–112, 115). It is possible that the Vpr-mediated DDR activates a pro-inflammatory state that promotes the establishment of a tissue-resident myeloid cell reservoir, whereby virus spreads efficiently due to persistent virion production and enhanced cell-to-cell contacts between HIV-infected myeloid cells and CD4+ T cells. In this way, the infection of myeloid cells is the bridge between the relatively hostile sites of virus acquisition (most notably the peripheral mucosal tissues) and the key target of HIV; CD4+ T cells. It seems likely that the true value of Vpr *in vivo* is its versatility, allowing for evasion of viral restriction both prior to and post integration in myeloid cells. Future studies will need to address the relative importance of each of the known Vpr functions *in vivo*.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Manipulation of Mononuclear Phagocytes by HIV: Implications for Early Transmission Events

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Mononuclear phagocytes are antigen presenting cells that play a key role in linking the innate and adaptive immune systems. In tissue, these consist of Langerhans cells, dendritic cells and macrophages, all of which express the key HIV entry receptors CD4 and CCR5 making them directly infectible with HIV. Mononuclear phagocytes are the first cells of the immune system to interact with invading pathogens such as HIV. Each cell type expresses a specific repertoire of pathogen binding receptors which triggers pathogen uptake and the release of innate immune cytokines. Langerhans cells and dendritic cells migrate to lymph nodes and present antigens to CD4 T cells, whereas macrophages remain tissue resident. Here we review how HIV-1 manipulates these cells by blocking their ability to produce innate immune cytokines and taking advantage of their antigen presenting cell function in order to gain transport to its primary target cells, CD4 T cells.

Keywords: HIV, mononuclear phagocyte, dendritc cell, macrophage, transmission, interferon

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INTRODUCTION

Mononuclear phagocytes (MNP) are a group of antigen presenting cells (APC) which include monocytes, Langerhans cells (LC), dendritic cells (DCs), and macrophages. Circulating blood monocytes and DCs are bone marrow derived whereas LCs and tissue resident macrophages, such as Kupffer cells in the liver and those in lungs, kidneys, are seeded during embryogenesis. However, both LCs and macrophages can be replenished by infiltrating monocytes. In tissue, MNPs are the first immune cells to interact with invading pathogens such. HIV is transmitted across the various skin or mucosal tissues that comprise the genital and anorectal tracts which differ in their anatomy. The foreskin, glans penis, labia, vagina, ectocervix, fossa navicularis, and anal canal contain a stratified squamous epithelium mostly consisting of differentiating layers of keratinocytes resulting in a physically impregnable barrier against incoming pathogens. However, HIV transmission across this surface has been shown in human *ex vivo* genital tissue explants from the vagina, ectocervix, and foreskin (1–6). In type I mucosal tissue (urethra, rectum, and endocervix) a single layer of columnar epithelium separates the lamina propria from the lumen and HIV has been found to cross this barrier in mucosal explants and interact with MNPs (7, 8).

The primary role of MNPs is to detect incoming pathogens via an array of pathogen binding receptors expressed on their surface. These include Toll-like receptors (TLR) and lectin receptors such as C-type lectin rectors (CLR) and Sialic acid-binding immunoglobulin-type lectins (Siglec). In response to pathogens binding TLRs, MNPs produce an array of innate immune cytokines

including type I and III interferons, chemokines, and proinflammatory cytokines. Type I and III interferons trigger the induction of a wide array of interferon stimulated genes (ISG), both in infected and bystander cells, which encode antiviral proteins to help cells combat the viral infection. Chemokines such CCL3-5, act to recruit other immune cells to the site of infection and proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1B, and IL-6, act to activate these cells to prepare them to elicit a potent immune response. Thus, many successful invading pathogens, such as HIV, have evolved mechanisms to block the ability of cells to produce innate immune cytokines, or evade them.

Following detection of pathogens via CLRs, MNPs rapidly endocytose (LCs and DC) or phagocytose (macrophages) them, destroying them via acid proteolysis. Macrophages remain in tissue, but LCs and DCs undergo a process of maturation triggering them to migrate to the draining lymph nodes where they present pathogen antigens to CD8 and CD4 T cells via MHC-I and –II, respectively. They then mediate an immune response against pathogens or immune tolerance toward commensal bacteria. As CD4 T cells are the main HIV target cells in which the virus undergoes active replication, LCs and DCs provide HIV with a delivery vehicle from the site of initial exposure to the lymph nodes where the virus has access to a high concentration of CD4 T cells. However, the virus must avoid destruction by acid proteolysis in the process. It is currently unclear if macrophages can perform a similar function within the mucosa at the site of initial exposure.

In this review, we describe how HIV-1 manipulates MNPs in order to facilitate successful transmission. We review how HIV-1 blocks interferon induction in MNPs while at the same time inducing the expression of specific ISG. We also describe how HIV-1 modulates DC maturation such that it triggers migration to draining lymph nodes but avoids destruction by acid proteolysis. Finally, we describe two independent mechanisms by which HIV-1 is transferred from MNPs to CD4 T cells.

MONONUCLEAR PHAGOCYTES SUBSETS AND SEXUAL TRANSMISSION OF HIV

MNPs consist of monocytes, LCs, DCs, and macrophages.

Monocytes

Monocytes are found in blood only and although there is some evidence that certain monocyte subsets (especially $CD16^+$ monocytes) may represent HIV target cells, they are not discussed in this review which is focused on HIV transmission within anogenital tissues where it now occurs in the vast majority of cases.

Dendritic Cells

Dendritic Cells are potent APCs and differentiate from bone marrow derived precursors. They can be split into three key subsets: plasmacytoid DCs (pDC) and two subsets of myeloid or conventional (c) DCs named cDC1 and cDC2.

pDCs

The primary role of pDCs is to migrate to inflamed tissue and secrete large quantities of Type 1 interferons to combat viral infections. However, they have also been shown to act as APCs and stimulate CD4 T cells, earning them their descriptor as a type of DC. These cells express the HIV entry receptor CD4 as well as the two key chemokine receptors CXCR4 and CCR5 and HIV can enter these cells via the classical CD4 entry pathway or can be taken up via dynamin endocytosis where the viral RNA is detected by TLR7 (9–11) triggering type I interferon induction. As they are found in inflamed tissues only they are not generally regarded as key players in initial HIV transmission, and will not be discussed in the review in detail which focuses on myeloid cells. However, we refer the reader to a recent review on the involvement of pDCs in infection and pathogenesis (12). However, two points should be noted. Firstly, pDCs have previously been defined as CD11c– $CD123^+$ $CD303/BDCA2^+$ cells but recently this group of cells has been shown to be made up of a heterologous population of *bona fide* pDCs which secrete Type I interferons, and a small myeloid population that can be distinguished by expression of Axl and Siglec-6, hence named AS DCs (13, 14). It is the AS DC myeloid component that act as APCs, but their potential role in HIV transmission and pathogenesis has yet to be investigated. Secondly, recently it has become clear that sexual transmission of HIV is strongly associated with inflammation (15–17), especially in Sub-Saharan Africa. This is a key concern as some current prevention strategies are not as effective in the context of an inflamed mucosa (18–20). Therefore, the role that pDCs, AS DCs, and other potential inflammatory MNPs should be investigated.

cDC1

cDC1 are present in the dermis, lamina propria, anogenital skin, and mucosa and were originally defined by their expression of CD141/ BDCA3. However, this marker can also be expressed on cDC2 under some circumstances and cDC1 are now better defined by their unique expression of CADM1, IRF8, Clec9a, and XCR1 (13). cDC1 are able to detect dead and dying cells via their unique CLR Clec9a which binds F-actin (21, 22) and importantly can cross-present antigen to CD8 cells or directly to CD4 T cells. Few studies have investigated these cells in the context of HIV but a recent study showed they express high levels of the retroviral restriction factor SAMHD1 and are resistant to infection (23). They will not be discussed further, however it is of note that poly(I:C) was recently shown to up regulate the expression of costimulatory molecules in cDC1 in response to HIV peptides making them important to consider for potential vaccine design (24).

cDC2

cDC2 are also present in the dermis and lamina propria and are more abundant than cDC1. They can be defined by their expression of CD1c, IRF4, and high expression of CD11c. In skin only they also express CD1a and a minor population have been shown to express the CLR langerin (25). cDC2 express the HIV-1 entry receptors CD4 and CCR5 as well as a wide range of pathogen binding CLRs and cDC2 cell counts are correlated

with a high risk of rapid disease progression during early HIV-1 infection (26). Despite this, until recently, these cells had not been studied in the context of HIV transmission. However, in a recent study Bertram et al. (27), showed that these cells are present at the epithelial surface of human anogenital tissues where they capture HIV and then transfer the virus to CD4 T cells. Furthermore, they showed that these cells predominate over LCs in these tissues and thus could be the primary HIV transmitting MNP. In another study DCs have been reported to exist in vaginal stratified squamous epithelium which the authors refer to as Vaginal Epithelial Dendritic Cells (VEDC) (28). These are almost certainly the cDC2 described by Bertram et al. (27).

Langerhans Cells

Langerhans Cells are found within the stratified squamous epithelium which forms the outermost layer of anogenital tissues comprised of skin (anal verge, labia, and outer foreskin) or Type II mucosa (vagina, cervix, inner foreskin, glans penis, fossa navicularis, and anal canal). These were originally believed to be a type of DC however more recently some groups have argued that they are more macrophage-like (6, 29–31). In terms of function they act like DCs in that they migrate out of tissue in response to pathogen exposure, and present antigens to CD4 T cells in the lymph node. However, ontologically they are more like macrophages as both cell types have an embryonic origin but can be reseeded after inflammation by two waves (32, 33). The first wave is a transient population that appear to be derived from circulating monocytes. The second wave establish long-term LCs derived from precursors of an unknown origin (34). In regards to this Bertram et al. recently described a human epidermal cell that is phenotypically almost identical to LCs but can be differentiated by their lower expression of CD33. These $CD33^{low}$ LC-like cells differ to LCs in their morphology and are also functionally very weak APCs. Thus, these $CD33^{low}$ cells could represent a LC precursor. DCs on the other hand are exclusively derived from bone marrow derived progenitors (31). Therefore, LCs are best grouped as their own MNP subset. Prior to the very recent studies by Bertram et al. (27) and Pena-Cruz et al. (28), LCs were believed to be the only MNP subset found within the epidermis and as such much research has been conducted on these cells as they have been considered to be the first cells that interact with HIV (6). Recently, an increase in "LCs" ($CD1a^+$ expressing cells) in human inner foreskin explants after 24 h virus exposure has been described (35). This was repeated with macaques *in vivo*, however, these cells may be more like the recently described cDC2/VEDCs than *bona fide* LCs as CD1a does not discriminate between LCs and cDC2 (27).

LCs bind HIV via their unique CLR langerin and can be infected via CD4 and CCR5 but their role in transmission is still controversial. Some groups argue they play a critical role in transmission by directly transferring HIV to CD4 T cells (1–3, 36), while another group has argued they form a natural barrier to HIV and degrade the virus in Birbeck granules (37, 38); the latter may be explained by isolation methods using trypsin which cleaves CD4, preventing infection. Importantly in anogenital tissue, $langerin^+$ cells have been shown to express CCR5 and CXCR4 in human foreskin. Higher CCR5 expression was found on $langerin^+$ cells present in the inner compared to the outer foreskin (39), so these cells are prime target cells for HIV.

Macrophages

The primary role of macrophages is the phagocytosis and killing of pathogens at the site of infection and in tissue homeostasis. They are relatively weak APCs, unlike DCs, but similar to LCs they are seeded in tissue during embryogenesis but can also be replenished within tissue by infiltrating monocytes. Traditionally they have been defined by their expression of CD14 and the transcription factor FXIIIA and high autofluorescence. Recently, four distinct subsets of macrophages (Mf1, Mf2, Mf3, and Mf4) have been defined in the human small intestine which have distinct phenotype, function, and anatomical location within the mucosa (40). It will be of interest to investigate whether these subsets interact with the virus early post anorectal exposure and if they can become infected.

Macrophages express relatively low levels of CD4 but moderate levels of CCR5 unlike circulating $CD14^+$ monocytes which are $CCR5^-$ (41). However, once they enter tissue and differentiate into macrophages they upregulate CCR5 and become susceptible to HIV infection. In contrast circulating $CD16^+$ monocytes are $CCR5^+$ and susceptible to HIV. Macrophages can be infected by cell-free HIV or cell-to-cell transmission from infected CD4 T cells via virologic synapses, or by selective phagocytosis of infected $CD4^+$ T cells, leading to efficient infection (42, 43). Macrophages can also bind to HIV via mannose receptor (MR) (44) and DC-SIGN which act as concentrating receptors prior to binding to CD4 and CCR5(45). The levels of productive infection of monocyte derived monocytes (MDMs) by clinical isolates is HIV strain and donor dependent as shown by studies with identical twins (41, 46). Furthermore, as HIV infection progresses to immunodeficiency and AIDS the CCR5 utilizing strains become more infectious for MDMs (47), this being predominantly determined by enhanced HIV envelope binding to CCR5 (see below) (5). Prior to the availability of combination antiretroviral therapy the ability of HIV, SIV, or SHIV to persist with noncytopathic infection in macrophages in brain, lymph nodes, gut, spleen, and marrow was well-described (48–52).

More recently there has been intense interest in macrophages for their potential secondary role as sites of persistent latent infection, after resting memory CD4 T cells, during combination ARV therapy (cART) (53). Recent (and older) studies in SIV-macaque models and in humans have shown persistent infectious virus in alveolar macrophages (43, 48), lymph nodes (54), spleen and liver before cART, and persistent viral DNA during ART, but infectious virus was not observed in macaque and human alveolar macrophages (43) or human liver (55) while under or after treatment with cART. In the brain, infectious HIV persists mainly in microglial cells and perivascular macrophages. Noninfectious HIV DNA is in astrocytes and is inversely proportional to peripheral CD4 T cell counts. The effect of cART, which often penetrates poorly into brain, seems to incompletely suppress HIV infectivity (56). Severe HIV-associated neurocognitive disorders (HAND)

has become less common in the cART era but milder forms of HAND persist. Whether this represents inadequate cART suppression of HIV in brain myeloid cells, or whether there is a modified form of latency, requires further studies. In blood CD16$^+$ monocytes express higher levels of CCR5 than CD14$^+$ CD16$^-$ monocytes. Their transport of virus into tissues and differentiation into macrophages bearing HIV, especially in to CD163 perivascular macrophages also needs further definition (57).

Are Non-autofluorescent CD14$^+$ Cells Macrophages or DCs?

It was previously believed that non-autofluorescent CD14 expressing tissue cells were DCs. However, in skin, these cells have recently been shown to be more transcriptionally similar to macrophages and have been redefined as monocyte-derived macrophages which are derived from CD14$^+$ monocytes that migrate into tissue from the blood (58). Thus, in skin, all CD14 expressing cells are now defined as macrophages. Both macrophage populations express Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)/CD209$^+$. They are concentrated in lymphoid aggregates more commonly found in inner than outer foreskin (39).

Whether or not intestinal non-autofluorescent CD14 expressing cells are also macrophages and not DCs is currently a very interesting topic in the field of HIV as there are a plethora of studies using intestinal tissue showing that these cells are key target cells for HIV via their CLR, DC-SIGN. These cells have been shown to extend dendritic process through the columnar epithelium to sample pathogens in the intestinal lumen (59, 60). They have been specifically shown to sample HIV in this fashion and then transfer the virus to CD4 T cells in the underlying lamina propria (4, 61). Similarly, these cells have been shown to be key HIV target cells in the rectum (62). Finally CD11c$^+$CD14$^+$ cells they have recently been shown to capture HIV in the female reproductive tract (63). Although they may be transcriptionally similar to macrophages, functionally they may be more similar to DCs and, like LCs, these may need to be redefined as a new type of MNP.

MONOCYTE DERIVED DENDRITIC CELLS AND MACROPHAGES

As MNPs are so difficult to isolate from tissue and also undergo maturation as a result of their extraction (64), by far the majority of studies have been carried out on *in vitro* derived monocyte-derived DCs (MDDC) and MDM. These cells can be generated in larger numbers in an immature state by culturing blood CD14$^+$ monocytes in IL-4 and GM-CSF for MDDCs, or in MCSF or human serum only for MDMs. Interestingly MDDCs were shown to be most transcriptionally similar to tissue CD14$^+$ MDMs (then referred to as CD14$^+$ DCs), which correlates with their mutual expression of DC-SIGN (65). By far the majority of studies described below have been conducted in these model MNPs.

DC TRANSFER OF HIV TO CD4 T CELLS

DCs have been shown to transfer HIV to T cells in two ways. First-phase transfer (also termed trans-infection) involves binding of a PRR on the MNP, holding the virus in a nondegradative compartment, then delivering it to a T cell once a virological synapse is formed. Second-phase transfer (also termed cis-infection) involves infection of the MNP via CD4 and CCR5 co-receptor CCR5 and *de novo* synthesis of the virus before transferring the virus to a T cell. Second-phase transfer can be blocked by pre-incubating the cells with inhibitors to entry or reverse-transcriptase (RT) (e.g., the CCR5-binding inhibitor Maraviroc, or the RT inhibitor AZT), whereas first phase cannot (36, 66).

First-phase transfer was initially attributed to the CLR DC-SIGN which has been shown to bind gp120 (67). After uptake some DC-associated HIV-1 evades the endocytic degradation pathway by trafficking to a tetraspanin (CD81$^-$) enriched protective environment from where infectious particles are specifically released to T cells upon DC: T cell contact (68). DC-SIGN, although present on MDDCs, has been shown in tissue to be expressed predominantly on macrophages (58). Other CLRs have now been also shown to bind HIV and implicated in first-phase transfer including: langerin (CD207) (36) expressed at high levels on Langerhans cells (LCs) and at lower levels on a subset of cDC2s (25), mannose receptor (MR or CD206), and DC immunoreceptor (DCIR or CLEC4A) (69).

Siglec-1 (or CD169) (70) reviewed in Akiyama et al. (71) and expressed on cells of myeloid lineage has been shown to take-up HIV into a storage compartment and enhance first-phase transfer by MDDCs, MDMs, and tissue-derived (tonsil) cDC2s (70). Siglec-1 has been shown to work in conjunction with tetherin to form these virus-containing compartments (72).

Tetherin (BST-2 or CD317) is an IFN-I inducible restriction factor that blocks the release of enveloped viral particles from infected cells. Tetherin has been shown to restrict the transfer of HIV-1 from myeloid cells to CD4$^+$ T cells (73–75). Tetherin is antagonized by SIV Nef in non-human primates (76–78). Humans, however, have a deletion in tetherin that prevents the SIV Nef antagonism. Interestingly, different HIV-1 strains have evolved to antagonize tetherin by utilizing different accessory proteins and counteracting tetherin to different degrees. The HIV-1 group M strains (responsible for the global AIDS epidemic) have gained the ability to utilize Vpu to antagonize tetherin (77, 79, 80). HIV-1 group O Nefs target a region of tetherin that leads to down modulation of tetherin but this had no effect on MDM:T cell transfer (81). Furthermore, HIV-1 group O strains have been identified that use both Nef and Vpu to target tetherin (82). Tetherin antagonism appears to play a major role in transmitted/founder strain resistance to type I IFN (83). A Vpu mutant virus that abrogated tetherin antagonism induced comparable gene expression to wild-type, whereas a Vpu *null* mutant induced very different gene expression in CD4$^+$ T cells (84).

Lectins can also be involved in second-phase transfer, which is dependent on productive infection of the MNP. Hijazi et al. found that DC-SIGN can increase the stability of the gp140: CD4

complex, enhancing infection (45) whereas langerin does not. DCIR (69) has been shown to enhance second-phase transfer and in MDMs Siglec-7 has been shown to enhance infection (85). Interestingly, MR expression, has been shown to be inhibited by HIV-1 in macrophages [as well as by HIV-2 and SIV reviewed in (86)]. Without downregulation of MR, HIV-1 virions were held, stuck on the surface of MDMs and downregulating MR allowed more virus to be released from the surface, aiding spread (87).

As productive infection is needed for second phase transfer of HIV, it is pertinent to examine what factors influence HIV productive infection of MNPs. It has long been identified that HIV-1 strains display tropism to different cell types, with some able to infect T cells but not myeloid cell types (macrophages having been the most studied). Strains that are able to infect myeloid cells or macrophages have been termed M-tropic. Earlier studies found macrophage-tropic viruses are able to utilize CCR5 and low levels of CD4 (88–90). This is possibly due to gp120 polymorphisms that increase macrophage tropism through enhanced interactions with CCR5 (91). Although this broadening of tropism my come at the cost of lower infectivity (92). Adding to the complexity, a recent study has highlighted that CCR5 exists in heterogeneous conformations and oligomerization states. Different subpopulations of CCR5 were preferred by divergent viruses (93) and importantly, macrophages and T cells expressed different subpopulations of CCR5 and it is likely another factor driving virus strains to be macrophage-tropic or T cell-tropic. It remains to be studied what subpopulations of CCR5 are expressed by all the various tissue myeloid cells that exist at the site of infection or if indeed this is the main determinant of M-tropism.

MANIPULATION OF MATURATION, MIGRATION, AND LYSOSOME FUNCTION BY HIV

After detecting pathogens via TLRs and CLR's DC undergo a process of maturation whereby their function changes from cells specialized in antigen capture to those specialized in antigen presentation. Thus, pathogen binding receptor expression is down-regulated leading to a reduced ability for anigen detection and endocytic uptake. Secondly, CCR7 is upregulated which allows for chemotactic migration to the lymph nodes along CCL19 and CCL21 gradients. Thirdly, various surface molecules are up-regulated that facilitate DCs–T cell interactions such as those involved in antigen presentation (MHC-II), T cell activation (CD40, CD80, CD83, CD86), and cell adhesion (CD54/ICAM-1). This allows for the formation of clusters of mature DCs and T cells within the lymph nodes. Such clusters require the formation of an immunological synapse (94, 95) which requires actin cytoskeleton remodeling of the and an interaction between LFA1 on the T cell and CD54 on the DC. Within the synapse costimulatory and antigen presentation molecules on the DC membrane, and their cognate receptors on the T cell membrane, become concentrated (94, 95), and this contact region facilitates T cell activation and antigen presentation. If the DCs are HIV infected with HIV then a virological synapse is formed with T cells instead, which allows for transfer of HIV from the DC to the T cells (96, 97). Though similar, the immunological and virological synapse (94, 95) differ in their complement of adhesion molecules and other proteins (98).

As DCs migrate from the site of pathogen exposure to the lymph nodes which are rich in the primary HIV target cells, CD4 T cells, this makes them an ideal delivery vehicle for HIV to exploit. Indeed, it is well-known that DCs enhance infection of T cells so efficiently that they become insensitive to the anti-viral drugs tenofovir and raltegravir (99). Mature DCs form DC:T cell conjugates more readily than immature DCs (96). However, there has been controversy in the literature as to whether HIV can trigger DC maturation, some arguing in favor (100–102) and some against (103–105). To address this controversy Harman et al. (102) carried out a detailed and through study and showed that HIV induces both MDDCs and *ex vivo* LCs (derived using collagenase) to undergo a process of partial maturation via two independent mechanism resulting in an increased migratory and T cell stimulatory capacity. As a key differential between the conflicting studies was the way in which HIV virus stocks were produced, they then went on to investigate this process more fully. By comparing raw virus stocks to highly purified ones a follow up study showed that one of the mechanisms of HIV induced maturation was driven by microvesicles in the viral inoculum and the other was dependent on productive infection of the DCs. They also showed that HIV drive increased CD54 (ICAM-1) expression which lead to increased DC: T cell interactions (106). Other studies have shown that cross-talk between infected MDDCs and T cells decreases the restriction factor SAMHD1 in the MDDCs, promoting efficient viral replication (107). This was dependent on viral synapses as blocking CD54 reduced the effect.

A key aspect of DC maturation is the destruction of the pathogen by acid proteolysis in lysosomes and subsequent loading of antigen fragments on MHC-II. This process is mediated by the cathepsin and cystatin families of proteins. Using microarrays Harman et al. were able to show in MDDCs that HIV specifically down-regulates the expression of specific cathepsin proteins that degrade pathogens and up-regulate specific cystatin proteins that act to negatively regulate this process. Furthermore, using enzyme function assays they showed that HIV was able to disrupt the enzymatic activity of cathepsins to inhibit lysosomal degradation and thus protect the virus from degradation by at least two independent mechanisms. These two effects of HIV on DC biology are part of a wider spectrum, shown by the fact that HIV-1 induces two waves of gene expression in MDDCs that correspond to the two phases of HIV transfer to T cells (108).

HIV-1 has also evolved mechanisms to avoid complement-mediated destruction by incorporating regulators of complement activation during the budding process and by binding the fluid-phase factor H (109, 110). Complement-opsonized HIV-1 triggered T592 phosphorylation of SAMHD1, allowing increased HIV production and initiating the strong maturation and co-stimulatory capacity of DCs (111).

MANIPULATION OF THE INTERFERON SYSTEM IN MONONUCLEAR PHAGOCYTES BY HIV

IFNs represent a family of critical effector cytokines that underpin both innate and adaptive antiviral immunity, and have been grouped into three distinct classes (112, 113). Type I IFNs (IFN-I) encompass IFN-α (which has thirteen human sub-types), IFN-β, IFN-ε, IFN-κ, and IFN-ω, which all signal through the IFNA receptor (114, 115). IFN-ε is constitutively expressed in the female reproductive tract (116) but it has also been shown to be produced in response to viral infection in both MDDCs and MDMs (117). Type II IFN consists of a single cytokine, IFN-γ, which is mainly secreted by T cells, NK cells and innate lymphoid cells. Type III IFNs consists of IFN-λ1-4 (118) and induce IFN-I-like antiviral activity as well as sharing features with the IL-10 family (113, 119, 120). Whilst IFN-I have a broad expression and activity range due to ubiquitous expression of the IFNA receptor, the expression of the IFN-λ receptor is generally limited to epithelial cell surfaces (121).

Type I and III IFNs can act through autocrine and paracrine mechanisms to induce the expression of numerous interferon-stimulated genes (ISGs) in both host and neighboring cells, collectively enacting an antiviral state. The induction of these ISGs can lead to degradation of viral RNA/DNA and the inhibition of viral gene expression, protein synthesis, virion assembly and release, with marked variation across cell types (122–124). In addition, IFN-I can enhance immune cell activation and effector functions, which includes promoting an antigen-presenting phenotype in DCs and macrophages (125, 126), stimulating $CD8^+$ T cell clonal expansion and NK cell activation (127–132) as well as B cell class switching and affinity maturation (133).

PATHWAYS OF IFN-I PRODUCTION IN MONONUCLEAR PHAGOCYTES

During viral infection, IFN-I production is usually triggered in MNPs through the recognition of viral nucleic acids by cytoplasmic sensors of RNA: retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), or DNA: mainly cyclic GMP-AMP synthase (cGAS), with some possible contribution from interferon-γ-inducible protein 16 (IFI16) and polyglutamine binding protein 1 (PQBP1) (134–136) (summarized in **Figure 1**). Whilst RIG-I and MDA5 signal through the adaptor mitochondrial antiviral-signaling protein (MAVS) upon recognition and binding of viral RNA, activation of cGAS, IFI16, or PQBP1 by viral DNA instead leads to signaling through the adaptor stimulator of IFN genes protein (STING). Both MAVS and STING activation result in the downstream clustering of TANK binding kinase-1 (TBK1), which forms a complex with TNF receptor-associated factor (TRAF3) and interferon-regulatory factor (IRF)-3 (137). TBK1 subsequently undergoes ubiquitination and trans-autophosphorylation, by which it becomes self-phosphorylated, and can then phosphorylate IRF-3 (138). Following phosphorylation, IRF-3 dimerises and translocates to the nucleus where it induces the expression of IFN-β by binding to IFN-stimulated response elements in the promoter of various genes, including *IFNB* and other ISGs. This then leads to autocrine and paracrine signaling through the IFNA receptor which triggers IRF7 transcription, mediating a positive feedback loop of IFN-I production (139). Along with IFN-β, multiple subtypes of IFN-α are produced during this second wave of gene transcription (140).

The importance of IFN-I on the course of HIV infection has been well-established but presents an apparent paradox on disease outcome, namely an apparent protective function during the earliest stages of infection, but contribution to pathogenesis during and after the transition to chronic infection. Studies performed in rhesus macaques and humanized mice (141–143) examining early timepoints after infection (up to 12 weeks post infection) have highlighted the importance of an early IFN-I response to SIV/HIV, with the inhibition of IFN-I signaling resulting in increased reservoir size and accelerated progression to end-stage disease. Indeed, exogenous addition of IFN-I prior to SIV infection in rhesus macaques delays SIV acquisition and necessitates an increased number of challenges for transmission to occur (142, 144). Furthermore, studies looking at transmitted HIV isolates have reported that founder viruses that establish infection are largely more resistance to the inhibitory effects of IFN-I and ISGs on viral replication compared to their non-transmitted counterparts isolated from the same individual (145, 146). This relative IFN resistance suggests that early IFN-I constitutes a major selective barrier against the initial virus the host is exposed to and helps to protect against infection during the acute phase. In complete contrast, protracted production of IFN-I into chronic HIV infection exacerbates disease progression (147, 148). This has been observed in non-pathogenic SIV infection of natural hosts such as African Green monkeys and Sooty Mangabeys where a strong IFN-I signature occurs early but then dissipates (148–150). On the other hand, pathogenic infection in rhesus macaques shows sustained IFN-I production, including through to chronic infection. A similar parallel can be observed with HIV-1 and the less pathogenic HIV-2, where HIV-2 induces a shorter burst of IFN-I production *in vitro* (151). Continued administration of IFN-I in rhesus macaques during SIV infection also leads to desensitized IFN signaling and increased $CD4^+$ T cell viral load and depletion, consistent with the immunopathology of chronic immune activation (142). These studies noting the disease association with excess long-term IFN-I are also well reflected in human individuals infected with HIV, where elevated plasma levels of IFN-α has been demonstrated to correlate with plasma viral load, hyperimmune activation and progression to AIDS (152–154).

EVASION AND INHIBITION OF THE IFN-I RESPONSE IN MONONUCLEAR PHAGOCYTES BY HIV

Aside from its genomic RNA, HIV also generates proviral cDNA and post-integration mRNA as part of its retroviral lifecycle,

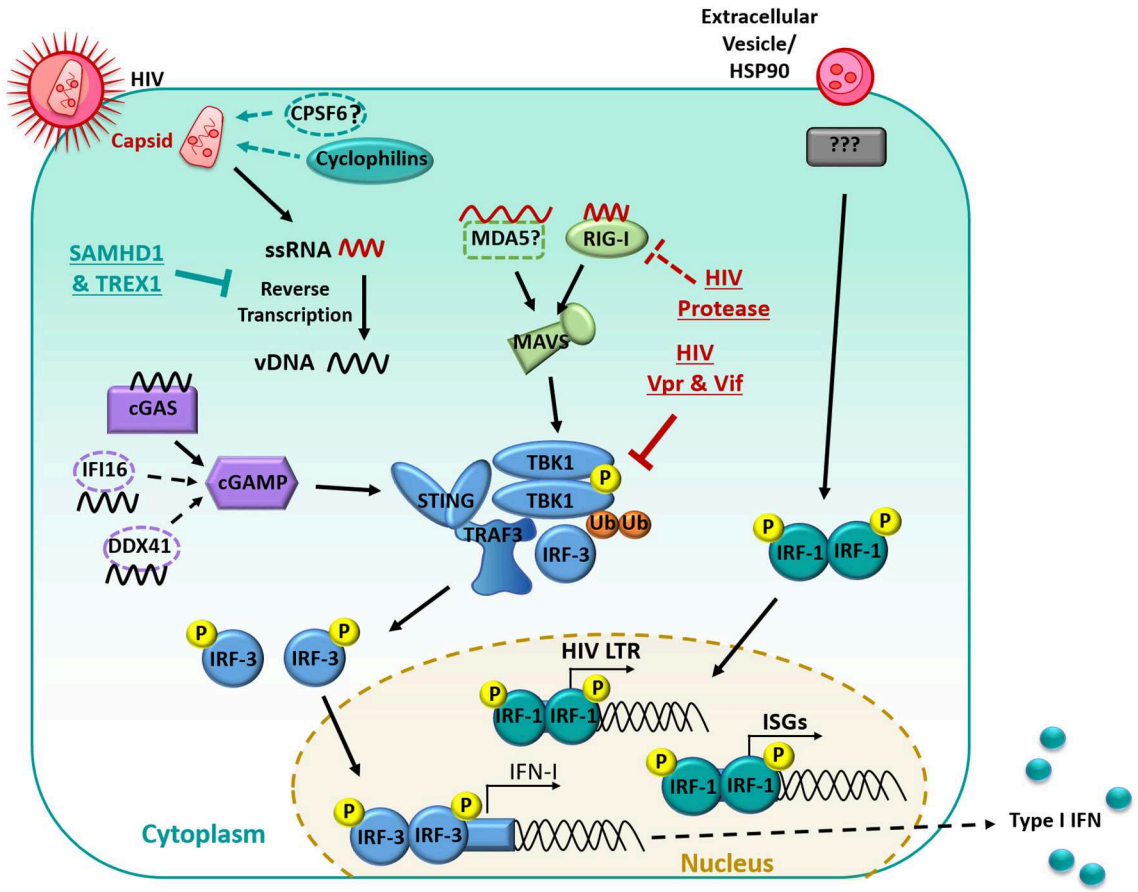

FIGURE 1 | Manipulation of the interferon signaling pathway in myeloid cells. HIV-1 infection is restricted by the host proteins SAMHD1 and TREX1, limiting Reverse transcription. RNA is detected primarily by RIG-I and signaled through MAVS. Reverse Transcribed viral DNA is primarily detected by cGAS and signals through cGAMP. PRR recognition signals are then transduced through STING which results in the downstream clustering of TBK1 which complexes with TRAF3 and IRF-3. TBK1 then undergoes ubiquitination and auto-phosphorylation and can then phosphorylate IRF-3. Phosphorylated IRF-3 then dimerises and translocates to the nucleus, where it induces the expression of IFN-I. HIV-1 limits the IFN response in many ways, including undergoing reverse transcription with in the capsid, the capsid also recruits host proteins (primarily cyclophilins) to shield HIV-1 from PRRs. The HIV-1 protease targets RIG-I for degradation. HIV-1 Vpr and Vif prevent the auto-phosphorylation of TBK1 and subsequent translocation of IRF3 to the nucleus. Extracellular vesicles in the HIV-1 inoculum can induce IRF-1 translocation to the nucleus. IRF-1 induces a subset of ISGs and an IRF-1 binding site is also located on the HIV-1 promoter which may enhance subsequent virus production.

which can all potentially stimulate host nucleic acid sensors to initiate IFN-I production. Given the importance of the early IFN response to viral control, HIV has unsurprisingly adopted various strategies aimed at evading immune recognition and inhibiting the induction of IFN-I in its target cells, including in myeloid DCs and macrophages. It is important to note that many studies exploring these inhibitory mechanisms have performed experiments in cell-lines that could not recapitulated across different cell-lines or in primary cells (155–159), or have relied upon the introduction of Vpx, the accessory protein encoded by HIV-2 and SIV that targets SAMHD1 (discussed in the next section) to infect DCs (155, 160). Given the complexity and functional redundancy within the IFN-I signaling pathway, whether these experiments accurately capture the interactions between HIV and MNPs is subject to debate.

Limited Early Detection of HIV

Firstly, there is a limited ability for host nucleic acid sensors to detect HIV RNA from the incoming virion or cDNA following reverse transcription. HIV undergoes reverse transcription within the capsid following its fusion-mediated entry into the cytoplasmic space, thus shielding the proviral cDNA from detection by host DNA sensors, and limiting host sensing of the viral RNA genome (161). In addition, the capsid only uncoats close to or directly at the nuclear pore, restricting the exposure of viral nucleic acids to the cytoplasmic sensors (161). Work performed in MDMs and the monocyte THP-1 cell line has also suggested that the capsid recruits host proteins (cyclophilins and CPSF6) that further shield HIV cDNA from cGAS sensing (155, 162), although CPSF6 has not been confirmed in follow-up studies. It has also been shown that only RIG-I but not MDA5 can detect HIV RNA leading to reduced downstream

signaling through MAVS (163), with a causal mechanism still to be elucidated.

Furthermore, various host restriction factors such as SAM domain and HD-containing protein 1 (SAMHD1) and three-prime repair exonuclease (TREX1) act to inhibit the genomic lifecycle of HIV, but also inadvertently limit the accumulation of proviral cDNA for sensing by cGAS and IFI16. SAMHD1 depletes the pool of intracellular dNTPs thereby minimizing the reverse transcription process, and also potentially degrades incoming HIV RNA via its ribonuclease activity (164, 165). Likewise, TREX1 degrades cytoplasmic and nuclear DNA (in macrophages) and so limits the copies of HIV cDNA for sensing by cGAS and downstream TBK1 signaling (166). Knockdown of TREX1 in a humanized mouse model enabled the restoration of IFN signaling and delayed the acquisition of HIV (167). The importance of these mechanisms that HIV employs to minimize its detection is highlighted in a recent study by Johnson et al. (160) which demonstrated that inhibition of SAMHD1 with Vpx during HIV infection in MDDCs was sufficient to induce expression of a small number of IRF-3-dependent genes, through an increased buildup of intracellular HIV cDNA which in turn allowed for low levels of cGAS stimulation and STING signaling. However, robust stimulation of the cGAS-STING pathway was only achieved at later timepoints or with additional signaling from another inflammatory pathway.

Inhibition of IFN S*ignaling* by HIV

HIV has also encoded multiple inhibitory mechanisms aimed at disrupting the key sensors and signaling proteins involved in the host IFN response. Multiple studies performed in our lab in MDDCs and MDMs have identified that HIV completely inhibits the induction of all type I and type III IFNs (IFN-α, -β, -ε, -κ, -ω, -λ) up to 96 h post infection, with a corresponding lack of nuclear translocation of IRF-3 (117, 124, 168). Harman et al. (168) demonstrated this could not be solely attributed to aberrant sensing by upstream pattern recognition receptors, given that TBK1 complexation with TRAF3 and IRF3 could still be observed. Instead, the HIV accessory proteins Vpr and Vif physically prevented the autophosphorylation of TBK1 thereby blocking its ability to phosphorylate and activate IRF-3 (168). Infection with Vpr- or Vif-deficient mutants restored the production of IFN-β in MDDCs and MDMs, suggesting that the targeting of TBK1 represents the major inhibitory mechanism of IFN induction by HIV. This phenomenon appears to be unique to myeloid cells, as IRF-3 is directly targeted for cellular degradation in $CD4^+$ T cells (169, 170), which does not appear to occur in MDDCs or MDMs (168, 171). As such, strategies that aim to restore IFN signaling by targeting Vpr/Vif or TBK1 may be promising avenues for the design of novel therapeutics.

In addition to the manipulation of TBK1, additional work using MDMs has demonstrated that the HIV protease appears to target RIG-I for lysosomal degradation (163), and that the adaptor protein IPS-1 functioning downstream of RIG-I is transcriptionally down-regulated by HIV (172), but both of these observations require further validation in other MNPs. Further work has also suggested the binding of HIV to DC-SIGN on the cell surface of MNPs can lead to the recruitment of mitotic kinase PLK1, which blocks downstream DEAD box RNA helicase (DDX3)-MAVS signaling (173), but recent reports confirming the importance of other lectin receptors in HIV binding, particularly Siglec1 (71, 72, 174, 175), have cast doubts on the *in vivo* relevance of this process.

In contrast to the global inhibition of type I IFNs in other MNPs (myeloid DCs and macrophages) by HIV, plasmacytoid DCs can secrete IFN-α/β in the *in vitro* presence of HIV and HIV-infected cells (9, 176, 177). pDCs express constitutively high levels of IRF-7 and sense HIV through TLR7 (9), which directly stimulates IRF-7 and type I IFN production and bypasses the requirement for TBK1/IRF-3 activation. It is worth noting that during the early stages of HIV infection in the anogenital mucosa, pDCs are recruited from circulation and have been detected in the cervical mucosa underlying the epithelium in SIV-infected rhesus macaques within 3–4 days (178, 179). Consequently, they likely represent one of the major sources of IFN during acute and chronic SIV and HIV infection (141, 178).

ISG Induction by HIV

Despite circumventing the production of IFN-I in MNPs, HIV infection still induces the expression of a distinct subset of ISGs in MDDCs and MDMs, in a biphasic manner (160, 171, 180). In the initial phase (between 0 and 24 h post infection), ∼25 ISGs are transiently induced by extracellular HIV vesicles in a cell-type specific manner, with the most up-regulated ISGs in MDMs being *Viperin* (also known as *RSAD2*), *IFIT1*, *IFIT2,* and *IFIT3* (124, 171), and *ISG15, IFIT1,* and *IFIT2* in MDDCs (160). The induction of these ISGs occurred independent of viral replication and could be mainly attributed to extracellular HIV vesicles present in incoming viral inoculum (171), which can also be found *in vivo* in the circulation of HIV-infected individuals (181, 182). This phase of ISG induction by vesicles appears to rely upon IRF-1 and was also independent of IFN-I, being unaffected by knockdown of adaptor proteins involved in IFN signaling (MyD88, TRIF, MAVS, and STING) downstream of toll-like receptor and cytosolic sensors (171). The preliminary round of ISG expression can be induced in MDMs by stimulation with "shed vesicles" from activated T cells (whether HIV infected or not), or the heat shock protein (HSP)90α, present in extracellular vesicles in HIV inoculum (106). The second-phase of ISG induction occurs around 72 h post infection and results from the sensing of newly transcribed HIV mRNA following Tat-driven initiation and elongation of proviral transcription in productively infected cells (160, 171). These mRNA transcripts are then detected by RIG-I, following by downstream MAVS signaling and IRF-1/IR7-mediated ISG induction, leading to much stronger and wider variety of ISGs being expressed. This model of ISG expression is consistent with previous studies that have observed a first phase of ISG production occurring pre-integration and a second, larger phase following integration (106, 108, 117, 136, 160, 168, 171, 180, 183).

Interestingly, the HIV promoter region contains similar IRF-1/IRF-7 binding sites to the ISGs induced in MDMs (168), which leads to speculation that up-regulation of IRF-1 and IRF-7 by HIV results in composite HIV transcription to produce *de novo* progeny but also ISG induction that limits explosive

viral replication. This may potentially allow HIV to achieve a low-level productive and non-cytopathic infection of MDMs that enables the virus to exploit these cells as a long-term viral reservoir. In addition, in their study of ISGs induced by HIV in MDDCs, Johnson et al. (160) also show that other inflammatory stimuli such as TLR stimulation combined with HIV infection can augment the IFN response. Whether these patterns of ISGs are altered, or indeed if IFN-I induction is restored during inflammation *in vivo* is important to resolve, given that genital inflammation is associated with enhanced HIV acquisition.

CONCLUDING REMARKS

The combined role of MNPs as both immune sentinels and APCs makes them the perfect vehicle for HIV to achieve transport from the site of infection in the anogenital epithelium to its primary target cells (CD4 T cells) in the submucosa or lymph nodes. In order to do this HIV must manipulate MNP biology in several important ways. Firstly, it shuts down the interferon response which blocks the ability of others cells in the surrounding area to enter an antiviral state. At the same time it induces the expression of specific ISGs contributing to a low persistent non-cytopathic infection. This allows the cells to deliver newly formed HIV virions to CD4 T cells after migration to the lymph nodes which occurs via at least two separate sequential mechanisms. HIV is also able to manipulate the DC maturation process such that it induces migration to the lymph nodes by upregulating CCR7 and enhances DC-T cell interactions (especially via ICAM-1) while at the same time avoiding destruction by disrupting the lysosomal degradation process.

In order to improve preventative strategies such as PrEP and develop an effective vaccine we need a better understanding of how the HIV penetrates and interacts with its target cells in both inflamed and uninflamed anogenital mucosa. As DCs are the key cells of the immune system that present antigens to T and cells to drive the respective adaptive immune responses they are key initial target cells for an HIV vaccine. A critical piece of information will be to determine which specific subsets of MNPs take up the virus and deliver it to which T or B cells. Once this has been determined specific adjuvants could be used for the targeted activation of the correct specific MNP subsets in mucosa (for mucosal vaccines) or draining lymph nodes (in systemic vaccines) to drive the required response.

Defining the interactions between HIV and its mucosal MNP target cells should also assist in developing PrEP regimes which work in inflamed mucosa. Thus, defining the inflammatory HIV target cells and their HIV binding receptor expression profiles, will aid in the development of blocking agents that can be used to block MNP infection in an inflamed mucosa.

AUTHOR CONTRIBUTIONS

KB and OT wrote the main body of the review with input from CR, ST, and NN. AH and AC guided design and construction of review.

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Lower Interferon Regulatory Factor-8 Expression in Peripheral Myeloid Cells Tracks With Adverse Central Nervous System Outcomes in Treated HIV Infection

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Cognitive dysfunction persists in 30–50% of chronically HIV-infected individuals despite combination antiretroviral therapy (ART). Although monocytes are implicated in poor cognitive performance, distinct biological mechanisms associated with cognitive dysfunction in HIV infection are unclear. We previously showed that a regulatory region of the *interferon regulatory factor-8* (*IRF8*) gene is hyper-methylated in HIV-infected individuals with cognitive impairment compared to those with normal cognition. Here, we investigated IRF-8 protein expression and assessed relationships with multiple parameters associated with brain health. Intracellular IRF-8 expression was measured in cryopreserved peripheral blood mononuclear cells from chronically HIV-infected individuals on ART using flow cytometry. Neuropsychological performance was assessed by generating domain-specific standardized (NPZ) scores, with a global score defined by aggregating individual domain scores. Regional brain volumes were obtained by magnetic resonance imaging and soluble inflammatory factors were assessed by immunosorbent assays. Non-parametric analyses were conducted and statistical significance was defined as $p < 0.05$. Twenty aviremic (HIV RNA<50 copies/ml) participants, 84% male, median age 51 [interquartile range (IQR) 46, 55], median CD4 count 548 [439, 700] were evaluated. IRF-8 expression was highest in plasmacytoid dendritic cells (pDCs). Assessing cognitive function, lower IRF-8 density in classical monocytes significantly correlated with worse NPZ_learning memory (LM; rho = 0.556) and NPZ_working memory (WM; rho = 0.612) scores, in intermediate monocytes with worse NPZ_LM (rho = 0.532) scores, and in non-classical monocytes, lower IRF-8 correlated with worse global NPZ (rho = 0.646), NPZ_LM (rho = 0.536), NPZ_WM (rho = 0.647), and NPZ_executive function (rho = 0.605) scores. In myeloid DCs (mDCs) lower IRF-8 correlated with worse NPZ_WM (rho = 0.48) scores and in pDCs with worse NPZ_WM (rho = 0.561) scores. Declines in IRF-8 in classical monocytes significantly correlated with smaller hippocampal volume (rho = 0.573) and in intermediate and non-classical monocytes with smaller cerebral white matter volume (rho = 0.509 and

rho = 0.473, respectively). IRF-8 density in DCs did not significantly correlate with brain volumes. Among biomarkers tested, higher soluble ICAM-1 levels significantly correlated with higher IRF-8 in all monocyte and DC subsets. These data may implicate IRF-8 as a novel transcription factor in the neuropathophysiology of brain abnormalities in treated HIV and serve as a potential therapeutic target to decrease the burden of cognitive dysfunction in this population.

Keywords: HIV-1, IRF-8, interferon, monocytes, dendritic cells, cognition, central nervous system, anti-retroviral therapy

INTRODUCTION

Cognitive performance is compromised in ∼30–50% of chronically HIV-infected individuals despite access to combination antiretroviral therapy (ART) (1, 2). These cognitive, behavioral, and motor deficits are not only widespread, but also impact everyday functioning, increase morbidity and mortality and have lasting critical public health effects (3–5). Since clinically approved therapies for HIV-associated cognitive impairment are not available (6), there is need to identify novel therapeutic targets.

The transmigration of both infected and uninfected monocytes into the central nervous system (CNS) is thought to be a significant mediator of the development of cognitive disorders during HIV infection, primarily by promoting viral seeding of CNS resident cells and promoting neuroinflammatory responses (7–14). Furthermore, myeloid cells, including monocytes and dendritic cells (DCs), play crucial roles in maintaining homeostasis along with inducing and controlling neuroinflammatory responses when recruited to the CNS (15–17). The implications of myeloid cells entering the CNS can differ depending on the pathological state and their peripheral phenotype, either inducing neuroinflammatory cytotoxic effects or promoting neural regeneration (18–20). Therefore, a better understanding of molecular mechanisms linking peripheral myeloid cells to the CNS is crucial to further elucidate the pathophysiology of HIV-associated brain injury.

Previously, we identified 1,032 differentially methylated loci in monocytes from persons with and without HIV-associated cognitive impairment. *IRF8*, the gene encoding for interferon regulatory factor-8 (IRF-8), had regions of significant hypermethylation in HIV-infected individuals with cognitive impairment compared to those with normal cognition, suggesting a potential role for this otherwise constitutively expressed transcription factor in HIV-related cognitive dysfunction (21). IRF-8 plays critical roles in the regulation of lineage commitment and differentiation during myeloid cell maturation and response to stimuli (22–24). For example, IRF-8 expression is elevated in the brains of a Alzheimer's Disease rodent model and in the context of accelerated aging and Alzheimer's Disease, IRF-8 was found to be one of the biomarkers with the highest correlation coefficient (25–27). Here, we wish to extend our *IRF8* epigenetic findings and evaluate IRF-8 protein expression in myeloid cells and the relationship to several measures of cognition, CNS injury, and inflammation in virally suppressed chronic HIV individuals on stable ART.

MATERIALS AND METHODS

Cohort Description

This study consisted of 20 chronically HIV infected individuals from the Hawaii Aging with HIV—Cardiovascular cohort study. The study, which has been previously described (28), was approved by the University of Hawaii Manoa Committee on Human Studies. Entry criteria to the study required subjects to have documentation of HIV infection, be ≥40 years, and to be on stable ART for≥6 months. Two study participants had a history of Hepatitis C infection. The selection of participants for our study was based on the availability of neuropsychological (NP) testing data and banked cryopreserved peripheral blood mononuclear cells (PBMCs) and plasma.

Neuropsychological Assessments

NP testing was conducted at the UH Clint Spencer Clinic by trained psychometrists. In order to minimize the risk of distraction and fatigue, NP testing was conducted in a quiet room and participants were provided breaks as needed throughout the testing session. The test battery was comprised of measures known to be sensitive to HIV infection including psychomotor speed, executive function, learning and memory, and working memory (29, 30). All raw NP scores were transformed to standard z-scores (NPZ score) using normative data (31, 32) and a global score was defined by aggregating the domain scores.

Regional Brain Volume Assessments by Magnetic Resonance Imaging

T1-weighted MRI data were processed with FreeSurfer (version 5.0, https://surfer.nmr.mgh.harvard.edu) (33–36) to obtain volumes of the caudate, putamen, hippocampus, amygdala, cortical gray matter, cerebral white matter, cerebellar gray matter, cerebellar white matter, and total subcortical gray matter, FreeSurfer's processing steam involves skull-stripping (37), intensity normalization (38), Talairach transformation, segmentation of subcortical white matter and deep gray matter (34, 35), and reconstruction of the cortical gray/white matter boundary and pial surface (33). Total regional volumes were computed by summing over the left and right hemispheres. An estimate of total intracranial volume (ICV) was obtained and used to normalize the regional volumes of interest (39). Each regional volume was expressed as a fraction of ICV (i.e., volume/ICV) to adjust for inter-individual head size variability.

IRF-8 Intracellular Staining

Cryopreserved PBMCs were placed in 96 well-polypropylene round bottom plates and stained with Live/Dead® Fixable Red Dead Cell Stain for 15 min at room temperature followed by a 30 min room temperature with conjugated monoclonal antibodies (mAbs) against CD3 [Brilliant Violet (BV)711], CD4 (PE-Texas Red), CD8 (PE-Cy5), CD7 (PE-Cy7), CD19 (PE-Cy7), CD20 (PE-Cy7), CD11c (AlexaFluor700), CD123 (FITC), CD11b (BV510), HLA-DR (APC-H7), CD14 (BV605), CD16 (BV421). Cells were then fixed and permeabilized with BD FACS Lysing Solution and Permeabilizing Solution 2 (BD Bioscience, San Jose, USA), respectively, then stained with an anti-IRF-8 antibody or isotype control (PerCP-eF710). Cells were fixed with 1% PFA and samples were acquired on a custom 4-laser BD LSRFortessa (BD Bioscience, San Jose, USA). Compensation and gating analyses were performed using FlowJo (FlowJo LCC, Ashland, USA). Reagents were purchased from BD Bioscience, San Jose, USA (mAbs CD123 Catalog Number [Cat] 558663, CD8 Cat 555368, CD19 Cat 557835, CD20 Cat 560735), Invitrogen, Carlsbad, USA (Live/Dead Stain, CD4 Cat MHCD0417), BioLegend, San Diego, USA (mAbs CD3 Cat 317328, CD7 Cat 343113, CD11b Cat 301333, HLA-DR Cat 307618, CD14 Cat 301834, CD16 Cat 302038) and eBioscience, San Diego, USA (mAbs CD11c Cat 56-0116-42, IRF-8 Cat 46-9852-80, isotype control Cat 46-4714-82). The implemented phenotyping gating strategy is shown in **Supplementary Figure 1**.

Quantification of Plasma Markers

The plasma soluble biomarkers, matrix metallopeptidase-9, myeloperoxidase, tissue plasminogen activator inhibitor-1, C-reactive protein, serum amyloid A, serum amyloid P, interleukin (IL)-1β, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α, soluble E-selectin, soluble vascular cell adhesion molecule-1, soluble intercellular cell adhesion molecule-1 (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor, interferon-gamma (IFN-γ), and N-terminal pro-brain natriuretic peptide were measured by Milliplex Human Cardiovascular Disease panels (EMD Millipore, Temecula, CA) as outlined in the manufacturer's protocols as previously described (40).

Statistical Analysis

Demographic and clinical characteristics were presented as a median and interquartile range (IQR) except for gender, which was presented as a percentage. Comparisons between continuous variables were carried out using Kruskal-Wallis tests, and for categorical variables, chi-squared tests. For IRF-8 outcomes, Kruskal Wallis tests were used to compare between groups. Associations between two continuous variables were evaluated by Spearman correlation. Statistical analyses were performed using R v3.2.2 or Prism GraphPad version 7 (GraphPad Software, San Diego, California).

RESULTS

Participant Characteristics and IRF-8 Expression in Blood

Clinical and demographic characteristics of the participants are shown in **Table 1**. The twenty participants included in this study had undetectable viral loads (<48 copies/ml), were 84% male, with a median age of 51 ([IQR 47, 55]), a median CD4 count of 548 [439, 700]), and had a range of cognitive performance [global score of −0.04 (−0.46, 0.55)]. IRF-8 density in each myeloid subset from HCV-infected and HCV-uninfected subjects are shown in **Supplementary Figure 2**.

Among the myeloid subsets analyzed, plasmacytoid DCs (pDCs) had the highest IRF-8 expression (geometric mean fluorescence intensity (GMF) 1501 [1273, 1651]), which was significantly higher than all 3 monocyte subsets and myeloid DCs (mDCs) (GMF 250 [222,277]) in concordance with a previous murine study (p's<0.001; **Figures 1A,B**) (41). Non-classical monocytes had the lowest IRF-8 expression (GMF 202 [192, 230]) compared to intermediate monocytes (GMF 244 [211, 273], not significant) and classical monocytes (GMF 270 [236, 289]; $p < 0.01$) (**Figures 1A,B**), while CD4+ and CD8+ T cells had undetectable IRF-8 levels (**Figure 1B**). Frequencies of each myeloid subset are show in **Supplementary Table 1**. IRF-8 expression in all myeloid subsets did not associate with age, CD4 count or nadir, CD8 count, CD4/CD8 ratio, or total self-reported years of infection or on ART (data not shown).

IRF-8 Density in Myeloid Cells Correlates With Cognitive Performance

Next, correlations of IRF-8 density in myeloid cells and neurocognitive functions were analyzed (**Figure 2** and **Supplementary Table 2**). Higher IRF-8 expression on classical

TABLE 1 | Characteristics of the study participants.

Clinical and Demographic Characteristics of Participants ($n = 20$)	
Gender (% male)	84% ($n = 17$)
Age (years)	51 [47, 55]
Education (years)	14 [14, 16]
CD4%	30 [25, 36]
CD4 Nadir* (cells/mm^3)	108 [34, 234]
CD4 Count (cells/mm^3)	548 [439, 700]
COB Count (cells/mm^3)	795 [611, 1140]
CD4/CD8 ratio	0.769 [0.491, 0.879]
HIV RNA (copies/ml)	48 [48, 48]
Years Infected*	15.7 [11.8, 17.3]
Years on ARV*	13.7 [10.8, 16.3]
NPZ Global (NPZ14)	−0.04 [−0.46, 0.55]

Median [IQR] presented for continuous variables and N (%) presented for categorical variables.
**Self-reported.*
Relevant demographic and clinical information of the study participants.

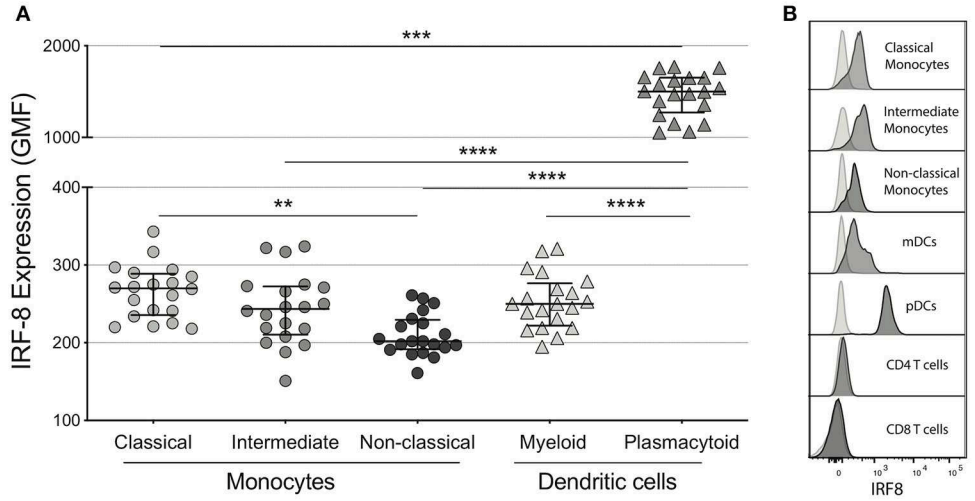

FIGURE 1 | IRF-8 expression on monocytes and dendritic cells. **(A)** Intracellular IRF-8 levels, expressed as geometric mean fluorescence intensity (GMF), on monocyte subsets (classical, intermediate, and non-classical) and dendritic cell (DC) subsets (myeloid and plasmacytoid). The hash mark indicates the median and error bars are the interquartile range. $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$. **(B)** Representative histograms of IRF-8 expression in each subset.

monocytes correlated with better NPZ_learning and memory (LM) (rho = 0.556, $p = 0.013$) and working memory (WM) (rho = 0.612, $p = 0.004$; **Figure 2A**) scores. Higher IRF-8 expression on intermediate monocytes also correlated with better NPZ_LM scores (rho = 0.532, $p = 0.019$; **Figure 2B**). Greater IRF-8 expression on non-classical monocytes also correlated with increased NP testing (global: rho = 0.646; $p = 0.004$; LM: rho = 0.536, $p = 0.018$, WM: rho = 0.647, $p = 0.002$ and executive function: rho = 0.605; $p = 0.005$; **Figure 2C**). Higher IRF-8 expression on both mDCs and pDCs also correlated with better NPZ_WM scores (rho = 0.484, $p = 0.030$ and rho = 0.561, $p = 0.010$, respectively; **Figures 2D,E**). Age did not show significant correlations with any NPZ testing data (data not shown).

IRF-8 Density in Monocytes Correlates With Brain Volumes

We analyzed correlations of IRF-8 density with regional brain volumes (**Figure 3** and **Supplementary Table 3**). Higher IRF-8 expression in classical monocytes correlated with larger ICV-adjusted hippocampal volume (rho = 0.573; $p = 0.008$; **Figure 3A**). Higher IRF-8 expression in intermediate and non-classical monocytes correlated with larger cerebral white matter volume corrected for ICV (rho = 0.509; $p = 0.022$ and rho = 0.473; $p = 0.035$, respectively; **Figures 3B,C**). DC IRF-8 expression did not correlate with regional brain volumes (**Supplementary Table 3**). Age did not correlate with volumes of hippocampus or cerebral white matter (data not shown).

IRF-8 Density in Myeloid Cells Correlates With Plasma Inflammatory Mediators

Finally, correlations of IRF-8 density and plasma cytokine levels were analyzed (**Figure 4** and **Supplementary Table 4**). Higher IRF-8 density in classical, intermediate and non-classical monocytes all correlated with higher sICAM-1 levels (rho = 0.756; $p = 0.0001$; rho = 0.600, $p = 0.005$ and rho = 0.534, $p = 0.015$, respectively; **Figure 4A**). Higher IRF-8 density in mDCs and pDCs also correlated with higher sICAM-1 (rho = 0.490, $p = 0.030$; rho = 0.490, $p = 0.028$, respectively; **Figures 4B,C**). Higher IRF-8 density in intermediate monocytes also correlated with higher IFN-γ levels (rho = 0.453; $p = 0.045$; **Figure 4D**). No correlations between IRF-8 and the other measured inflammatory mediators were observed (**Supplementary Table 4**). However, separately, higher sICAM-1 levels correlated with higher learning memory NPZ scores (rho = 0.484, $p = 0.036$) (**Supplementary Table 5**). Higher IL-8 and MMP-9 levels correlated with lower NPZ_LM scores (rho = −0.523, $p = 0.022$) and NPZ_WM (rho = −0.502, $p = 0.024$) scores, respectively (**Supplementary Table 5**). Actual values of NPZ scores and soluble inflammatory markers are shown in **Supplementary Tables 6**, **7**, respectively.

DISCUSSION

Given our intriguing methylation data demonstrating a link between the *IRF8* gene and HIV-associated cognitive impairment, the protein expression of intracellular IRF-8 was investigated in peripheral myeloid cells in virally suppressed HIV-infected individuals with multi-dimensional measures of cognitive performance and brain volumes. Although constitutive IRF-8 expression has been previously reported in myeloid cells, to our knowledge, our data are the first to link reduced IRF-8 protein expression in monocytes and DCs to worse NP testing performance and smaller brain volumes in virally suppressed HIV-infected individuals, suggesting this transcription factor is relevant in the neuropathology of brain health in treated HIV

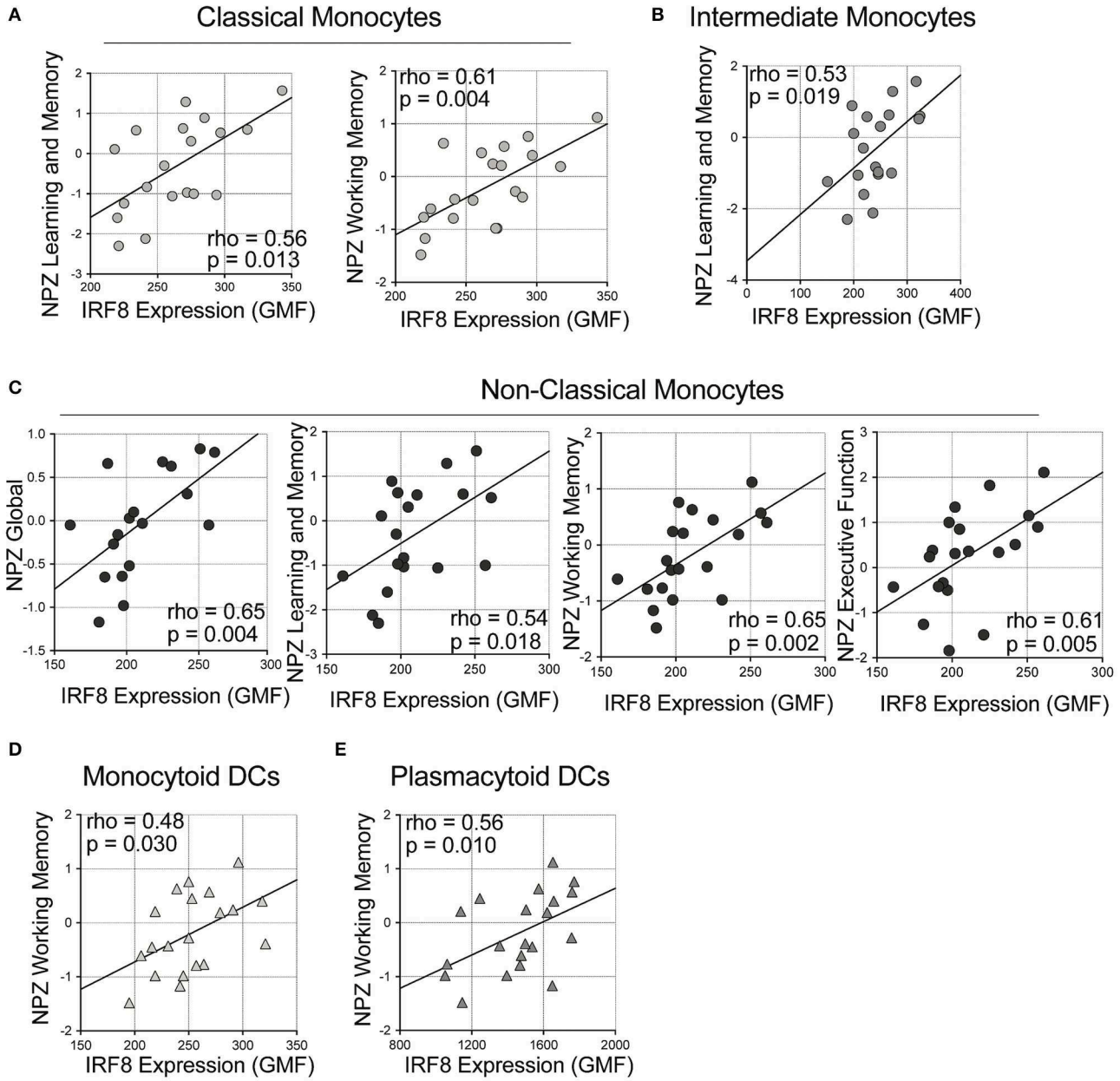

FIGURE 2 | Correlations between IRF-8 expression on myeloid cells and neuropsychological testing. Correlations between IRF-8 expression of **(A)** classical, **(B)** intermediate, and **(C)** non-classical monocytes, and **(D)** myeloid and **(E)** plasmacytoid dendritic cells and NPZ scores.

possibly by secretion of cytokines beneficial for CNS function and control of proviral quiescence of HIV.

Both in humans and mice, *IRF8* gene mutations are associated with depletions in circulating monocytes and dendritic cells (42) with IRF-8 driving myeloid differentiation. The maintenance of the monocytic homeostatic pool is not affected by deletion of *IRF-8* in mice (23, 43, 44). In fact, in our cohort, total and subset myeloid, both monocyte and DC, frequencies were not affected by IRF-8 expression (data not shown).

The functional consequences of higher IRF-8 expression as well as the relationship to CNS measures merits further study. Indicative of systemic inflammation, the presence of inflammatory cytokines can drive IRF-8 expression, which may further influence functional differences in monocyte and DC subsets (45–47). Indeed, IRF-8 is essential for controlling infection in both mice and humans (48, 49). Mutations in *IRF8* are associated with increased susceptibility to *M. tuberculosis* infection (47). Moreover, IRF-8 is crucial for the optimal activation of NLRC4 inflammasome and deletion of the gene induce susceptibility to *Salmonella Typhimurium* and *Burkholderia thailandensis* in mice (49). Finally, in the context of viral control, IRF-8 in the cytosol is crucial for NF-kB activation

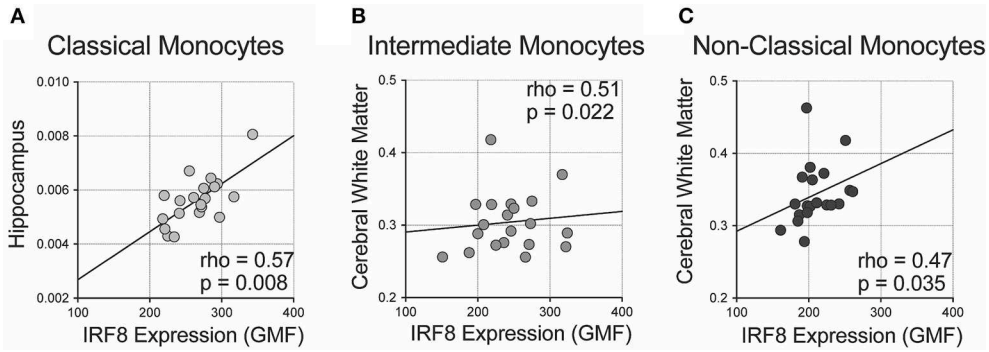

FIGURE 3 | Correlations between IRF-8 expression on myeloid cells and regional brain volumes. Correlations between IRF-8 expression of **(A)** classical, **(B)** intermediate, and **(C)** non-classical monocytes and regional brain volumes corrected for intracranial volume.

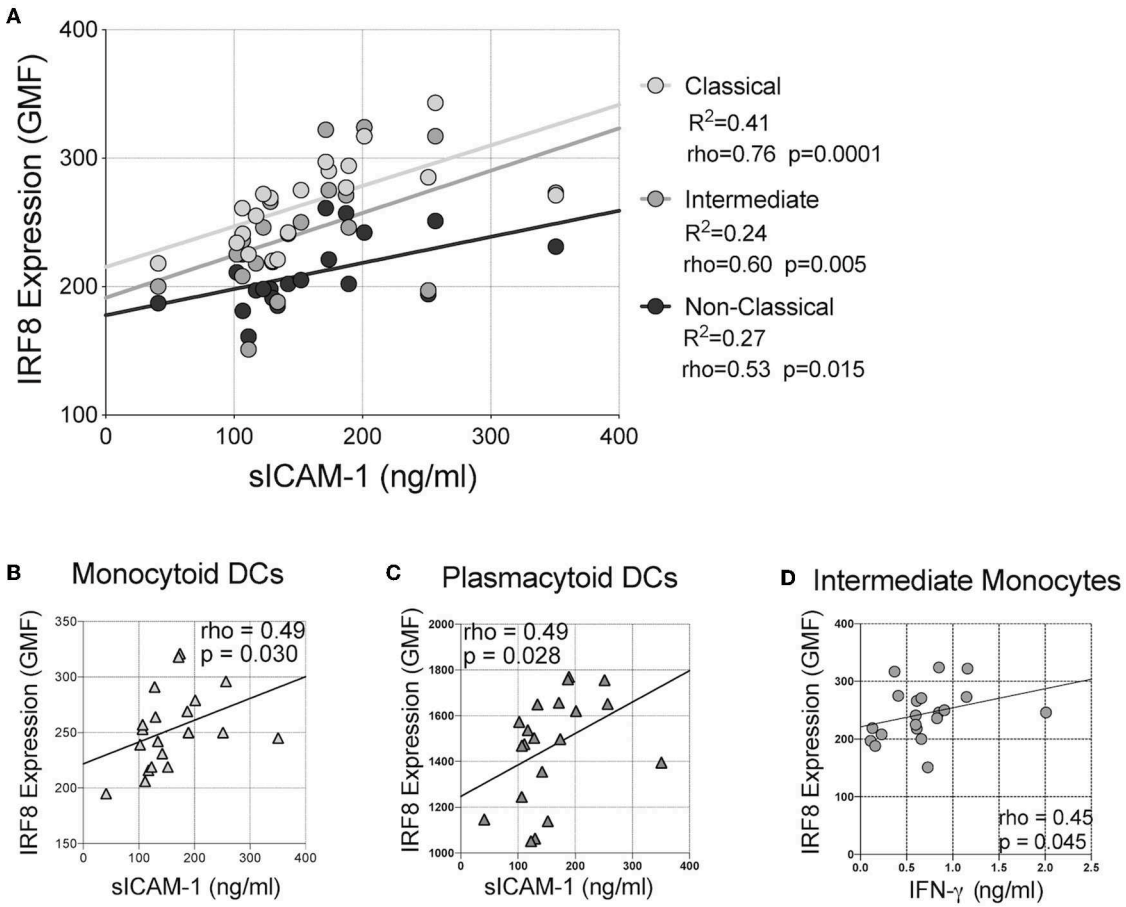

FIGURE 4 | Correlations between IRF-8 expression on myeloid cells and soluble inflammatory markers. Correlations between IRF-8 expression of **(A)** classical, intermediate, and non-classical monocytes, **(B)** myeloid and **(C)** plasmacytoid DCs and soluble intercellular cell adhesion molecule-1 (sICAM-1). **(D)** Correlation between IRF-8 expression in intermediate monocytes and plasma interferon-gamma (IFN-γ) level.

via the TLR9-MyD88 dependent pathway (50). IRF-8 also acts as a transcription factor for the induction of *Il12b* gene upon TLR stimulation (51, 52). In addition, monocyte cytokine responses to pathogenic stimuli, such as interferon-beta (IFN-β) secretion, can also be synergistically regulated by IRF-8 and IRF-3 binding of the ETS/IRF composite element of the IFN-β promoter (53).

Although inflammatory responses are historically viewed as undesirable in the context of pathological conditions,

recent studies, on the other hand, have shown the importance of inflammatory responses that bridge subsequent anti-inflammatory responses, which suggests complicated nature of inflammation (54, 55). In HIV-infected individuals, a previous report highlighted positive correlations of higher IFN-γ, MCP-1, and TNF-α level to higher volumes of the putamen, pallidum and amygdala, whereas higher IL-1β, IL-6, and IL-18 plasma levels were related to lower volumes of the putamen, pallidum, thalamus, hippocampus, and amygdala (56). In line with this report, our data showed the positive correlation between IRF-8, which expression is driven by cytokines including IFN-γ, and brain volume. In a cutaneous wound healing murine model, the down-regulation of IRF-8 by *in vivo* miRNA-induced silencing complex administration decreased transcription of inflammatory mediators associated with M1 macrophages (IL-1β, IL-6, iNOS, TNF-α) and impaired wound healing at the site of injury (57). Yuan et al. has previously reported that plasma soluble ICAM-5 level was increased in HIV-infected subjects with cognitive impairment compared to HIV-infected subjects with normal cognition (58). Since sICAM-5 suppresses immune activation status (58) whereas sICAM-1 activates the immune system, this supports our results (59). Intriguingly, we found a correlation such that higher plasma sICAM-1 levels correlate with better learning memory NPZ scores (**Supplementary Table 5**). Furthermore, our findings point toward an interesting correlation found among MMP-9 and learning memory NPZ score such that higher MMP-9 correlated with lower learning memory NPZ scores (**Supplementary Table 5**). Similarly, higher IL-8 negatively correlated with working memory scores (**Supplementary Table 5**). MMP-9 has previously been described as inhibiting IL-23 mediated pro-inflammatory responses in dendritic cells while IL-8 is essential for mounting a sufficient T helper cell response (60–62). It has been described that IRF-8 is involved in CCL4 expression which in turn promotes MMP-9 expression in macrophages (63). Additional studies into networked IRF-8, MMP-9, IL-23, and IL-8 activities to decipher the nature of inflammation in people living with HIV are warranted.

Additionally, IRF-8 activity may regulate HIV provirus production. *In vitro* HIV infection of Jurkat cells has been shown to significantly increase IRF-1 gene expression (42). However, when HIV-infected Jurkat cells were stably transfected with IRF-8, p24 production was decreased compared to the control vector, due to the ability of IRF-8 to inhibit the binding of IRF-1 to Tat, suggesting that IRF-8 is a dominant negative regulator of IRF-1 activity and can block HIV-1 transcription (42). When the chronically HIV-infected pro-monocytic cell line, U1, was treated with a histone deacetylase inhibitor, a concomitant decrease in IRF-8 gene and protein expression was observed along with an increase in gag gene expression (64). This decreased expression of IRF-8 following reactivation of latency suggests a role of IRF-8 in maintaining of proviral quiescence of HIV. Higher IRF-8 levels in the myeloid cells may permit better control of HIV levels within the monocytes which may be a principle mechanism that may maintain viral quiescence and limit neuroinflammation leading to CNS injury manifesting as cognitive impairment (65).

Our observed associations between IRF-8 density and both cognitive performance and regional brain volumes are supported by functional relationships between the relevant regions and NP domains. Consistent with its relationship to better working memory and learning and memory, higher IRF-8 expression in classical monocytes correlated with larger volumes of hippocampus. The involvement of the hippocampus in working memory is well-known (66): in Alzheimer's disease, decline in this domain is associated with hippocampal atrophy (67), as is learning and memory impairment (68). Hippocampal volume has also been positively correlated with memory performance in healthy young individuals (69). The active role played by white matter in learning and memory (70) supports the correlations of IRF-8 expression in intermediate and non-classical monocytes with cerebral white matter volume and with learning and memory. Similarly, correlations of non-classical monocyte expression of IRF-8 with cerebral white matter volume, working memory, executive function, and global NP performance are in line with the literature relating white matter reductions to cognitive decline (71).

Correlations linking the periphery and brain might reflect monocytes migrating into the tissues and carrying out a protective role with regards to HIV infection. IRF-8 expression in monocytes and DCs tracking with NP performance suggests a role for IRF-8 in HIV-related cognitive dysfunction and may highlight a potential therapeutic target. In Vogt-Koyanagi-Harada disease (VKH), monocyte-derived dendritic cells (MDDCs) from active VKH patients have decreased IRF-8 mRNA expression in association with higher methylation levels compared with normal controls or individuals with inactive VKH. Treating DCs the demethylation reagent, 5-Aza-2'-deoxycytidine (DAC), increased IRF-8 mRNA expression by reducing the methylation level of the IRF-8 gene (72). Hypermethylation of IRF-8 in myeloid cells in the context of VKH is similar to our previously published methylation data in HIV (21). Pharmacological interventions targeting demethylation of IRF8 may offer a novel strategy in the context HIV-associated cognitive dysfunction. This may be feasible with the use of microRNA interference as evidenced by murine studies that have enriched pDC frequencies with administration of miR-103 (73). An evaluation of IRF-8 regulatory activity and characterization of affected genomic elements will be beneficial to understand precise mechanisms of IRF-8 function in myeloid cells. Lin et al. demonstrated identification of DC lineage-specific transcription factor regulatory networks for IRF-8 with studies examining consecutive changes of stage-specific expression for key DC regulators are associated with specific histone modifications in promoter and enhancer sequences (74).

Here we report the lowest levels of IRF-8 in peripheral myeloid cells from virally suppressed individuals with chronic HIV correlates with worse cognitive performance. The conclusions of this study are limited by the small sample size of only 20 participants. In addition, the design of this study did not include an HIV-uninfected control group, which limits our ability to conclusively define a causal link between IRF-8 expression and HIV infection. Future longitudinal studies are also necessary

to examine the causal relationships between the high IRF-8 expression and HIV-related cognition dysfunction.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The study, which has been previously described (25), was approved by the University of Hawaii Manoa Committee on Human Studies. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MD'A, KK, MC, DO-A, DC, CS, and LN conceived and designed the study. MD'A, KK, and TP performed the experiments. MD'A, KK, TP, TF, EL, and VK analyzed the data. MD'A, TF, and EL wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02789/full#supplementary-material

Supplementary Figure 1 | Representative gating strategy for T cells, monocytes, dendritic cells using flow cytometry. After excluding doublets and dead cells, $CD4^+$ and $CD8^+$ T cells were identified from the $CD3^+$ cell population. From the $CD3^-$ population, after the exclusion natural killer (NK) and B cells (CD7, CD19, or CD20 positive cells), monocytes, positive for HLA-DR and CD11b, were subset by CD14 and CD16 expression: Classical monocytes ($CD14^{++}CD16^-$), intermediate monocytes ($CD14^{++}CD16^+$) and non-classical monocytes ($CD14^+CD16^+$). Dendritic cells (DCs; $CD3^-$ $CD7^-$ $CD19^-$ $CD20^-$ $CD14^-$ $HLA\text{-}DR^+$) were subset into myeloid ($CD11c^+$) or plasmacytoid ($CD123^+$) DCs. IRF-8 expression was then assessed on T cells subsets ($CD4^+$ and $CD8^+$), DC subsets and monocyte subsets.

Supplementary Figure 2 | IRF-8 density in each myeloid subset from HCV+ and HCV- subjects. Intracellular IRF-8 expression in **(A)** monocyte each subset and **(B)** DC each subset from HCV+ and HCV- subjects.

Supplementary Table 1 | Frequencies of each myeloid subset. After performing gating strategy as detailed in **Supplementary Figure 1**, frequencies of myeloid subsets with respect to total PBMCs are given here.

Supplementary Table 2 | Complete analyses of correlations of IRF-8 density and neuropsychological testing correlation. Correlations using Kruskal Wallis testing was used to evaluate associations between IRF-8 expression density and measures of neuropsychological tests, including: learning memory, working memory, executive function, psychomotor, and a composite neuropsychological score of the four domains referred to as global. The values reflected in **Figure 2** are reflected here in table format as this table.

Supplementary Table 3 | Complete analyses of correlations of IRF-8 density and regional brain volumes. Correlations using Kruskal Wallis testing was used to evaluate associations between IRF-8 expression density and regional brain volumes listed in the table. Values reflected in **Figure 3** are reflected here in table format as this table.

Supplementary Table 4 | Complete analyses of correlations of IRF-8 density and soluble inflammatory markers. Correlations using Kruskal Wallis testing was used to evaluate associations between IRF-8 expression density and soluble inflammatory markers, including: soluble E-Selectin (sE-Selectin), soluble vascular cell adhesion protein 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), metallomatrixprotein-9 (MMP-9), myeloperoxidase (MPO), plasminogen activator inhibitor-1 (tPAI-1), C-reactive protein (CRP), serum amyloid A (SAA), serum amyloid P component (SAP), interleukins (IL-1β, IL-6, IL-8, IL-10), Tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), interferon gamma (IFN-γ). Values reflected in **Figure 4** are reflected here in table format as this table.

Supplementary Table 5 | Complete analyses of correlations of neuropsychological testing and soluble inflammatory markers. Correlations using Kruskal Wallis testing was used to evaluate associations between neuropsychological testing scores and regional soluble inflammatory markers listed in the table.

Supplementary Table 6 | Values of NPZ scores in each subject. Actual values of NPZ scores in each subject are shown in the table.

Supplementary Table 7 | Values of soluble inflammatory cytokine levels in each subject. Actual values of soluble inflammatory cytokine levels measured in each subject are shown in the table.

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Myeloid Cells Enriched for a Dendritic Cell Population From People Living With HIV Have Altered Gene Expression Not Restored by Antiretroviral Therapy

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Antiretroviral therapy (ART) for human immunodeficiency virus (HIV) infections has been designed to optimize CD4 T-cell survival and limit HIV replication. Cell types other than CD4 T cells such as monocytes/macrophage, dendritic cells, and granulocytes (collectively known as myeloid cells), are generally not considered in the development of ART protocols. Myeloid dendritic cells (mDCs) are the most potent inducers of CD4 T-cell activation and central to the regulation of immune responses. mDCs in the blood are decreased in number, altered in function, and implicated in promoting HIV latency in people living with HIV (PLWH). We found that cells enriched for mDC in PLWH had transcriptional changes compared to mDC from HIV uninfected individuals, some of which were not completely restored by ART. In contrast, other mDC functions such as interleukin-1 signaling and type I interferon pathways were restored by ART. Some of the transcriptional changes in mDC not completely reversed by ART were enriched in genes that are classically associated with cells of the monocyte/macrophage lineage, but new single-cell RNA sequencing studies show that they are also expressed by a subset of mDC. A cellular enzyme, acyloxyacyl hydrolase (AOAH), important for lipopolysaccharide (LPS) detoxification, had increased transcription in mDC of PLWH, not restored by ART. It is possible that one reason ART is not completely successful in PLWH is the failure to phenotypically change the mDCs. Thus, inability of ART to be completely effective might involve myeloid cells and the failure to restore mDC function as measured by gene transcription. We suggest that mDC and myeloid cells should be considered in future combination ART development.

Keywords: human immunodeficiency virus (HIV), antiretroviral therapy (ART), pathogenesis, dendritic cells, myeloid cells, monocytes

INTRODUCTION

Dendritic cells (DCs) and monocytes are innate immune cells of the myeloid lineage that play critical roles in protection against microbial infections (1, 2). DCs internalize microbes in endocytic compartments where microbes are degraded into components. The resulting antigens are presented to T and B cells to stimulate immune responses against that microbe. In contrast, monocytes, which

differentiate into macrophage within tissues, are specialized to degrade and scavenge microbes from the host organism (1). Monocytes/macrophage can also present antigens; however, it is important to note that DCs are >100-fold more potent in activating T cells through their antigen-presenting functions than are monocytes/macrophage (1).

DC and monocytes are generated in the bone marrow during hematopoiesis. These two cell types share a common hematopoietic progenitor, the monocyte and DC precursor (MDP) (3–5). Two branches of differentiation are thought to arise from the MDP: one branch to monocytes and another branch to a common DC progenitor (CDP) (6, 7). As we are focusing on DC, it is important to know that the CDP generates at least three DC subpopulations. The three major DC populations are the myeloid DC type 1 (mDC1), 2 (mDC2), and plasmacytoid DC (pDC) (5, 8). mDC2 is the most frequent and is known as conventional DC (6, 7, 9). Recent single-cell RNA sequencing (scRNAseq) of mDC from healthy humans revealed that the mDC2 population is comprised of two transcriptionally distinct subsets, one newly defined, which expressed certain genes traditionally associated with monocyte/macrophage (10). Herein, we concentrate our studies on mDC2, also known as conventional DC type 2 (cDC2) or CD1c$^+$ mDC, the largest DC subset. This subset will be called mDC in our studies. We will focus on this subset in the peripheral blood before cells migrate to different tissue types and are designated by some as "precursor-DC" (pre-DC) [reviewed in Collin and Bigley (9)].

A major effect of HIV-1 (herein called HIV) infections is the loss of CD4 T cells, the primary target of direct HIV infection. While HIV does not significantly infect mDC *in vivo* (11), mDCs are altered in function (12) and decreased in number (13–17) in the blood in untreated people living with HIV (PLWH) and simian immunodeficiency virus (SIV)-infected macaques (18, 19). Increased HIV RNA viral loads and disease progression are associated with loss of blood mDC (13–16, 18). There is some indication that mDC may be an important co-factor in the efficient infection of CD4 T cells as *in vitro* studies show they bind virus on their cell surface and are able to transfer virus to CD4 T cells in a mode called "trans" infection (20, 21) [reviewed in Manches et al. (22)].

Antiretroviral therapy (ART) has been developed to limit HIV replication and prevent the loss of CD4 T cells. Unfortunately, ART is not always efficacious as some PLWH fail to reconstitute their CD4 T-cell numbers and become susceptible to opportunistic infections. One component of ART failure may be a result of the incomplete restoration of blood mDC count and function. One can speculate that myeloid cells, and specifically mDC, play a role in HIV persistence. First, plasma levels of two soluble myeloid cell surface molecules, CD14 and CD163, correlate with adverse events, co-morbidities, and disease progression in both ART-treated and treatment-naïve (TN) people living with HIV (PLWH) (23–31) and SIV-infected macaques (32). CD14 and CD163 are shed by myeloid cells (particularly monocytes) after binding to bacterial ligands. It is thought that this myeloid cell surface molecule shedding occurs, in part, because of the elevated levels of the gram-negative bacterial endotoxin, lipopolysaccharide (LPS), in the blood of PLWH. Increased LPS and other bacterial components in the blood of PLWH (33–35) are hypothesized to be a result of increased gastrointestinal (G.I.) tract permeability in PLWH (36) [reviewed in Brenchley and Douek (37, 38)]. Second, generalized T-cell immune activation occurs with chronic HIV infections (39–42) and correlates with HIV disease progression. This immune activation is associated with ART failure, yet its causes remain unexplained. It is possible that mDC, in close contact with T cells, play a role in such immune activation. Thus, due to their close association with T cells, and their changes in PLWH, mDC may be important in sustaining generalized T-cell immune activation that occurs in PLWH. Third, *Mycobacterium tuberculosis* (MTB) is a major opportunistic infection (O.I.) in PLWH. While the incidence of MTB is significantly reduced after ART, by ca. sixty-five percent (43, 44), it is not completely eliminated and still occurs at higher frequencies worldwide in PLWH than in the population at large (43, 44). Studies in mice suggest that mDC are important for immune responses to and clearance of MTB [reviewed in Durai and Murphy (45)], and therefore, their decreased numbers may be a factor in MTB susceptibility PLWH. Thus, mDC loss or alterations may be related to the increased susceptibility to secondary infections in PLWH.

Our work, described herein, was designed to determine whether mDC gene expression is altered in untreated or ART-treated PLWH. While the loss of CD4 T cells has been the dominant explanation for the immune dysfunction that occurs during HIV infections, including susceptibility to O.I.s such as MTB, defects in mDC may also play a role. Some limitations and/or failure of ART might be attributable to changes in mDC and/or myeloid cells.

MATERIALS AND METHODS

Study Participants

All participants included in this study provided written informed consent. This study was approved by either the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases or the Vaccine and Gene Therapy Institute Florida (VGTIFL), as appropriate. In order to obtain a sufficient number of mDC, apheresis was obtained from each donor. Participants were pre-screened for their ability to undergo apheresis. Each participant was required to be in good health and have within, or close to, normal levels of circulating CD4 T cells. Apheresis was performed for all subjects.

Myeloid Dendritic Cell and Monocyte Purification

mDCs were directly isolated from donors as follows: We identified mDC using the following markers: CD1c$^+$, HLA-DR$^{\text{intermediate to high}}$, CD11c$^{\text{high}}$, CD14$^-$, CD19$^-$, CD3$^-$, CD123$^-$, and BDCA 4$^-$ (summarized in **Supplementary Figure 1**). The leukapheresis product was elutriated (Elutra, Gambro) to separate myeloid cells from lymphocytes, according to the manufacturer's protocol (46, 47). The elutriated myeloid cells were then used for the separation of mDC and CD14$^+$ monocytes by a magnetic

bead-enrichment protocol (DC Enrichment Kit, Miltenyi) and platform (Automacs, Miltenyi) for the isolation of DC. Briefly, the elutriated myeloid cells were incubated with Fc receptor block and BDCA-4 antibody conjugated to magnetic beads for 15 min at 4°C. Cells were washed and then centrifuged at 1,650 rpm for 3 min. The cells were resuspended in wash buffer and depleted of BDCA-4+ plasmacytoid DCs by magnetic bead separation. The remaining cells were washed and incubated with a magnetic bead-conjugated CD19 antibody and a biotin-conjugated CD1c antibody for 15 min at 4°C. Cells were then washed and centrifuged for 3 min at 1,650 rpm and resuspended in wash buffer (PBS with 2% BSA and 0.09% azide). The cells were depleted of $CD19^+$ B cells. The remaining cells were centrifuged and incubated with an anti-biotin antibody to capture $CD1c^+$ cells. In some cases, when elutriation did not result in cell fractions that were highly enriched for monocytic cells, as determined by an automated cell counter (Countess, Invitrogen), cells were further separated by Ficoll and then enriched by antibody-conjugated bead separation followed by FACS sorting with the same procedures as described for the elutriated-only product. This was performed for two samples. For all samples, monocytes were isolated from the cell fraction remaining after magnetic bead-conjugated antibody depletion of $CD1c^+$ cells, B cells, and plasmacytoid DC, as per the manufacturer's protocol (Miltenyi).

It is known that using bead enrichment alone yields DC preparations that are contaminated with other cell types (48), and for this reason, we used a subsequent FACS purification step. The magnetic bead enriched mDCs or monocyte populations were further purified by fluorescence-activated cell sorting (FACS; Becton Dickinson, FACSARIA). Viable mDC, as measured by the viability dye, aqua (Invitrogen), were sorted on the basis of size (side scatter and forward scatter) and the following markers (obtained from BD Biosciences): $CD11c^{high}$, HLA-$DR^{intermediate\ to\ high}$, $CD14^-$, $CD123^-$, $CD3^-$, CD56–, and $CD19^-$. Monocytes were sorted on the basis of size (side scatter and forward scatter), viability (aqua vs. side scatter), and the following markers: HLA-$DR^{\ intermediate\ to\ high}$, $CD14^{hi}$, $CD3^-$, $CD56^-$, $CD123^-$, and $CD19^-$ (**Supplementary Figure 1**). We showed that monocytes were confirmed to be ≥97% pure by post-sort analysis and estimate the mDC had the same level of purity, although there were not sufficient mDCs to analyze by post-sort analysis. Cells were sorted directly into RLT buffer (guanidinium thiocynate) with 1% β-mercaptoethanol (Qiagen) and stored at −80°C until RNA isolation. All cells were sorted as replicates, in at least duplicates.

mRNA Extraction and cRNA Generation

RNA was extracted from RLT Buffer (Qiagen)-treated mDC using the RNeasy Micro kit (Qiagen), according to the manufacturer's protocol. Total RNA was checked for quantity and quality using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and Experion automated electrophoresis system (Bio-Rad Laboratories). Samples that were not of sufficient quality and/or quantity were not further studied. mRNA from each sample was amplified using the MessageAmp II aRNA Amplification kit (Ambion) according to the manufacturer's protocol. This involved oligo DT primers and optimized MMLV RT, T7 RNA polymerase, and biotinylated nucleotides.

Microarray Analyses

Subsequently, 750 ng of the biotinylated amplified cRNA was hybridized to the HumanRef-8 v3.0 or Human Transcriptome (HT)12_V4_Beadchip Microarrays (Illumina) at 58°C for 20 h and then quantified using the iScan System (Illumina).

Statistical Analyses

The statistical analysis of the microarray data was performed essentially as described (49). Analysis of the Genome Studio output data was conducted using the R statistical language (R Development Core Team) and software packages from Bioconductor (50). First, arrays displaying unusually low median intensity or low correlation relative to the bulk of the arrays were discarded from the rest of the analysis. Quantile normalization was applied followed by a $\log_2$ transformation. Some samples were acquired at different times, using different versions of the HT gene arrays. For this reason, batch correction was employed to normalize for interarray variability. Batch effect subtraction was performed using the ComBat procedure (51). The LIMMA package (Bioconductor) (52) was used to fit a linear model to each probe and to perform a (moderated) Student's *t* test on various differences of interest.

Ingenuity pathways analysis (IPA, Qiagen) and gene set enrichment (GSEA) were used to identify pathways altered in the samples being compared. Two-dimensional scaling analysis was done in Bioconductor. The expected proportions of false positives, the false discovery rates (FDR), were estimated from the unadjusted *p*-value using the Benjamini and Hochberg method (53). The microarray data are available through the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession no. GSE139559.

Quantification of Intracellular IL-1α and Phosphorylated p38 and IκKγ

Peripheral blood mononuclear cells (PBMC) from study subjects were isolated by density gradient centrifugation (Histopaque, GE Healthcare) of aphereses obtained from TN HIV-infected or HIV-uninfected individuals. PBMC were stained with the following cell surface molecules: HLA-DR APC-Cy7 (BD Biosciences), CD11c APC (BD), CD14 Pacific Blue (BD), CD3 PerCP-Cy5.5 (BD), and CD19 ECD (Beckman Coulter) for 30 min and washed twice in FACS buffer (2% fetal bovine serum in PBS). The samples were then resuspended in complete media (RPMI 1640) and stimulated with interleukin-1 (IL-1; 10 ng/ml; R&D Systems) for 15 min at 37°C. Following the incubation, the samples were centrifuged at 1,800 rpm for 5 min at RT and then fixed with pre-warmed BD Cytofix buffer (BD Biosciences) for 10 min at 37°C. The fixed samples were then permeabilized in cold BD Phosphoflow™ Perm Buffer III (BD Biosciences). Samples were washed in FACS buffer and then stained with BD Phosphoflow™ phosphorylation intracellular p38-P and IκKγ-P antibodies (BD Biosciences). After two additional washes with FACS buffer, the samples were analyzed by flow cytometry using an LSR II (BD) and DIVA software. mDCs were identified by

TABLE 1 | Study participants for mDC isolations.

HIV status	Age (years)	Gender	Treatment	Estimated time since HIV± (years)	Time since ART (years)	Viral RNA copies/ml	CD4 T cells/μl
HIVp1	28	M	ART	2	2	<50	447
HIVp2	52	M	ART	15	13	392	654
HIVp3	51	F	**TN**	**12**	**NA**	**5,970**	**641**
HIVp4	41	M	ART	12	6	<50	619
HIVp5	21	M	**TN**	**1**	**NA**	**304, 742**	**513**
HIVp6	52	M	**TN**	**20**	**NA**	**19,998**	**430**
HIVn1, 2, 3, 4	§	§	NA	NA	NA	NA	*

*The cohort included antiretroviral therapy (ART)-treated and treatment-naïve (TN) (in bold) people living with HIV (PLWH) as well as HIV-uninfected persons. HIVp, HIV positive; HIVn, HIV negative; NA, not applicable; §, unavailable; *within normal ranges for CD4 T cells.*

expression of the following markers: HLA-DR$^{\text{intermediate to high}}$, CD11c$^{\text{high}}$, CD14$^-$, CD3$^-$, CD19$^-$, CD56$^-$, and CD123$^-$. Analyses were performed using Flow Jo version 10 (Tree Star) to assess IL-1α levels, and p38 and IκKγ phosphorylation levels after flow cytometry gating on mDC populations.

Intracellular IL-1α expression was determined by intracellular staining with a PE-conjugated antibody to IL-1α (BD Pharmingen). PBMCs were stimulated with CL097 (1 μg/ml; a TLR 8 ssRNA analog; Invivogen) or LPS (100 ng/ml; Sigma-Aldrich) for 16 h. Cells were stained with antibodies to the aforementioned cell surface molecules for 30 min and washed twice in FACS wash buffer, after centrifugation at 1,800 rpm for 5 min followed by a fixation in 2% PFA in PBS. Then cells were permeabilized by a wash with 0.05% saponin in PBS followed by centrifugation at 1,800 rpm for 5 min. Cells were then stained with IL-1α antibody in PBS with 0.05% saponin for 30 min at room temperature. Cells were then washed twice in FACS wash buffer and then fixed in 2% PFA in PBS. Sample data were collected as described above.

TABLE 2 | Strategy for the isolation of mDC from PLWH and HIV uninfected persons.

Cell surface marker	Enrichment	FACS
CD1c$^+$	x	
BDCA-4$^-$	x	
CD19$^-$	x	
HLA-DR$^{\text{intermediate–high}}$		x
CD11c$^+$		x
CD14$^-$		x
Lin (CD3$^-$, CD56$^-$, CD19$^-$, CD123$^-$)		x

RESULTS

Isolation of mDC From PLWH and HIV-Uninfected Study Participants

The participants are a cross section of HIV-infected persons who were seen at the Vaccine Research Center Clinic, NIAID, NIH (Bethesda, MD) and who were infected with HIV for times ranging from an estimated 1 to 20 years and who met the criteria of having within, or close to, normal levels of CD4 T cells in the blood, i.e., ∼500–1,500 CD4 T cells/μl (**Table 1**). Three of the six HIV-infected persons were untreated, while the others received ART regimens for times ranging between 2 and 13 years. The estimated time since HIV infection was between 1 and 20 years for the TN participants and between 2 and 15 years for the ART-treated participants. All ART-treated PLWH had been treated at least 2 years before enrolling in this study. Two of the ART-treated participants were virally suppressed, <50 plasma viral RNA copies/ml, and one ART-treated individual had a plasma viral RNA load of ∼400 copies/ml, which is considered a relatively low viral load. Since only one time point was examined, it is not known whether the ca. 400 copies/ml represents an ongoing low level of viral replication or a "viral blip." Viral blips are known to occur in ∼20% of ART-treated PLWH (54).

Of the untreated HIV-infected participants, plasma viral RNA loads were of a wide range, from 6,000 to 300,000 copies/ml (**Table 1**). It is generally accepted that long-term non-progressors (LTNPs) are PLWH who have been HIV infected for ≥7 years and maintain CD4 T cells ≥500 cells/μl of blood [reviewed in Gurdasani et al. (55)]. Subject HIVp3 had been infected for an estimated 12 years and had CD4 T-cell levels of ca. 640 cells/μl and, thus, is considered an LTNP. Participant HIVp6 was HIV infected for over 20 years and had CD4 T cells of 430 cells/μl and, thus, is not considered an LTNP. The third untreated HIV-infected participant, HIVp5, was infected for only 1 year. In addition to the HIV-infected participants, four HIV uninfected healthy subjects, with CD4 T-cell levels within the normal range, whose blood was available through the NIH Clinical Center, were also studied as controls (**Table 1**). All individuals were subjected to apheresis.

As mDCs are sensitive to cryopreservation, we obtained mRNAs from freshly isolated mDCs as well as from monocytes, as described (see Materials and Methods section). We identified mDC using the following markers: CD1c$^+$, HLA-DR$^{\text{intermediate to high}}$, CD11c$^{\text{high}}$, CD14$^-$, CD19$^-$, CD123$^-$, CD56$^-$, and BDCA 4$^-$ (**Table 2** and **Supplementary Figure 1**). Monocytes were identified by the following markers: CD1c$^-$, HLA-DR$^{\text{intermediate to high}}$, CD14$^{\text{high}}$, CD19$^-$, CD123$^-$, CD56–, and BDCA-4$^-$ (**Supplementary Figure 1**). We isolated mRNA

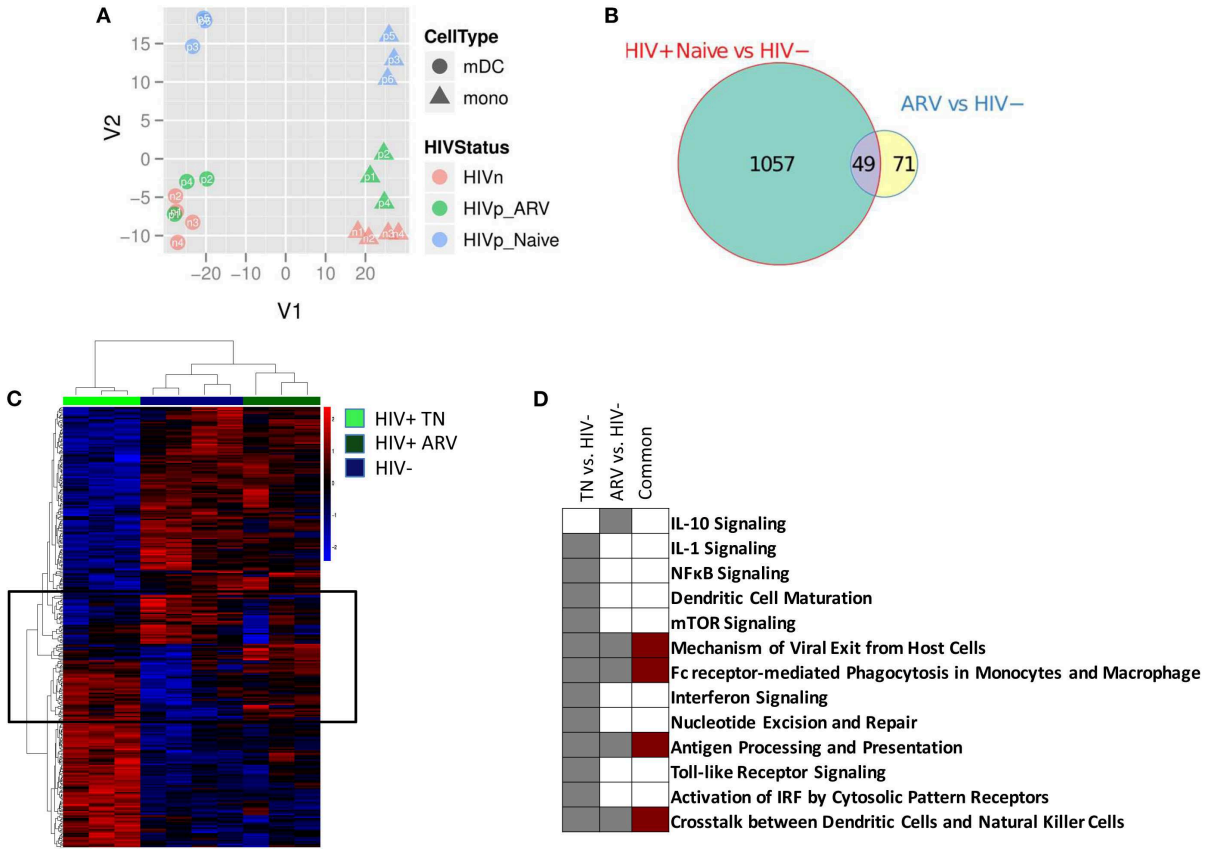

FIGURE 1 | Myeloid dendritic cells (mDCs) from treatment-naïve (TN) and antiretroviral (ARV)-treated people living with HIV (PLWH) have a different gene transcription compared to mDCs from HIV-uninfected individuals. **(A)** Principal component analysis of the gene expression profiles for the top-most variable genes between mDCs and monocytes from TN PLWH, ARV-treated PLWH, and HIV-uninfected individuals. **(B)** The Venn diagram indicates the number of common and unique genes ($|FC| > 2$ and $p < 0.05$) between the mDC contrasts from PLWH and HIV-uninfected persons. **(C)** Heatmaps of differentially expressed genes of mDC from treatment-naive (TN) or ARV-treated PLWH. Differentially expressed genes with $|FC| > 2$ and $p < 0.05$ are shown. Highlighted on the heatmap, indicated by a box, are those genes commonly altered in mDC from TN and ARV-treated PLWH compared to HIV-uninfected persons. **(D)** Pathway alterations in mDC from TN and ARV-treated PLWH compared to mDC from HIV uninfected persons. Ingenuity pathway analysis (IPA), as described in the Materials and Methods section, was used to identify those pathways enriched in the differentially expressed genes in each contrast. The complete list of pathways associated with these contrasts is provided in the **Supplementary Materials**, **Supplementary Figure 2**. ARV, antiretroviral therapy; HIVn, HIV negative; HIVp_Naive, HIV positive, treatment naïve; HIVp_ARV, HIV positive, antiretroviral-treated.

from these purified cells, generated cRNA libraries, and performed gene expression array analyses.

Altered Gene Expression in mDC From Untreated and ART-treated PLWH

We compared the gene expression profiles of mDC and monocytes from treatment-naïve (TN) PLWH, ART-treated PLWH, and those from HIV-uninfected individuals. The principal component analysis (PCA) describes the variation in gene expression between samples of interest. Using the PCA, we found that cell type-specific gene expression represented the greatest variation between the samples. This is graphically represented in PC1 (or the x axis) in **Figure 1A**, with mDCs and monocytes separating from each other, for all individuals (**Figure 1A**). Other gene expression variations between samples are accounted for in PC2 (or the y axis). Samples grouped together based on HIV treatment status in PC2. mDCs and monocytes from TN PLWH grouped together in PC2, as did mDCs and monocytes from ART-treated PLWH, and as did those from HIV-uninfected persons (**Figure 1A**). However, mDCs and monocytes from TN individuals had the greatest divergence from all of the other groups. For example, mDCs and monocytes from ART-treated individuals clustered more closely to those from HIV-uninfected individuals than those from TN PLWH. The transcriptional profile for mDCs and monocytes from ART-treated HIV-infected individuals, however, segregated distinctly from HIV-uninfected individuals (**Figure 1A**).

We performed a differential gene expression (DEG) analysis between mDC from TN or ART-treated PLWH to those from HIV-uninfected persons (using a supervised analysis). We found that 1,106 genes were differentially expressed between mDCs from TN PLWH compared to those of HIV-uninfected individuals (FC, fold change ≥ 2, $p \leq 0.05$; **Figure 1B**). We also found that 120 genes were differentially expressed in mDCs

from ART-treated HIV PLWH compared to those of HIV-uninfected individuals (**Figure 1B**). Of the 120 genes altered in mDCs from ART-treated PLWH compared to those of HIV-uninfected individuals, 71 of these were unique to ART treatment, while 49 of these were shared with mDCs from TN PLWH (**Figure 1B**).

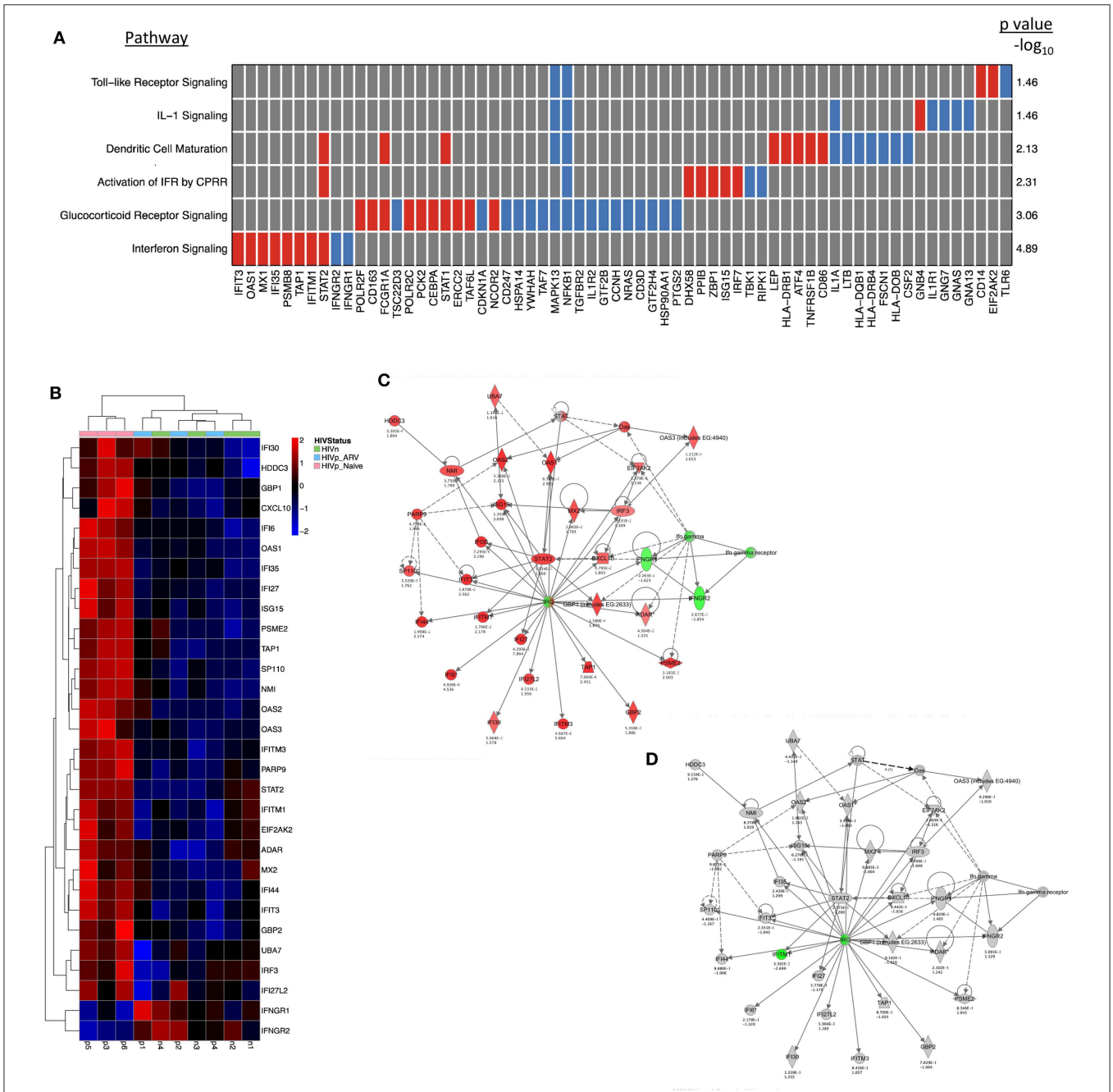

FIGURE 2 | Interferon signaling is the most significantly altered pathway in mDCs from untreated PLWH compared to HIV-uninfected individuals and is mostly restored to HIV-uninfected levels in mDCs from ARV-treated PLWH. mDCs from treatment-naïve (TN) PLWH have type I IFN pathway transcriptional signatures. **(A)** A pathway heatmap showing pathways altered, including the IFN pathway, the most significantly altered pathway, in mDCs from TN PLWH compared to HIV-uninfected persons. **(B)** Heatmap showing interferon-response genes in the ingenuity pathway analysis (IPA) network for antimicrobial and inflammatory response for mDCs from TN and ARV-treated PLWH and HIV-uninfected individuals. **(C,D)** The same IPA genes in their networks, showing those gene alterations in **(C)** mDCs from TN PLWH and **(D)** ARV-treated PLWH relative to HIV-uninfected persons. In ARV-treated PLWH, type I IFN gene transcription is mostly restored to HIV-uninfected levels; however, some **(D)** type I IFN genes are reversed in expression and downregulated relative to HIV-uninfected levels. Numbers below each gene node indicate the *p* value and below that (log_2) fold change of that gene's expression relative to HIV-uninfected levels.

mDC Biological Pathways Are Altered in Untreated and ART-Treated PLWH

When evaluating the differentially expressed genes in the mDCs from TN PLWH compared to those from HIV-uninfected persons, we found that the mDCs from ART-treated PLWH grouped more closely to the mDCs from HIV-uninfected individuals (**Figure 1C**). However, mDCs from ART PLWH formed a distinct group or branching from HIV-uninfected individuals, as depicted in the heatmap shown (**Figure 1C**). The ART profile was more akin to the HIV-uninfected profile, but there are groups of genes that differed from those of HIV-uninfected individuals, many of which were in common with those of TN HIV-infected persons (**Figure 1C**). This was also the case for the $CD14^+$ monocytes (data not shown). We then determined whether the genes that changed in the three groups were included in specific biological pathways. We used the ingenuity pathways analysis (IPA) database to identify such pathways. The most highly represented pathway alteration in mDCs in TN PLWH relative to HIV-uninfected persons was the interferon pathway (**Figure 1D** and **Supplementary Figure 2**). We also found significant alterations in other pathways such as the viral nucleotide excision repair and mechanisms of viral exit from host cell pathways. There were also alterations in the antigen processing and presentation and mTOR pathways as well as in the IL-1 signaling pathway (summarized in **Figure 1D** and **Supplementary Figure 2**).

In mDCs from ART-treated PLWH, the IL-4 and IL-10 pathways were altered relative to HIV-uninfected persons, and these were not significantly altered in TN PLWH (**Figure 1D** and **Supplementary Figure 2**). Pathway alterations shared between mDCs from ART-treated and TN PLWH included the FC receptor-mediated endocytosis pathway and antigen processing and presentation (**Figure 1D**). Thus, while the majority of pathway changes in mDC appeared to be reversed by ART, there were some pathways that were only found in the mDCs from ART-PLWH and some pathways that were shared between the mDCs from ART and TN PLWH relative to HIV-uninfected individuals (**Figure 1D** and **Supplementary Figure 2**).

A Type I IFN Transcriptional Signature in mDCs From TN PLWH Is Mostly Reversed by ART

We observed that mDCs from TN HIV-infected individuals had profound transcriptional upregulation of a type I interferon (IFN) response (**Figures 2A–C**). When we scrutinized networks of IFN-inducible genes of mDCs from TN PLWH, we found a significant induction of many of the IFN-inducible genes (**Figures 2A–C**) including several that are components of the innate cell intrinsic antiviral machinery (MX1, OAS1, IFIT3). STAT1 and STAT2, which activate type I IFN transcriptional networks, with STAT1 also involved in type II IFN responses, were uniformly upregulated in mDC from TN PLWH (**Figures 2A–C**). Surprisingly, other components of the IFNγ (type II IFN) pathway such as IFN gamma receptor 1 (IFNγR1) and 2 (IFNγR2) were slightly downmodulated in mDCs from TN HIV-infected subjects (**Figures 2A,C**).

The type I IFN pathway in the mDCs from ART-treated PLWH was similar to the mDCs from HIV-uninfected persons. However, the mDCs from ART-treated PLWH did not exhibit complete restoration of the type I IFN pathway to expression levels observed in HIV-uninfected individuals (**Figures 2B,D**). Three genes (IFITM1, IRF7, and IRF9), which are involved in regulation and transcriptional activation of interferon responses, were all reversed in expression change, and were downregulated relative to that of mDC from HIV-uninfected individuals (**Figures 2B,D**).

IL-1 Signaling Is Suppressed in mDCs From TN PLWH

We identified transcriptional downmodulation of the IL-1 pathway in TN HIV-infected individuals (**Figures 3A–C**). We found several genes that encode cytokine receptors belonging to the interleukin 1 receptor family, including IL1RA, and IL1R2 as well as IL1A, itself, were downregulated in mDCs from TN PLWH compared to HIV-uninfected individuals (**Figures 3A,B**). The IL-1 signaling pathway plays a critical role in response to microbial infection [reviewed in Mayer-Barber and Yan (56)]. In order to validate some of the transcriptional changes seen, we looked at IL-1α protein levels in activated mDC TN PLWH (**Figures 4A–C**). We sought to determine whether mDCs from TN HIV-infected individuals were impaired in IL-1α production after microbial stimulation or in IL-1 signaling pathways downstream of IL-1α stimulation. In order to address this, we first tested mDCs obtained from TN PLWH or HIV-uninfected persons, before or after microbial exposure *in vitro*. We measured intracellular IL-1α production in mDCs from HIV-uninfected individuals and compared this to that of TN HIV-infected individuals. We found no significant difference in basal levels of IL-1α protein expression (**Figure 4A**). However, upon stimulation with a single-stranded RNA toll-like receptor (TLR) 7/8 agonist, CL097 ($p < 0.01$), or TLR4 (and CD14) agonist LPS ($p < 0.001$), we found a statistically significant decline in IL-1α production among HIV-infected TN individuals compared to that of HIV-uninfected individuals (**Figure 4A**).

IL-1α stimulation activates the NF-κB pathway, which is essential for innate immune cell function [reviewed in Caamano and Hunter (57) and Dorrington and Fraser (58)]. Activation of NF-κB requires phosphorylation of p38 (MAPK) and IκK. Thus, we quantified levels of phosphorylated p38 and IκKγ after IL-1α stimulation of mDC, to determine whether activation of the NF-κB pathway by IL-1α stimulation was impaired in mDCs from TN PLWH as we had observed in gene transcription. Following stimulation of mDCs from HIV-infected TN individuals with IL-1α, we also observed a statistically significant suppression of p38 MAPK activation and NF-κB activation by IκKγ phosphorylation between HIV-uninfected and TN HIV-infected individuals ($p = 0.052$ and $p = 0.005$, respectively) (**Figures 4B,C**). Similarly, one previous study demonstrated suppressed IκKγ phosphorylation after TLR ligand stimulation in DCs from healthy individuals in an HIV plasma-transfer model (12). We found that mDCs from chronic TN HIV infections have suppressed IL-1α activation in

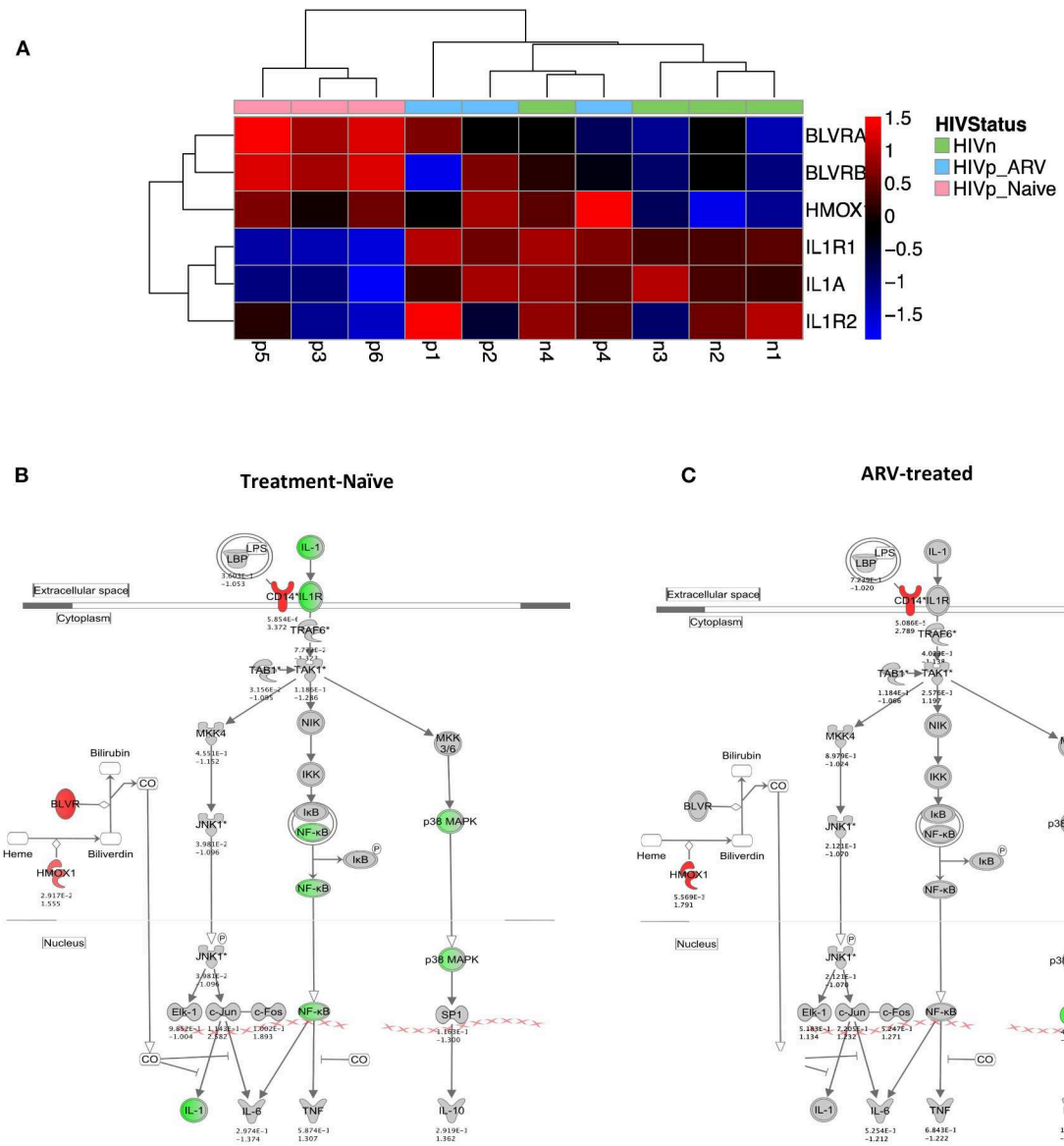

FIGURE 3 | The IL-1 signaling pathway is transcriptionally suppressed in mDC from treatment-naïve PLWH. **(A)** Heatmap and **(B)** network illustrating key IL-1 pathway genes and their modulators. **(A,B)** IL-1α and its receptors are transcriptionally suppressed in mDCs from TN PLWH compared to ARV-treated or HIV-uninfected persons. **(B,C)** Negative regulators of IL-1 signaling, including hemoxygenase (HMOX-1) and CD14, components of the IL-10 pathway, are **(B)** upregulated in mDCs from TN PLWH. **(C)** In mDCs from ARV-treated PLWH, levels of suppressors of IL-1 signaling are not completely restored to levels of mDCs from HIV-uninfected persons. **(B,C)** Shown is the ingenuity pathway analysis (IPA) pathway, showing both IL-1- and IL-10-associated genes. Numbers below each gene node indicate *p* value and below that (log_2) fold change of that gene's expression relative to HIV-uninfected levels.

response to microbial stimulation and an impaired IL-1 pathway in response to IL-1α stimulation (**Figures 4A–C**).

IL-1 Signaling Pathway Is Mostly Restored in mDCs From ART-Treated PLWH

Three known endogenous suppressors of the IL-1 pathway, Biliverdin A and B (BLVRA and BLVRB) and hemoxygenase-1 (HMOX-1) are associated with the IL-10 pathway. All three were upregulated in TN and ART-treated PLWH compared to HIV-uninfected individuals (**Figures 3A,C**). Unlike the IL-1 receptor family genes in mDCs from ART-treated individuals that were like (grouped with) HIV-uninfected individuals in expression patterns (**Figure 3A**), the BLVR genes, and most significantly, the HMOX-1 gene expression, were more akin to mDCs from TN PLWH than from HIV-uninfected persons (**Figures 3A,C**). Therefore, ART is mostly reversing the IL-1 pathway gene transcription to HIV-uninfected levels, but not all of the suppressors of the IL-1 pathway, in mDCs.

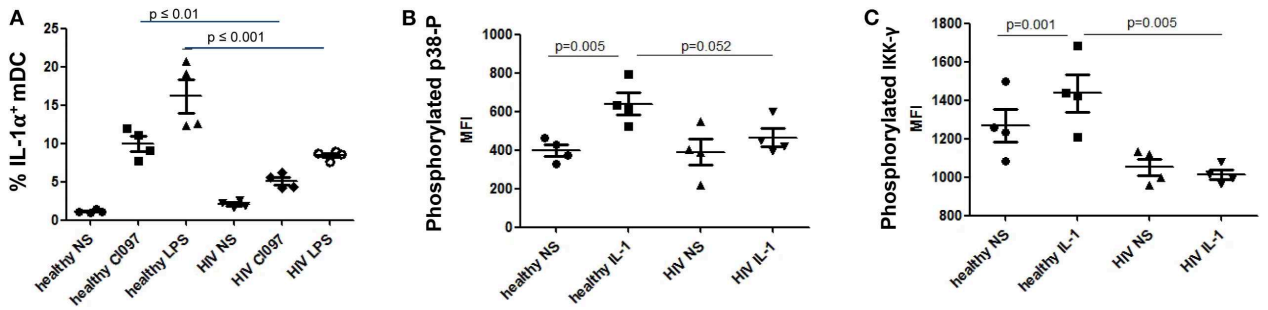

FIGURE 4 | Diminished interleukin-1 (IL-1α) production and suppressed NF-κB pathways in mDCs from treatment-naïve PLWH after IL-1α or microbial stimulation. Impaired **(A)** IL-1α production following microbial stimulation of mDCs from untreated PLWH. **(B)** IL-1-induced signaling in mDCs from untreated PLWH show decreased NF-κB activation as indicated by **(B)** p38 phosphorylation and **(C)** IKKγ phosphorylation. Phosphorylated p38 and IKKγ were measured by flow cytometry, and the mean fluorescence intensity (MFI) of the specific antibodies are reported for mDC, as described in the Methods and Materials section. CL097, a TLR 7/8 agonist. IL-1 refers to IL-1α-stimulated cells, as described in the Materials and Methods section. NS, no stimulation; LPS, lipopolysaccharide.

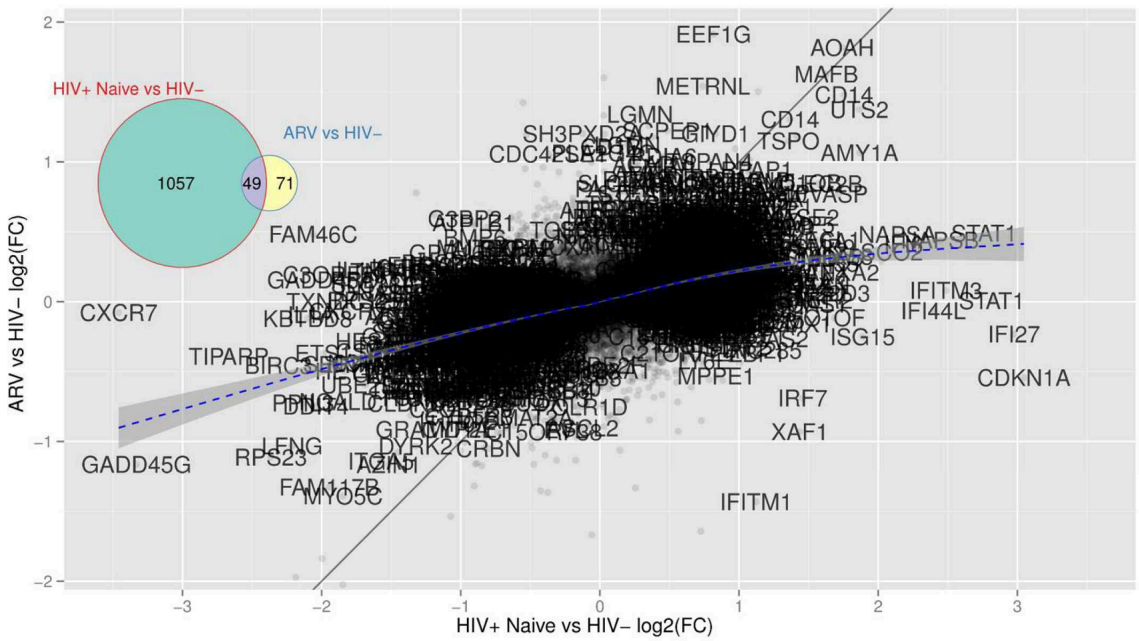

FIGURE 5 | mDCs from TN PLWH have transcriptional alterations not restored in mDCs from ARV-treated PLWH to HIV-uninfected levels. This scatterplot compares mDC gene expression from TN or ARV-treated PLWH relative to HIV-uninfected persons expressed as (log_2) fold changes. There are far more genes with high fold changes in the TN than in the ARV group, indicating that mDCs from ARV-treated PLWH is closer to the HIV-uninfected group. There are genes "maintained," i.e., HIV-elicited genes that are unaffected by the therapy (AOAH, MAFB, CD14, all those close to the identity line or "common" genes) and genes whose differential expression is strongly suppressed by the therapy (all those close to the x-axis and, therefore, unique to TN PLWH), and those that are an effect of the therapy alone (close to the y-axis and unique to ARV). A gene like IFITM1 is reversed by therapy as it lies close to the y = –x line.

mDC Gene Transcription Including CD14 Is Not Restored by ART in PLWH and Indicates Altered Myeloid Cell Differentiation Pathways

Similar to HMOX-1, a gene that was altered in mDCs from both TN and ART PLWH relative to HIV-uninfected individuals, we found that there were other genes commonly altered in HIV infection relative to HIV-uninfected persons. We examined those genes commonly altered in both TN and ART-treated PLWH. The multidimensional scaling plot (MDS) (**Figure 5**) shows those genes commonly altered in mDCs from untreated and ART-treated PLWH as well as those not restored to HIV-uninfected levels with ART treatment. Strikingly, there exists a group of genes that are commonly upregulated and relatively unaffected in expression levels in TN and ART-treated PLWH. Some of the genes included CD14, acyloxyacyl hydrolase (AOAH), and MAFB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B). A gene such as IFITM1 is upregulated in mDCs from TN PLWH but downregulated in ART-treated

TABLE 3 | List of genes whose transcription is commonly altered in mDCs from TN and ART-treated PLWH relative to HIV-uninfected persons.

AAMP	POLR1D
CECR5	FAM109A
STAB1	BECN1
LRPAP1	SEPT3
GTPBP8	COASY
CYP2E1	NT5DC3
SLC2A9	SFRS5
HK3	MAFB
MFSD7	CENTD2
ZNF362	AFF3
DGCR8	NCALD
EMILIN2	TBC1D9B
SFRS6	CKAP4
HLA-DRB5	MGAT4B
HSPC111	C1QB
AOAH	RIOK1
HLA-DRB1	MYO5C
PRPF31	TOMM7
SYTL3	ARMCX1
CD163	TSPO
FAM117B	GIYD1
CD14	FAM89A
GRK6	TSPAN4
ARS2	ATP6V0B
WAC	

PLWH compared to HIV-uninfected individuals (**Figure 5**). The complete set of 49 genes not restored by ART is shown (**Table 3**).

The genes were commonly altered in mDCs from ART-treated and TN HIV-infected persons mapped to myeloid cell lineage and differentiation (**Supplementary Figure 3**), using GSEA analysis, which annotates genes according to modules (datasets annotated by function and coordinated expression). The modules commonly altered in mDC from TN and ART-treated PLWH were (1) myeloid lineage and (2) inflammation I and (3) inflammation II (**Supplementary Figure 3**). Prominently, some of those genes coordinately upregulated are those most classically associated with a monocyte/macrophage lineage including CD14, CD163, C1QB, and MAFB, and inflammatory response, such as AOAH (**Figure 6** and **Supplementary Figure 3**), also associated with LPS response, as further discussed in the Discussion section. Overall, these results indicate that ART does not completely return transcription in mDCs from ART PLWH to levels seen in HIV-uninfected people.

Summary

Our data analyzing mDC gene expression indicated that two major pathways were mostly, but not completely, reversed by ART. These pathways are the IL-1 and type I IFN pathways. Surprisingly, some components of the type I IFN pathway were transcriptionally downregulated in mDCs from ART PLWH compared to HIV-uninfected persons. There were transcriptional changes common to mDCs of ART-treated and TN PLWH. Those genes mapped to myeloid cell differentiation and inflammation pathways and included AOAH, and to genes classically associated with a monocyte/macrophage lineage and included CD14, C1QB, CD163, and MAFB. Since recent data from others indicate that there is a subset of mDCs that express these RNA transcripts classically associated with monocyte/macrophage, further studies will need to be done to determine whether the changes we observe are due to alterations in monocytes/macrophages in PLWH that render them more phenotypical like mDCs or expansion of the newly delineated mDC "inflammatory" subpopulation in PLWH. Since our populations of mDC might include monocytic/macrophage precursors, future studies on more purified mDCs remain to be done to determine whether these are monocytes/macrophages or a subpopulation of mDCs. In either case, we found that ART does not restore myeloid cell transcription to that of HIV-uninfected persons.

DISCUSSION

This work, indicating changes in mDC associated with HIV infections, need to be considered in the development of ART. In this report, we compared gene expression in mDCs from untreated and ART-treated PLWH, who were chronically HIV infected ($\geq$1 year post infection), compared to HIV-uninfected persons. Surprisingly, we found differences in gene expression in mDCs from ART-treated as well as untreated PLWH compared to that of uninfected healthy persons. This indicates that ART does not completely restore transcriptional changes that occur with HIV infections. The data presented herein reveal those 49 genes whose altered expression is common to ART and untreated HIV infections. Some of these genes are primarily considered to be indicative of a classical monocyte/macrophage lineage rather than a DC lineage. These genes include CD14, CD163, and the transcription factor, MAFB. We isolated mDCs by the canonical markers used to delineate DC at the time of the study. Our method employed an antibody-conjugated bead enrichment for $CD1c^+$ myeloid cells followed by FACS for mDCs based on size, $CD11c^+$, HLA-$DR^{intermediate-high}$, and lineage negative ($CD3^-$, $CD19^-$, $CD56^-$) as well as $CD14^-$ markers.

One explanation for our finding is related to a recent study of single-cell RNA sequencing (scRNAseq) of DC (10). This study showed that the $CD1c^+$ mDC that we studied here is made up of two transcriptionally distinct mDC subpopulations in healthy individuals (10). The previously unidentified minor mDC subpopulation called "$CD1c^+$_B," or "inflammatory cDC2" expresses monocyte/macrophage genes including CD14 RNA in the absence of CD14 protein on the cell surface, which is what we found in our study. Villani et al. also found higher levels of CD163 and HMOX-1 in this mDC subpopulation than in classical $CD1c^+$ mDC [reviewed in Collin and Bigley (9)], similar to genes we observed whose transcription was increased in mDCs from PLWH. Thus, it is possible that the mDCs from PLWH have an increase in this "inflammatory" mDC subpopulation. Another report showed a $CD1c^+$ mDC subpopulation that expressed higher levels of MAFB, a transcription factor that had previously been ascribed to monocyte lineage that we also observed with increased transcription in ART-treated and TN PLWH (9, 59). This leads to the possibility that this newly identified

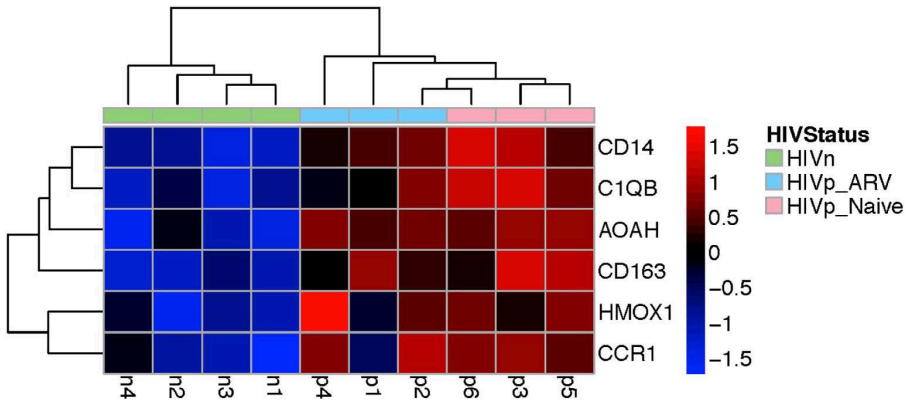

FIGURE 6 | Genes commonly upregulated in mDCs from TN and ARV-treated PLWH included those classically associated with the monocyte/macrophage lineage, myeloid cell differentiation, and inflammatory response. This heatmap shows a subset of those genes commonly upregulated in mDCs from TN or ARV-treated PLWH relative to HIV-uninfected persons. **Supplementary Figure 3** illustrates, in more detail, the annotated functions of the genes shown.

mDC population that expresses genes previously associated with monocyte/macrophage, and whose role in health and disease has not yet been established, is an mDC subpopulation expanded in ART-treated and TN HIV infections.

Of those 49 genes altered in mDC from untreated and ART-treated PLWH, we observed some of the highest increases in transcription of the acyloxyacyl hydrolase (AOAH) gene (fold change = ∼4). AOAH is a host lipase, which deacylates the gram-negative bacterial outer membrane component, LPS, rendering it less immunostimulatory to the host [reviewed in Munford et al. (60)]. AOAH is primarily expressed by antigen-presenting and phagocytic cells, including mDCs and monocytes (60, 61). AOAH mRNA is increased with LPS exposure of macrophage *in vitro* and *in vivo* (59). AOAH prevents prolonged responses to LPS when overexpressed in mouse DC (62, 63) and protects mice from inflammation-induced injury during gram-negative bacterial infections (59) as well as from gram-negative bacterial infection (63). Thus, the role of AOAH in HIV infections is somewhat paradoxical as PLWH are more susceptible to secondary infections, yet AOAH is associated with protection from gram-negative bacterial infections, at least in murine studies. Research has found a short nucleotide polymorphism (SNP) in an intron of the AOAH gene to be correlated with HIV RNA levels in PLWH (64). It may be that AOAH causes changes in mDC or myeloid cells that alters their ability to stimulate antiviral immunity, but this will need to be addressed in future studies targeted at understanding the role of AOAH in PLWH.

Some systems indicate that type I IFNs suppress IL-1 pathways, leading to increased susceptibility to bacterial and fungal infections (65–67). A type I IFN transcriptional profile in blood cells occurs in active MTB infection and is associated with MTB disease severity (68). The increased type I IFN transcriptional profile, alongside a suppressed IL-1 signaling pathway that we observed in mDC from TN PLWH, could be involved in enhancement of O.I.s such as MTB infections in PLWH. In this study, we found that ART restored type I IFN and IL-1 signaling pathways to near-HIV-uninfected levels. However, we found ∼70 genes altered in myeloid cells enriched for mDC in ART-treated PLWH compared to HIV-uninfected persons that were not in common with mDCs from untreated PLWH. Given the use of widespread use of pre-exposure prophylaxis (PrEP), these ART-specific changes should be further investigated for the effects of ART alone on myeloid cells.

The CD14 cell surface protein in HIV-uninfected persons has been used as a marker to differentiate blood monocytes and mDCs since monocytes have CD14 on their cell surface, whereas mDC generally do not. Here, we show a myeloid cell population that expresses CD14 RNA in the absence of significant levels of CD14 protein on the cell surface in PLWH. There are at least two ways to explain these results other than expansion of the newly described CD14 RNA-expressing mDC subpopulation in PLWH, as described above. CD14 is either shed from cells or internalized after binding to LPS (69, 70). This calls into question the utility of using the CD14 antibody as a marker to exclude, identify, or differentiate myeloid cell types in PLWH. Alternatively, a $CD14^{low}$ population of monocytes, known to be expanded in both ART-treated and untreated PLWH, albeit to a lesser extent after ART (71–73), may have been included in our mDC population in PLWH. Inclusion of an additional cell surface marker, CD16, will help exclude $CD14^{low}$ monocytes from the mDC population as $CD14^{low}$ monocytes co-express CD16 monocytes (74). For these reasons, we will refer to the myeloid cell population we have isolated as myeloid cells enriched for mDC. Importantly, we found common transcriptional changes in an enriched myeloid cell population in both ART and TN PLWH compared to HIV-uninfected individuals. Given more recent findings of two classes of mDC, it should be possible in the future to determine whether this newly defined mDC subpopulation is expanded in untreated and ART-treated PLWH, or whether CD14 surface protein is internalized or shed from CD14 RNA-expressing cells that bind LPS in PLWH.

We found that ART does not restore gene transcription in mDCs from PLWH to HIV-uninfected levels. It may be that this represents changes in myeloid cell subpopulations that occur in TN and ART-treated PLWH. Even so, these transcriptional

changes are not restored to HIV-uninfected levels with ART. It is known that high levels of CD14 and CD163 in the blood correlate with poor outcomes in including cardiovascular adverse events, fibrosis, and neurological abnormalities in ART-treated as well as TN PLWH [reviewed in Anzinger et al. (29)]. The mDC population that we have identified here may be responsible for the heightened levels of these proteins observed in PLWH, and thus targeting this myeloid cell population in ART would be important. Preliminary data from our lab indicate that plasma LPS levels correlate with CD14 RNA levels in the enriched mDC population in PLWH (unpublished data). This is consistent with the hypothesis that LPS is related to the mDC transcriptional changes that we observed in PLWH. Those 49 genes we have identified in mDCs that remain altered in PLWH with ART and have not previously been identified in biological studies in PLWH, such as AOAH, are candidates for further investigation in their role in HIV pathogenesis.

The participants in this study were pre-screened for near-normal CD4 T-cell counts and overall good health to be eligible for apheresis for the isolation of mDC. Therefore, the TN PLWH in this study were skewed toward long-term non-progressors, who represent only ∼5–15% of the PLWH population (75), and therefore, this cohort does not reflect all TN PLWH. The majority of PLWH are progressors, who have a decline in CD4 T cells to ≤350 cells/μl within the first 7 years post-HIV infection, at which time ART is initiated. It is not yet known whether the mDC transcriptional changes we observed are also seen in HIV progressors before ART treatment.

One of the ART-treated PLWH in this study was not completely virally suppressed, having plasma viral RNA loads of ∼400 copies/ml. As we only collected measurements at one time point for the participants, we cannot ascertain whether this is a viral blip or ongoing viremia. In the future, it would be important to look at mDC samples from HIV progressors pre- and post-ART to determine whether the residual changes that we observed in ART-treated participants are reflected in longitudinal samples from HIV progressors.

One barrier to effective ART is the persistence of CD4 T cells that harbor latent HIV proviruses. It has been shown that mDC promote latency and HIV persistence in CD4 T cells (76, 77). Therefore, mDC should be investigated as targets of latency-reversing agents (LRAs), the current drugs used to eliminate latently HIV-infected cells in ART PLWH, since the effects of these drugs on mDC have not been delineated. Of note, the cohort we studied included PLWH who had been taking ART for over 12 years and individuals HIV infected for up to 20 years. Thus, changes in mDCs were not restored to baseline by ART despite long times post HIV infection and ART initiation. In the future, new ART protocols should be developed to target these myeloid and mDC alterations. The findings described here, of transcriptional changes in myeloid cells enriched for mDCs from PLWH, including AOAH, should be further investigated for their relationship to O.I.s, correlates of HIV disease progression, and role in HIV persistence.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession no. GSE139559, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139559.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Institute of Allergy and Infectious Diseases or the Vaccine and Gene Therapy Institute Florida. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM was responsible for conceptualizing, writing the manuscript, and performing the experiments. YZ performed the experiments and contributed to the methods. DD and RS were responsible for conceptualizing the experiments as well as advising and funding of the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00261/full#supplementary-material

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Conventional Dendritic Cells and Slan$^+$ Monocytes During HIV-2 Infection

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HIV-2 infection is characterized by low viremia and slow disease progression as compared to HIV-1 infection. Circulating CD14^{++}CD16$^+$ monocytes were found to accumulate and CD11c$^+$ conventional dendritic cells (cDC) to be depleted in a Portuguese cohort of people living with HIV-2 (PLWHIV-2), compared to blood bank healthy donors (HD). We studied more precisely classical monocytes; CD16$^+$ inflammatory (intermediate, non-classical and slan$^+$ monocytes, known to accumulate during viremic HIV-1 infection); cDC1, important for cross-presentation, and cDC2, both depleted during HIV-1 infection. We analyzed by flow cytometry these PBMC subsets from Paris area residents: 29 asymptomatic, untreated PLWHIV-2 from the IMMUNOVIR-2 study, part of the ANRS-CO5 HIV-2 cohort: 19 long-term non-progressors (LTNP; infection ≥8 years, undetectable viral load, stable CD4 counts≥500/μL; 17 of West-African origin -WA), and 10 non-LTNP (P; progressive infection; 9 WA); and 30 age-and sex-matched controls: 16 blood bank HD with unknown geographical origin, and 10 HD of WA origin (GeoHD). We measured plasma bacterial translocation markers by ELISA. Non-classical monocyte counts were higher in GeoHD than in HD (54 vs. 32 cells/μL, $p = 0.0002$). Slan$^+$ monocyte counts were twice as high in GeoHD than in HD (WA: 28 vs. 13 cells/μL, $p = 0.0002$). Thus cell counts were compared only between participants of WA origin. They were similar in LTNP, P and GeoHD, indicating that there were no HIV-2 related differences. cDC counts did not show major differences between the groups. Interestingly, inflammatory monocyte counts correlated with plasma sCD14 and LBP only in PLWHIV-2, especially LTNP, and not in GeoHD. In conclusion, in LTNP PLWHIV-2, inflammatory monocyte counts correlated with LBP or sCD14 plasma levels, indicating a potential innate immune response to subclinical bacterial translocation. As GeoHD had higher inflammatory monocyte counts than HD, our data also show that specific controls are important to refine innate immunity studies.

Keywords: HIV-2, monocytes, slan$^+$ monocytes, dendritic cells, cDC1, cDC2, controls

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INTRODUCTION

HIV-2 infection (1) is mostly prevalent in West Africa, and in populations emigrated from West Africa to Portugal and France (1–3). Eighty-seven per cent of the participants included in the French ANRS CO5 cohort originate from this geographical area. Compared to HIV-1 infection, HIV-2 infection is characterized by undetectable-to-low viremia, slower $CD4^+$ T-cell decline and activation, slower disease progression (4, 5). The proportion of long-term non progressors (LTNP) is higher during HIV-2 than during HIV-1 infection (4). However people living with HIV-2 (PLWHIV-2) develop viral reservoirs with similar levels of HIV DNA in their PBMCs as people living with HIV-1 (PLWHIV-1) (6), and untreated HIV-2 infection can lead to AIDS, as current treatment options are scarcer than for HIV-1 infection (5).

Monocytes and conventional dendritic cells (DC) derive from common hematopoietic precursors. They are phagocytic, antigen-presenting cells and they secrete cytokines with effector, polarizing, inflammatory and reparatory functions. Monocytes represent 10–20% of peripheral blood mononuclear cells (PBMC). They are identified by their morphology and the expression of CD14. Among circulating monocytes, classical monocytes with a $CD14^{bright}$, $CD16^-$ phenotype represent the major population (90% of monocytes). Other monocytes, which will be called "inflammatory monocytes" along this text, express the FcγIII-receptor CD16, a receptor induced by microbial or inflammatory stimuli, conferring inflammatory, scavenger and cytotoxic functions. They comprise intermediate monocytes ($CD14^{bright}CD16^+$) and non-classical monocytes ($CD14^+$, $CD16^+$) (7). Circulating non-classical monocyte ($CD14^+CD16^+$) counts are higher in both asymptomatic chronically HIV-1-infected people with high viremia and in people developing AIDS than in people with low viremia or in uninfected donors (8, 9). They were found to be more permissive to HIV-1 infection *in vivo* and *in vitro* than other monocytes and to correlate with plasma sCD14 levels and microbial translocation (10, 11). Their accumulation is mostly due to a subpopulation of $slan^+$ monocytes, inflammatory monocytes bearing the 6-Sulfo LacNAc (slan) glycosylation variant of the P selectin glycoprotein ligand 1 (PSGL-1) (12–14). Although these cells were originally described as a DC subpopulation, transcriptomic approaches have shown that they coclustered with monocytes and not with $CD1c^+$ DCs (15, 16). Circulating $slan^+$ monocytes produce higher TNF-α amounts than other monocyte subpopulations, and higher TNF-α amounts in HIV-1 viremic than in non-viremic people (13). $Slan^+$ monocytes accumulate in viremic people (13) as well as in cART virosuppressed people who have experienced low CD4 count nadirs (14), but they are depleted during AIDS (17). They are known to be present in the lesions of major inflammatory chronic diseases like Crohn's disease, psoriasis or rheumatoid arthritis, which respond to therapeutic TNF-α blockade (18, 19). During HIV-2 infection, $CD16^+$ monocyte numbers were shown to increase significantly among $CD14^{bright}$ monocytes (20), but $slan^+$ monocytes had never been specifically studied.

Myeloid or conventional DC (cDC), originally characterized as $CD11c^{++}CD14^-$ cells, negative for other lineage markers, are depleted from the circulation during HIV-1 infection (21). They were found to be progressively depleted from the circulation during chronic HIV-2 infection (20). They were further delineated into two major populations: cDC1 express the chemokine receptor XCR1, thrombospondin (CD141), and receptors for dead cells like Clec9, and are the equivalent of the murine CD8-α^+ DC population, specialized in antigen cross-presentation to $CD8^+$ T cells; cDC2 express the non-classical HLA Class I molecule CD1c as well as SIRP-α, and are believed to stimulate mostly $CD4^+$ T lymphocytes (13, 22–25). Circulating cDC1 and cDC2 are both depleted during HIV-1 infection (13) and had not been specifically studied during HIV-2 infection.

During HIV-1 infection, the intestinal epithelium is durably damaged, allowing microbial product translocation into the circulation and the lymphoid system (26). This translocation has been associated with progression to non-AIDS-related complications like diabetes or cardiovascular diseases (26, 27). Plasma markers of bacterial translocation include lipopolysaccharides (LPS), major components of enteric Gram-negative bacterial outer membrane, which are classically measured in the plasma using the limulus assay but with low sensitivity and high variability. CD14, the main co-receptor for LPS detection, is shed from the surface of classical monocytes as a soluble molecule into the plasma. Microbial translocation and correlation with soluble CD14 (sCD14) levels were found in PLWHIV-1 from the USA, Canada or Finland (11, 26–28). In the SMART study on chronically HIV-1 infected participants, plasma sCD14 levels represented the only biomarker correlated with all-cause mortality, with a relative risk of 6 times in those with the highest quartile of CD14 levels, compared to those with the lowest quartile, after adjustment for CD4 cell counts, HIV-1 RNA levels and other inflammatory markers (29). CD163, a receptor involved in the clearance of hemoglobin, is also cleaved from monocytes or macrophages upon inflammation, leading to increased soluble CD163 (sCD163) plasma concentration. *In vitro*, it is expressed on M2-type monocytes stimulated with LPS and IL-4 or IL-13 (30). In PLWHIV-1, sCD163 plasma levels were found to be higher than in controls, to correlate positively with viral loads as well as with $CD14^+CD16^+$ monocyte counts, and inversely with the expression of CD163 on these cells and with $CD4^+$ T cell counts. Also, sCD163 represents a marker of neurocognitive impairment or atheromatous plaque formation in people living with HIV (17, 31–33). LPS-binding protein (LBP), an acute-phase reactant secreted by epithelial intestinal or hepatic cells in response to inflammation, mediates binding of the Lipid A part of LPS to CD14. Circulating LBP levels correlate positively with plasma sCD14 levels during chronic HIV-1 infection (26, 27).

A recently uncovered particularity during HIV-2 infection is the lack of intestinal mucosa disruption, and the conserved

Abbreviations: cDC, conventional dendritic cells (type 1, cDC1; type 2, cDC2); EFS, Etablissement Français du Sang (French Blood Bank); GeoHD, healthy donors matched for geographical origin with PLWHIV-2 from the LTNP and P groups; HD, healthy donors (the abbreviation in the text is for healthy donors from the blood bank (EFS); LBP, Lipopolysaccharide-binding protein; LTNP, long-term non-progressors; P, progressors; PLWHIV, people living with HIV; WA, West-African.

balance between Th17 cells and regulatory T cells (34). Paradoxically, high levels of plasma sCD14 were found in the Portuguese HIV-2-infected cohort, as well as a significant activation of monocytes and DC, with high levels of HLA-DR and CD86 expression (20), like during HIV-1 infection (35), but LBP levels were similar to those of controls. In the ANRS CO5 HIV-2 cohort ($n = 71$), no elevation in plasma sCD14 was noted compared to healthy donors, but sCD14 levels correlated with IL-6 and high sensitivity C-reactive protein levels, and inversely with $CD4^+$ T cell counts. Moreover, participants with plasma sCD14 levels >1.74 μg/mL had a 3.59 higher risk of disease progression than the others ($p = 0.004$), after adjustment for CD4 counts (36), like HIV-1 infected participants of the SMART study (29). sCD163 plasma levels were shown to decrease after cART in HIV-1-, and less in HIV-2-infected people from the Bissau HIV cohort (37, 38), but these data were not compared to those from healthy controls.

Here, we studied cDC1, cDC2 and monocyte subpopulations including $slan^+$ monocytes, in HIV-2 infected participants from the IMMUNOVIR-2 study part of the ANRS-CO5 HIV-2 cohort, in correlation with viral loads and gut translocation markers. We distinguished long-term non-progressors (LTNP) and non-LTNPs (P, including progressive and intermediate evolutive profiles). Moreover, as PLWHIV-2 from the cohort were living in the Paris area, but originated mostly from West-Africa, we compared them not only to local Blood Bank healthy donors (HD), as usually done in many studies performed on HIV-2 infection, but also to local HD matched for their geographical origin, i.e., originating from West-Africa (GeoHD), in order to control the influence of ethnicity on our data.

RESULTS

Participant Characteristics

All the participants were residents in the Paris area. Twenty-nine participants to the French ANRS-CO5 HIV-2 cohort, adult, asymptomatic and naïve of antiretroviral treatment were included into two groups depending on progression of HIV-2 infection: 19 Long-Term Non-Progressors (LTNP), including 17 from West-Africa, or 10 Non-LTNP (i.e., with progressive infection) (P), including 9 from West-Africa (**Table 1**). Long Term non progression was defined by asymptomatic infection for at least 8 years, with at least three CD4 cell counts or plasma viral load (pVL) measures during the 5 past years, stable CD4 cell counts (CD4) $\geq 500/mm^3$ since at least 5 years without a rapid decrease in the CD4 cell count slope (i.e., >50 cells/year) during the last 3 years). Thirty healthy donors, age- and sex- matched with the $HIV\text{-}2^+$ participants, 16 from the French blood bank [Etablissement Français du Sang (EFS)] (HD), and 10 additionally matched with most $HIV2^+$ participants for their West-African origin (GeoHD), were enrolled as control groups. Demographic and biological characteristics of $HIV\text{-}2^+$ participants and HD are presented in **Table 1**. The HIV-2 P group was on average older (53 years) than the GeoHD group (42 years), a statistical difference not significant when considering only West African participants (**Table 1**). The sex-ratio varied from 0.78 to 1.83 between the groups. As expected, median $CD4^+$ T cell counts were lower in HIV-2 P than in LTNP (in West African participants: 586 vs. 895 cells/μL $p = 0.029$).

Numbers and Percentages of Monocytes During HIV-2 Progressive vs. Non-progressive Infection Compared to Matched Controls From the Blood Bank or From West-African Origin

Monocytes were classified according to their CD14 and CD16 expression (**Supplementary Figure 1**, CD14 vs. CD16 plot) into three subsets: classical monocytes (blue: $CD14^{++}CD16^-$), intermediate monocytes (gray: $CD14^+CD16^+$) and non-classical monocytes (yellow: $CD14^+CD16^{\pm}$), the latter expressing slan (yellow) or not (dark green). First, we compared monocyte

TABLE 1 | Demographic and clinical characteristics of the HIV-2-infected and uninfected donors participating in this study.

	HD	GeoHD	HIV-2 LTNP	HIV-2 P	*p*	Dunn or Mann-Whitney *p*
N	16		19	10	NA	NA
West Africans	*NA*	*10*	*17*	*9*		
Age, years (median, [IQR])	50 [41–55]		50 [40–54]	53 [45–59]	**0.018 (K-W)**	GeoHD vs. P, $p = $ **0.015**
		42 [29–48]	*50 [40–54]*	*53 [44–61]*	*0.069 (K-W)*	*GeoHD vs.* P, $p = 0.06$
Sex (F/M), (ratio)	7/9 (0.78)		11/8 (1.38)	5/5 (1.00)	0.835 (Chi^2)	
		6/4 (1.50)	*11/6 (1.83)*	*5/4 (1.25)*	*0.669 (Chi^2)*	
Geographic origin	NA		2 France	1 France	0.316 (Chi^2)	
		10 West Africa	17 West Africa	9 West Africa		
CD4, cell/μl (median [IQR])	NA	NA	895 [820–1203]	502 [423–858]		P vs. LTNP, $p = $ **0.018**
			895 [823–1187]	*586 [356–936]*		P *vs. LTNP*, $p = $ ***0.029***
Viral load, copies/ml (median [IQR])	NA	NA	<40 [<40–40], $n = 17$	<40 [<40–117], $n = 9$		

HD, healthy donors from French blood bank (EFS); GeoHD, healthy donors of West-African origin; HIV-2 LTNP, long-term non-progressor HIV-2-infected donors; HIV-2 P, non-LTNP HIV-2 infected donors. Italics, West-African origin. NA, not available. IQR, interquartile range. K-W, Kruskall-Wallis. Chi^2, Chi square test. Bold, $p < 0.05$.

subset relative and absolute counts in GeoHD (West-African origin) and HD (blood bank). The percentages and absolute counts of classical monocytes and intermediate monocytes were similar between GeoHD and HD (**Table 2** and **Figures 1A–D**). Surprisingly, the percentages and absolute counts of non-classical monocytes were significantly higher in GeoHD than in HD (2.4 vs. 1.4%, Mann-Whitney test $p = 0.004$ and 54.1 vs. 31.8 cells/μL, $p = 0.0002$, respectively; **Table 2** and **Figures 1E,F**). Slan$^+$ monocyte percentages and absolute counts were significantly higher in GeoHD compared to HD (1.4 vs. 0.77%, $p = 0.005$ and 27.9/μL vs. 13.1/μL, $p = 0.0002$, respectively; **Table 2** and **Figures 1G,H**). Thereafter, cell counts were compared only between participants of West-African origin: we compared GeoHD with HIV-2 LTNP ($n = 17$) and P ($n = 9$) of West-African origin (**Table 2** and **Figure 2**). Classical and intermediate monocyte percentages and counts were similar between these groups (medians for classical monocytes: 14.0 in GeoHD, 12.1 in HIV-2 LTNP and 8.8 cells/μL in HIV-2 P), as well as the non-classical monocyte (54.1 in GeoHD, 61.5 in HIV-2 LTNP and 49.8 cells/μL in HIV-2 P) and the slan-monocyte counts (27.9 in GeoHD, 36.6 in HIV-2 LTNP and 32.8 cells/μL in HIV-2 P). This was surprising because when compared to HD from the local blood bank, as in (20), non-classical monocyte counts were twice as high in HIV-2 LTNP from all origins ($n = 19$; 61.5 vs. 31.8 cells/μL, Dunn's post test $p = 0.01$), and slan$^+$ monocytes too (36.5 vs. 13.1, $p = 0.004$). In HIV-2 LTNP from West-African origin ($n = 17$), non-classical and slan$^+$ cell counts were also significantly higher than in HD. No correlation of non-classical or slan$^+$ monocyte counts was found with either VL or proviral loads (**Supplementary Figure 2**). Thus, non-classical monocytes and among them, slan$^+$ monocyte counts were higher in GeoHD than in HD, but not between PLWHIV-2 and controls matched for geographical origin, indicating that the difference was related to ethnicity rather than to HIV-2 infection.

Numbers and Percentages of Conventional DC During HIV-2 Progressive vs. Non-progressive Infection Compared to Matched Controls From the Blood Bank or of West-African Origin

Conventional DC were characterized according to the preferential expression of the surface markers CD141 (BDCA3) on cDC1 or CD1c (BDCA1) on cDC2, among CD14low, CD16$^-$, HLA-DR$^+$, CD19$^-$ cells (**Supplementary Figure 1**). Percentages among total CD45$^+$ PBMC and absolute counts per μL blood for the two subsets of cDC were compared among the different groups of HIV-2-infected and uninfected individuals.

cDC1

Conventional DC1 percentages were similar between HD and GeoHD (**Table 2**, **Figures 3A,B**) as well as between PLWHIV-2 and GeoHD (**Table 2**, **Figures 3E,F**). Without one outlier participant, cDC1 percentages would have been lower in HIV-2 P than in GeoHD (0.034 vs. 0.068% Dunn's post-test $p = 0.029$, **Figure 3E**), cDC1 absolute counts followed a similar tendency (0.78 vs. 1.66 cells/μL, Dunn's $p = 0.066$, **Figure 3F**).

cDC2

Conventional cDC2 percentages and counts were similar between HD and GeoHD and between PLWHIV-2 and GeoHD (**Table 3**, **Figures 3C,D,G,H**).

TABLE 2 | Monocyte subset percentages among CD45$^+$ PBMC and absolute counts in HIV-2- infected and uninfected donors.

		HD	GeoHD	HIV-2 LTNP	HIV-2 P	Mann-Whitney *p* (HD/ GeoHD)	Kruskal-Wallis *p* (GeoHD/ LTNP/P)
Classical Monocytes	%, Median [IQR]	12.4 [10.8–17.1]		12.1 [8.4–14.2]	9.3 [6.9–11.1]		**0.044**
			14.0 [10.3–14.5]	*12.06 [8.4–14.2]*	*8.8 [6.5–10.0]*	*0.957*	*0.066*
	Cell/μl, Median [IQR]	255 [210–339]		335 [211–464]	234 [115–339]		0.230
			279 [233–354]	*277 [210–394]*	209 [114–352]	*0.655*	*0.334*
Intermediate Monocytes	%, Median [IQR]	0.45 [0.34–0.86]		0.60 [0.44–0.86]	0.49 [0.39–0.90]		0.540
			1.07 [0.52–1.15]	*0.60 [0.42–0.84]*	*0.51 [0.39–0.96*]	*0.240*	*0.299*
	Cell/μl, Median [IQR]	9.14 [6.66–18.91]		17.7 [11.3–25.7]	11.6 [7.7–30.6]		0.629
			20.0 [9.62–27.1]	*17.6 [10.5–24.1]*	*12.2 [8.0–33.4]*	*0.121*	*0.905*
Non-classical Monocytes	%, Median [IQR]	1.4[0.9–1.8]		2.86 [1.1–3.3]	2.0 [1.4–3.8]		0.955
			2.4 [2.0–3.3]	*2.86 [1.1–3.3]*	*2.0 [1.1–4.3]*	***0.004***	*0.937*
	Cell/μl, Median [IQR]	31.8 [17.9–39.6]	*54.1 [43.4–70.5]*	61.5 [26.3–82.4]	49.8 [30.0–118]	***0.0002***	0.820
				61.5 [24.6–85.4]	*49.8 [18.5–94.6]*		*0.961*
Slan+ Monocytes	%, Median [IQR]	0.77 [0.37–1.00]		1.3 [0.7–2.1]	1.3 [0.9–2.1]		0.981
			1.4 [1.0–1.8]	*1.3 [0.6–2.2]*	*1.3 [0.7–2.1]*	***0.005***	*0.943*
	Cell/μl, Median [IQR]	13.1 [7.6–20.1]		36.6 [16.9–52.4]	32.8 [18.6–46.3]		0.813
			27.9 [22.3–38.8]	*36.6 [16.3–54.3]*	*32.8 [10.7–34.6]*	***0.0002***	*0.967*

HD, healthy donors from French blood bank (EFS); GeoHD, healthy donors of West-African origin; HIV-2 LTNP, long-term non-progressor HIV-2-infected donors; HIV-2 P, non-LTNP HIV-2 infected donors. Italics, West-African origin, as in ***Figure 2****. IQR, interquartile range. Bold, p < 0.05.*

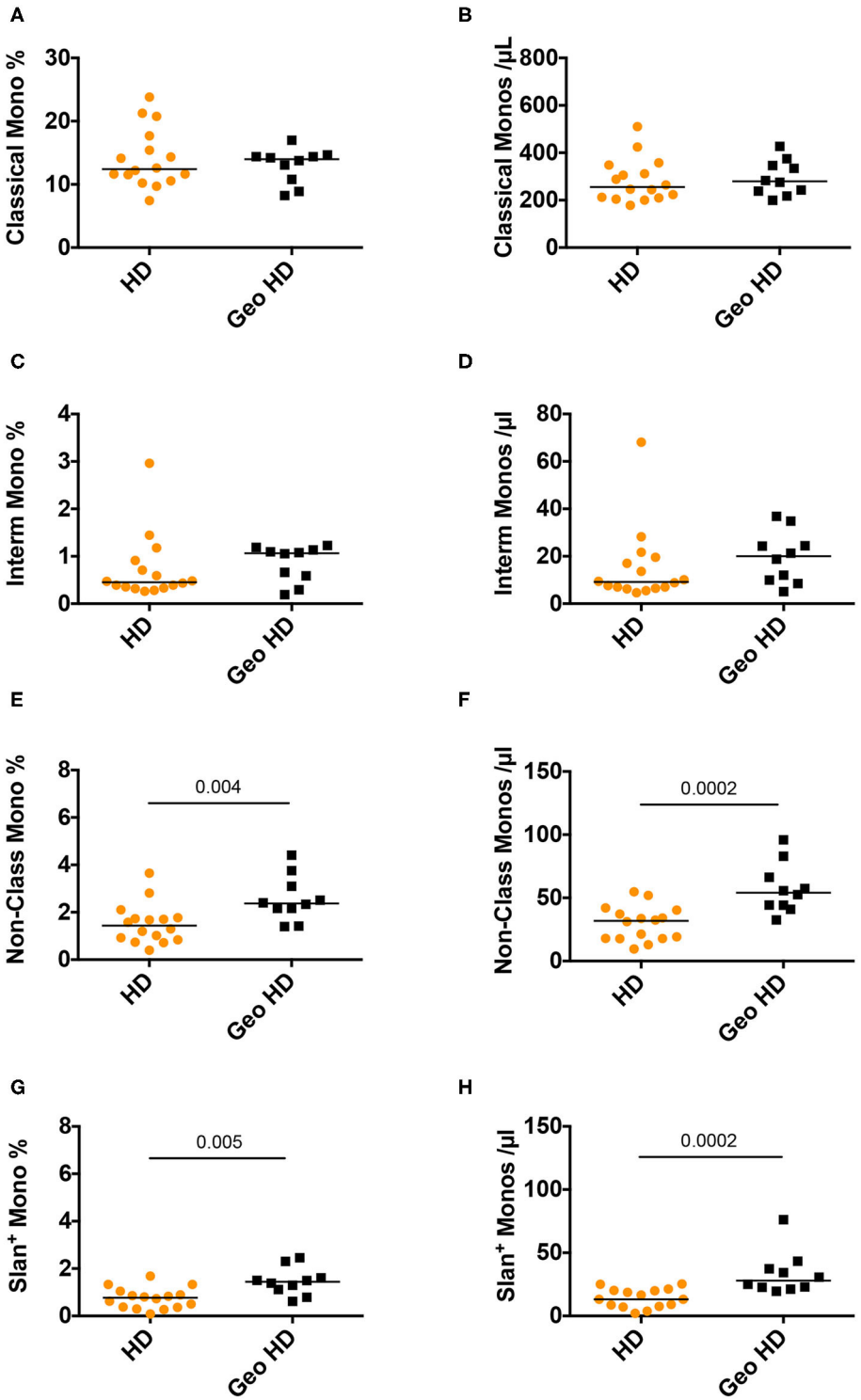

FIGURE 1 | Monocyte subset percentages among CD45$^+$ PBMC and absolute counts in controls from the blood bank (HD) compared to controls of West-African origin (GeoHD). **(A)** Classical monocyte (CD14^{++}CD16$^-$) percentages among CD45$^+$ PBMC and **(B)** absolute counts/µL blood, **(C)** intermediate monocyte CD14$^+$CD16$^+$ percentages and **(D)** absolute counts / µL blood, **(E)** non-classical monocyte (CD14$^{\pm}$CD16$^+$) percentages and **(F)** absolute counts, **(G)** slan$^+$ monocyte percentages and **(H)** absolute counts, in the two groups of age- and sex- matched healthy donors residing in the Paris area, one from the French Blood Bank (EFS) (HD) and the other of West-African origin (GeoHD). Orange: unknown origin, Black: West-African origin. Horizontal bars represent median values. Mann-Whitney's post-test *p* are represented above the horizontal line connecting the compared groups. Kruskall-Wallis tests and descriptive statistics can be found in **Table 2** (in *italics* for West-African participants).

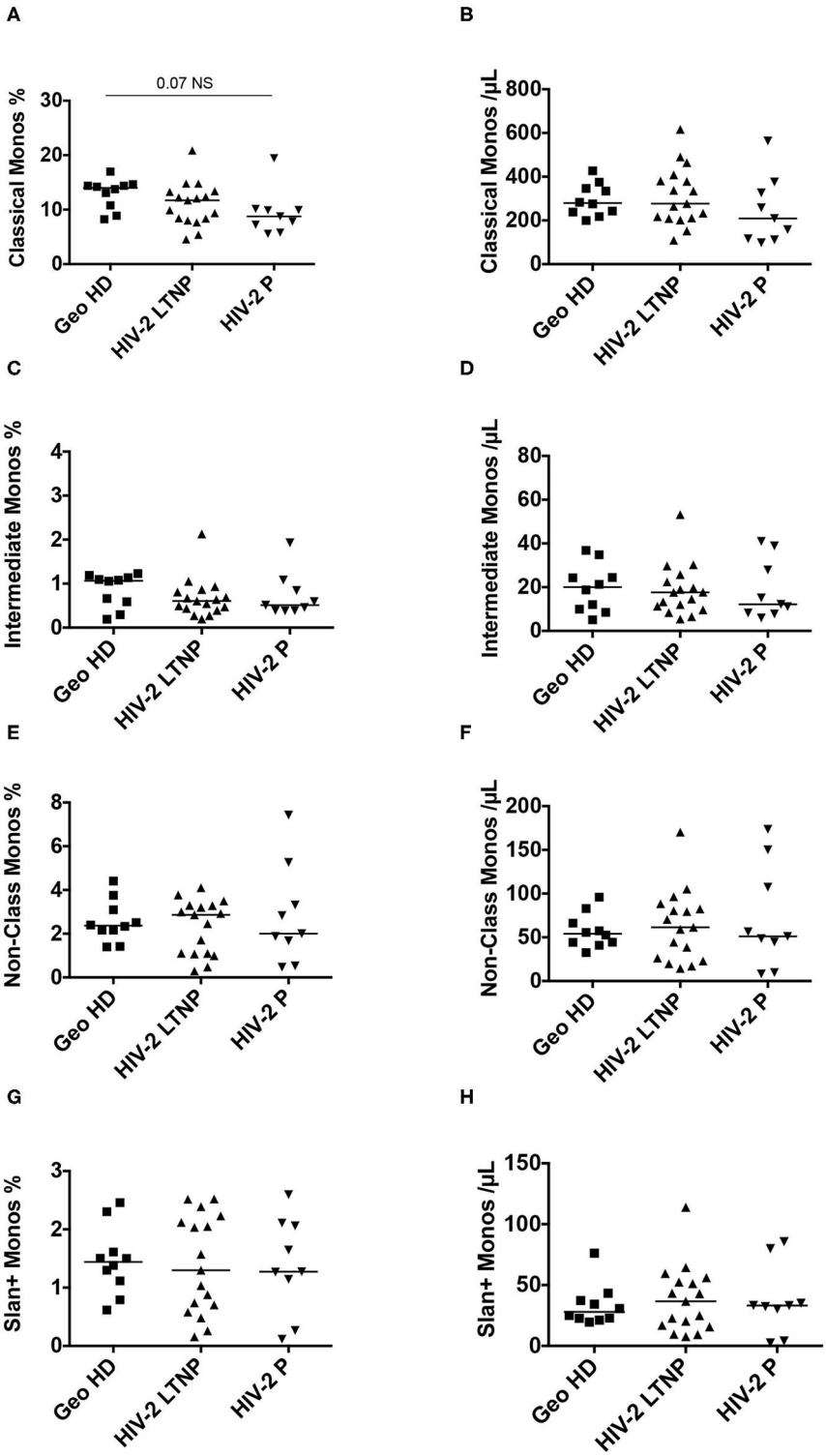

FIGURE 2 | Monocyte subset percentages among $CD45^+$ PBMC and absolute counts in HIV-2-infected participants (LTNP and P) and uninfected controls (GeoHD), all of West African origin. **(A)** Classical monocyte ($CD14^{++}CD16^-$) percentages among $CD45^+$ PBMC and **(B)** absolute counts/µL blood, **(C)** intermediate monocyte $CD14^+CD16^+$ percentages and **(D)** absolute counts/µL blood, **(E)** non-classical monocyte ($CD14^{\pm}CD16^+$) percentages and **(F)** absolute counts, **(G)** $slan^+$ monocyte percentages and **(H)** absolute counts, in GeoHD, HIV-2 LTNP and P groups, all of West-African origin. The data for the GeoHD group are the same as in **Figure 1** Dunn's post-test *p* is represented above the horizontal line connecting the compared groups. Horizontal bars represent median values. Kruskall-Wallis tests and descriptive statistics can be found in **Table 2**, in *italics*.

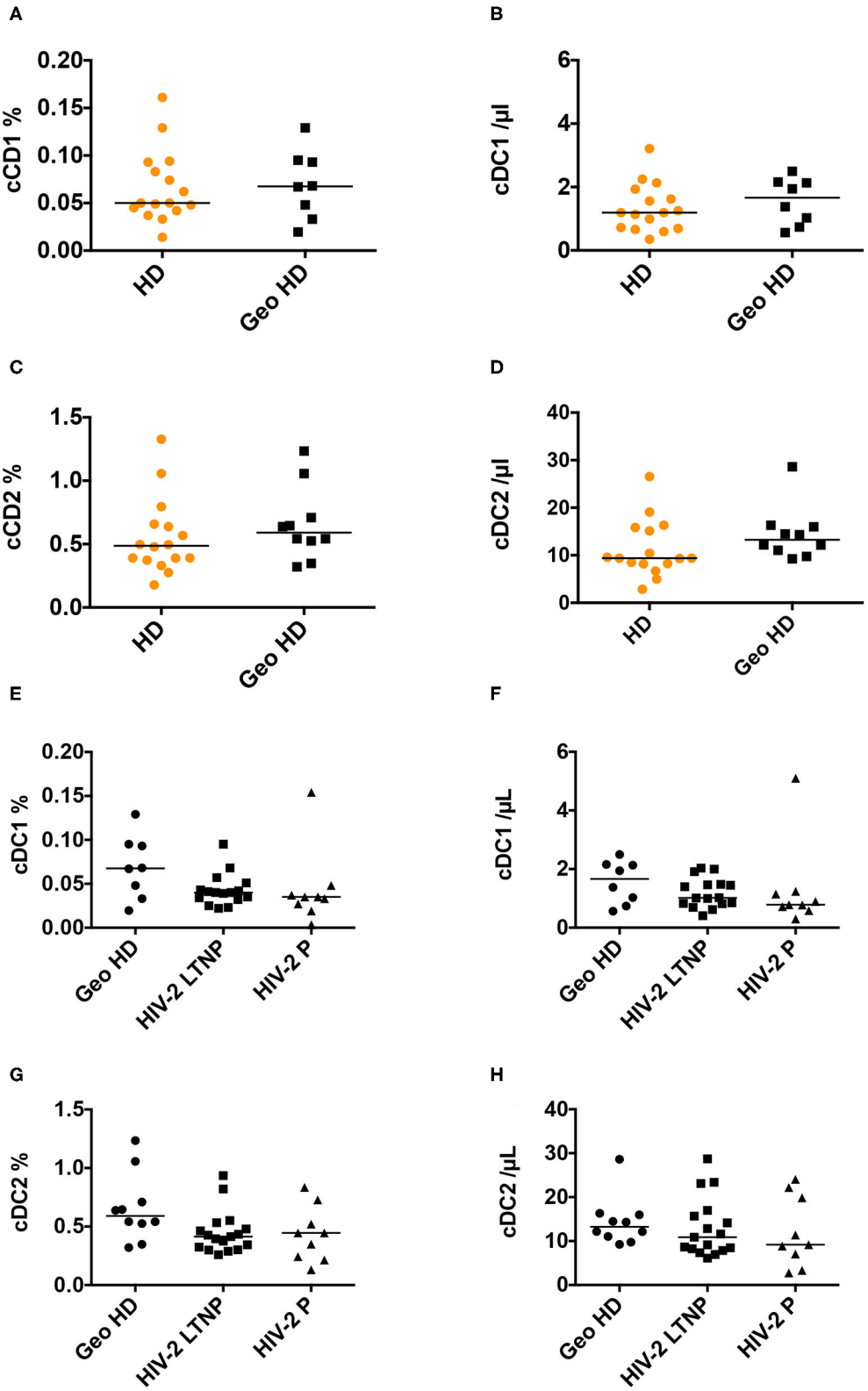

FIGURE 3 | Conventional cDC1 and cDC2 percentages among CD45$^+$ PBMC and absolute counts. **(A)** Conventional DC1 percentages among CD45$^+$ PBMC and **(B)** absolute counts/µL blood, **(C)** cDC2 percentages and **(D)** absolute counts/µL blood, in HD and GeoHD. Orange: unknown origin, Black: West-African origin. **(E)** cDC1 percentages and **(F)** absolute counts, **(G)** cDC2 percentages and **(H)** absolute counts, in GeoHD, HIV-2 LTNP and P, all of West-African origin. Horizontal bars represent median values. Mann-Whitney tests, Kruskall-Wallis tests and descriptive statistics can be found in **Table 3** (in *italics* for West-African participants).

TABLE 3 | Conventional cDC1 and cDC2 percentages among CD45$^+$ PBMC and absolute counts in HIV-2-infected and uninfected donors.

		HD	GeoHD	HIV-2 LTNP	HIV-2 P	Mann-Whitney *p* (HD/GeoHD)	Kruskal-Wallis *p* (GeoHD/LTNP/P)
cDC1	Median [IQR], %	0.050 [0.043–0.091]		0.040 [0.032–0.045]	0.034 [0.025–0.040]		0.060
			0.068 [0.043–0.091]	*0.040 [0.033–0.049]*	*0.035 [0.023–0.042]*	*0.776*	*0.119*
	Median [IQR], cell/μl	1.190 [0.700–1.850]		1.020 [0.786–1.470]	0.777 [0.564–1.170]		0.086
			1.66 [0.81–2.15]	*1.020 [0.820–1.470]*	*0.782 [0.650–1.19]*	*0.444*	*0.213*
cDC2	Median [IQR], %	0.487 [0.377–0.653]		0.414 [0.302–0.533]	0.409 [0.236–0.573]		0.425
			0.590 [0.482–0.797]	*0.414 [0.314–0.507]*	*0.446 [0.229–0.625]*	*0.280*	*0.066*
	Median [IQR], cell/μl	9.38 [8.21–15.68]		10.90 [7.85–16.98]	9.03 [6.11–20.44]		0.702
			13.25 [10.74–16.06]	*10.90 [8.05–16.33]*	*9.19 [5.18–21.02]*	*0.084*	*0.336*

HD, healthy donors from French blood bank (EFS); GeoHD, healthy donors of West-African origin; HIV-2 LTNP, long-term non-progressor HIV-2-infected donors; HIV-2 P, non-LTNP HIV-2. Italics, West-African origin, as in ***Figures 3E–H****. IQR, interquartile range.*

Effect of Sex on the Numeration of Monocytes and DC

As men and women have different innate immune responses (39), and as there were differences in gender ratios between the different groups of donors, we compared between female and male individuals the numerations of these populations where significant differences in myeloid cell counts were noted. **Supplementary Figure 3** shows that there was no difference according to participant's sex for classical monocyte percentages or slan$^+$ monocyte counts, but that the median count of non-classical monocytes was higher in women than in men (53 vs. 38 cells/μL, Mann Whitney's $p = 0.02$), which might bear on the medians obtained for West-African LTNP whose female/male ratio was the highest.

Correlation Between Bacterial Translocation and Myeloid Activation

Plasma markers of bacterial translocation and myeloid activation sCD14, sCD163, and LBP were measured in the plasma from HIV-2-infected people and GeoHD. As shown in **Table 4** and **Figure 4**, there was no difference in the levels of these soluble markers between the groups. In addition, we measured the plasma concentrations of GM-CSF, an inflammatory cytokine produced by myeloid and lymphoid cells. GM-CSF plasma levels can be higher, but moderately, in asymptomatic chronically HIV-1-infected people than in controls (40), and can be high during acute HIV-1 infection, predicting a lower viral setpoint (41). *In vitro*, GM-CSF induces the differentiation of classical monocytes into slan$^+$ monocytes (13). In the plasma from all of the HIV-2 infected donors and African individuals studied here, GM-CSF levels were normal, i.e., below 4 pg/mL, the detection limit of the test (except GeoHD #17 at 6 pg/mL, who had the second highest slan$^+$ -monocyte count in this group at 43 cells/μL).

Correlations between plasmatic markers of bacterial translocation and myeloid cell counts within each group are shown in **Figure 5** for West African participant samples. A positive correlation was observed between intermediate monocyte counts and both sCD14 and LBP plasma levels in all HIV-2-infected donors (LTNP+P) (Spearman correlation $r = 0.45$, $p = 0.039$, and $r = 0.53$, $p = 0.019$, respectively, **Figures 5A,B**) but not in LTNP donors alone. Similarly, LBP levels correlated positively with non-classical monocytes ($r = 0.62$, $p = 0.0047$) and with slan$^+$ monocytes ($r = 0.57$, $p = 0.011$; **Figures 5C,D**) in LTNP + P; this correlation was sustained in LTNP alone ($r = 0.71$, $p = 0.0058$ and $r = 0.77$, $p = 0.0019$; **Figures 5C,D**).

TABLE 4 | Comparison of sCD14, sCD163 and LBP plasma levels in HIV-2 infected participants of West-African origin and GeoHD.

Group		GeoHD	HIV-2 LTNP	HIV-2 P	Kruskal-Wallis *p*
sCD14	ng/mL, Median [IQR]	*949 [847–1,059]*	*1,004 [926–1,124]*	*938 [769–1,020]*	*0.504*
sCD163	ng/mL, Median [IQR]	*280 [155–605]*	*446 [336–603]*	*490 [352–1,010]*	*0.210*
LBP	ng/mL, Median [IQR]	*5,200 [4,185–5,793]*	*5,241 [4,031–5,822]*	*2,734 [1,755–8,385]*	*0.437*

GeoHD, healthy donors; HIV-2 infected donors; HIV-2 LTNP, long-term non-progressor HIV-2-infected donors; HIV-2 P, non-LTNP HIV-2 infected donors. All donors of West-African origin, as in ***Figure 4****. Italics, West-African origin. IQR, interquartile range.*

DISCUSSION

Our work establishes, in untreated HIV-2 infected participants from the IMMUNOVIR-2 study, the blood counts of sub-populations of monocytes or cDC that were shown previously to be affected in HIV-1 infected individuals. PLWHIV-2 from this study lived in the Paris area, and originated mostly from West-Africa like the majority of PLWHIV-2. Therefore, to control for the influence of ethnicity on our data, we compared the counts from PLWHIV-2 of West-African origin not only with those from age- and sex-matched donors from the Blood Bank (HD), but also to those from age- and sex-matched healthy donors of West-African origin (GeoHD). The percentages and

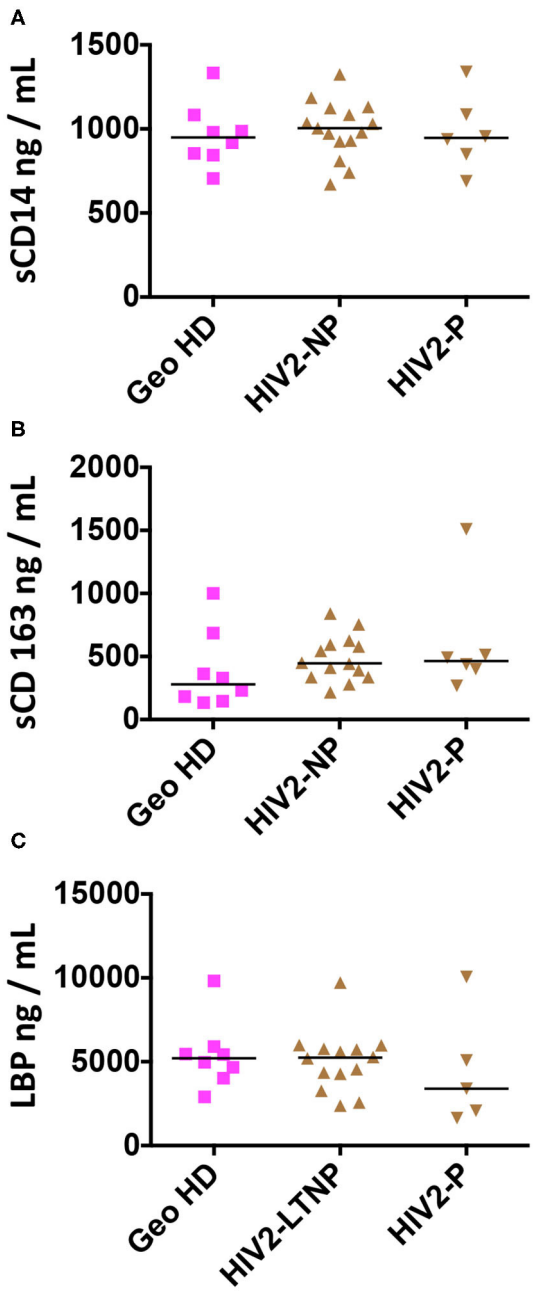

FIGURE 4 | Plasma concentrations of sCD14, sCD163 and LBP in HIV-2-infected participants and uninfected controls (GeoHD), all of West African origin. Plasma concentrations of **(A)** sCD14, **(B)** sCD163, and **(C)** LBP. Horizontal bars represent median values. Kruskall-Wallis test and descriptive statistics can be found in **Table 4**.

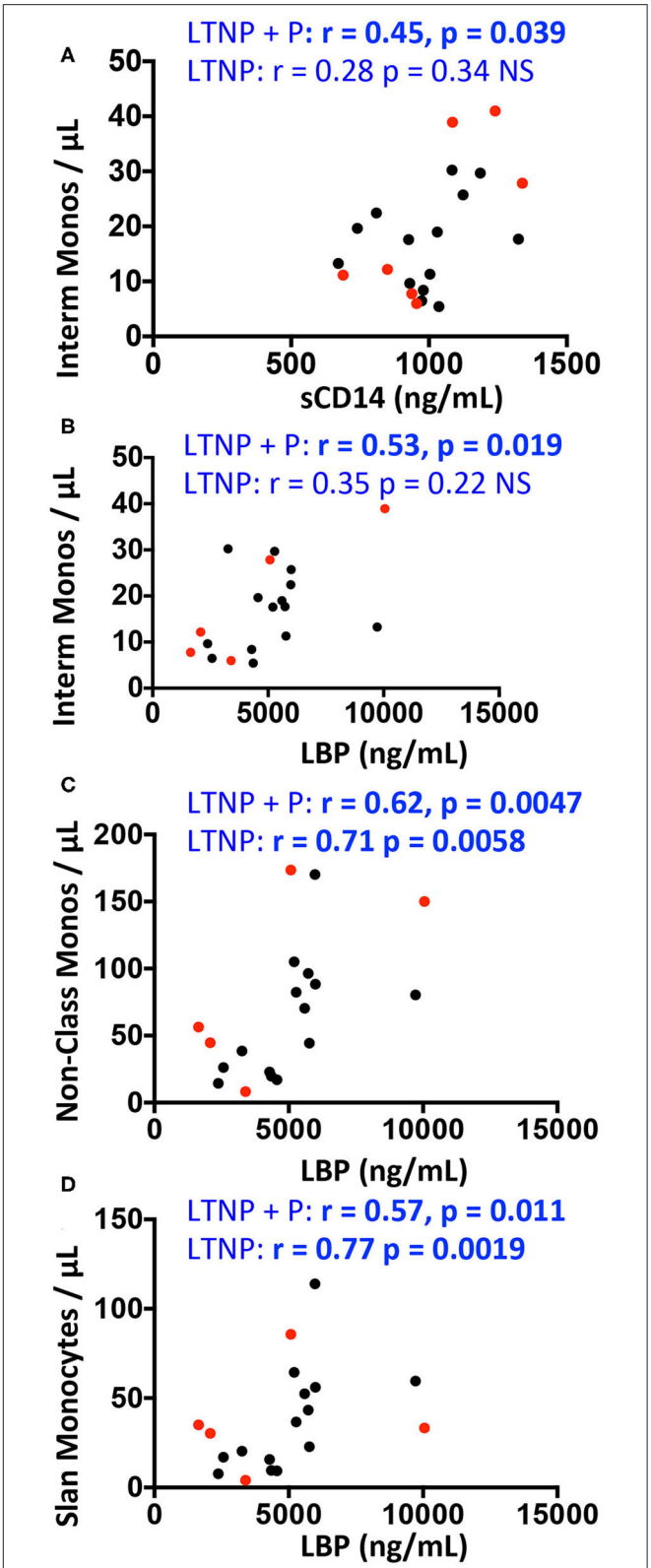

FIGURE 5 | Correlations between monocyte counts and plasma concentrations of sCD14, sCD163, and LBP in HIV-2 infected participants and uninfected controls (GeoHD), all of West-African origin. Data from PLWHIV-2

(Continued)

counts of circulating inflammatory non-classical monocytes, and especially slan$^+$ monocytes, were higher in GeoHD than in HD. Thus, even if the counts of circulating inflammatory non-classical monocytes, and especially slan$^+$ monocytes, were higher in long-term non progressor (LTNP) PLWHIV-2 than in HD, they were not significantly different from those of GeoHD. In the first

FIGURE 5 | from **Figure 2** were correlated to data from **Figure 4** by Spearman's test. The significant correlations are depicted. **(A)** Intermediate monocytes/μL vs. sCD14 plasma concentration. **(B–D)** Intermediate, non-classical or slan monocytes/μL vs. LBP. Correlations are given for all PLWHIV-2 (LTNP + P) or for LTNP alone. Progressor PLWHIV-2 (P) are indicated in red.

study on myeloid cells performed during HIV-2 infection in Portugal, high levels of $CD14^{bright}CD16^{+}$ monocytes had been found during HIV-2 chronic infection compared to controls from the local blood bank in Lisbon, Portugal (20). These events corresponded to the intermediate monocytes and to part of the non-classical monocytes measured in our work. The female/male sex ratios were lower in our cohort than in the Portuguese cohort (1.83 or 1.25, respectively, vs. 2.22), but our global comparison of non-classical monocyte counts showing higher counts in females than in males did not contradict the conclusions of both studies. Thus, in the present study, HIV-2 did not have any impact on monocyte subpopulations when infected people were compared to geographically-matched controls. For conventional DC (cDC1 and cDC2) populations, measured for the first time in HIV-2-infected people, no statistical difference was found between GeoHD and HD, neither between PLWHIV-2 and GeoHD. In the Portuguese cohort, compared to local blood bank donors, low counts of myeloid DC, defined as (CD16, CD14, CD3, and CD20)-negative, CD123-negative, $HLA\text{-}DR^{+}$, $CD11c^{+}$ cells, had been found, and they correlated with viral loads and with plasma sCD14 levels. The ages of the participants were comparable (50 or 53 vs. 52 years), and our global cDC1% did not show any gender-related difference. Compared to the Portuguese cohort, the IMMUNOVIR-2 cohort had a similar proportion of participants with undetectable HIV-2 viremia (25/29 vs. 21/25), but with lower thresholds (40 or 100 vs. 200 copies/mL), lower maximal VL at sampling (117 vs. 26,263 copies/mL) and higher CD4 counts [LTNP: 895 CD4 T cells/μL, P: 502, number of participants with CD4 < 350/μL: 2, vs. in the Portuguese cohort 538 cells/μL and 1/3 of the participants with CD4 <350/μL (20)]. Thus, the MMUNOVIR-2 cohort had a lower global progression than the Portuguese cohort, which may explain the absence of defect in DC population numbers, even though they were defined more precisely.

As we had found high plasma sCD14 only in HIV-1 infected individuals with high viremia (13), and as HIV-2-infected participants from the IMMUNOVIR-2 study had undetectable to low viremia, we were not surprised that they had normal translocation markers compared to GeoHD. Neither DC or monocytic population counts nor plasmatic markers of bacterial translocation were correlated in this study to viral loads, proviral loads or to progressor status. This is comparable to the results previously obtained in the larger ANRS CO5 cohort, where no differences in sCD14 plasma levels were found between progressors and non-progressors, although sCD14 levels were predictive of progression (36). Differently from the HIV-2 infected Portuguese cohort (20), we did not find any correlations between plasma bacterial translocation markers and DC counts. However, we found correlations between inflammatory monocytes expressing surface CD16 (intermediate, non-classical or $slan^{+}$ monocytes) and plasmatic markers of bacterial translocation, i.e., LBP or sCD14 levels, like in former studies in PLWHIV-1 (11). We showed previously that $CD14^{bright}$ classical monocytes can differentiate *in vitro* into $slan^{+}$ monocytes in the presence of M-CSF and GM-CSF (13), the latter occasionally found to be detectable in the plasma during acute or chronic HIV-1 infection (40, 41). However, in the plasma from all of the HIV-2 infected participants studied here, GM-CSF levels were undetectable. Similarly, this cytokine remained undetectable in the plasma of all but one GeoHD. The distinction between P and LTNP is rarely made in HIV-2-infected cohorts despite its potential interest. The correlations between inflammatory monocyte counts and plasma LBP levels as a sign of microbial translocation found in all HIV-2 infected donors (LTNP + P) were sustained in LTNP alone, and not in P alone. This is counter-intuitive, as microbial translocation and plasmatic inflammation markers are known to increase with age and with HIV-1 infection independently (28). This may be due to the low number of progressor participants, as some of them were in fact non-LTNP, with a relatively low level of HIV VL compared to other cohorts, as discussed above. Alternatively, a protective role might be attributed to inflammatory monocytes (defining here $CD16^{+}$ monocytes) against the progression of HIV-2 infection toward AIDS, i.e., in terms of viral loads or CD4 T cell counts. Indeed, these cells have antibody-dependent cellular phagocytic and cytotoxic effector functions (42). Therefore it is possible that despite apparent integrity of the intestinal mucosa (34), microbial translocation may occur more often in HIV-2 infected individuals than in healthy donors, inducing stimulation of classical monocytes by cytokines locally (without systemic dissemination of GM-CSF), sCD14 shedding, and CD16 and slan surface expression. Thus, circulating inflammatory monocyte counts may be a sensitive sign for intestinal mucosa intermittent leakage.

One very interesting lesson from this study is the comparison of the two control groups. The counts of circulating inflammatory non-classical monocytes, and especially $slan^{+}$ monocytes, were significantly higher in the GeoHD group originating from West-Africa than in the HD group. The HD control group matched for sex and age with HIV-2 infected individuals was recruited at a local blood bank, as usual in many HIV-2 infection studies. French legal rules require that blood banks do not collect any data related to ethnicity. However, the origins of the population in the Paris area are very diverse. Here, for the IMMUNOVIR-2 participants and after specific approval from the ethical board, we collected data on birth country, nationality (original or acquired) and the country where the contamination occurred. A similar effort was done in the Portuguese cohort by the classification into "black" or "white" individuals, which did not give rise to any difference between the two groups for plasma LPS or for any of the data levels (20). In other studies, higher percentages of $CD14^{+}CD16^{+}$ monocytes and lower percentages of $CD14^{+}CD16^{-}$ monocytes were found in Africans than in Caucasians, perhaps in relation with former exposure to pathogens such as *Plasmodium falciparum*

and *Schistosoma haematobium* (43). Higher frequencies of $CD14^+CD16^+$ "proinflammatory" monocytes, with higher expression of HLA-DR and PD-L1, were also found in a cohort of 50 healthy volunteer from Entebbe, Uganda than in a cohort of 50 healthy volunteers of similar ages from Lausanne, Switzerland (44). This difference was not related to gender differences. It was accompanied by higher $CD16^+HLA\text{-}DR^+$ "exhausted" NK cell frequencies, higher effector memory $CD4^+$ and $CD8^+$ T cell frequencies, and a more activated B cell compartment. Importantly, proinflammatory monocyte frequencies correlated negatively with these volunteer's neutralizing antibody responses to a live virus vaccine, the licensed yellow fever vaccine 17D (YF-17D). Other reports on adaptive or innate immunity during HIV-2 infection were performed in West Africa, with local controls (4, 45, 46). Comparisons of innate immunity parameters during HIV-1 infection were also performed between different countries. For instance, significantly higher plasma sCD14 levels were found in cART-naive HIV-1-infected donors from Mexico compared to similar donors from South Africa, along with other differences in inflammatory biomarkers (47). Untreated, PLWHIV-1 from Entebbe, Uganda, were found to have low $CD11c^+$ and $CD11c^-$ DC counts like PLWHIV-1 from the UK, compared to non-African laboratory workers from the UK (48). These cohorts recruited before the cART era had high viral loads and low circulating CD4 T cell counts. It was claimed that in Africa, baseline sCD14 plasma levels were lower, and LBP similar, in 86 HIV-uninfected donors from Uganda (who seroconverted and were studied longitudinally) compared to HIV-uninfected donors from the US (median 53 years old, 62.5% African-Americans, 50% female), and that they did not change significantly during HIV-1 infection (49). This was contested by other studies finding microbial translocation during HIV-1 infection in Kenya (50) or in India (51).

Origin differences, whether between countries but also between socio-economical groups, may have an impact on innate immunity baseline and pathological parameters through many factors. (1) Gender induces differences depending either on gene products or on hormones (39). (2) Aging clearly has an impact on microbial translocation and inflammation (28). (3) Coinfections with opportunistic or other pathogens, hygiene, or microbial and climatic environment and (4) Medications (antibiotics, anticancer drugs, immune suppressors, and proton pump inhibitors...) may also induce microbial translocation. (5) Food and substance intake, particularly alcohol intake (52) and high fat diet, are known to have a direct impact on microbial translocation and on the emergence of inflammation and metabolic syndrome, as shown experimentally in non-human primate SIV infection models (53). (6) All these factors may also affect the development of the immune system during early life, including fetal life, with differential expansion and polarization of adaptive and innate immune cell populations, compartment seeding and epigenetic imprinting. (7) Even epigenetic imprinting from previous generations may influence the innate immune response.

Our study has several strengths including (1) the choice of a cohort of donors from the same geographical origin as most PLWHIV-2, i.e., West Africa, and its comparison to a state-of-the-art control cohort from the local Blood Bank, as usually done in non-West African studies; (2) the identification of long-term non progressors compared to progressors in the HIV-2 infected cohort; (3) the homogeneity of the HIV-2 cohort, all asymptomatic, tested before any antiretroviral treatment, and all without opportunistic infections like tuberculosis, which could have confounded inflammatory parameters (47); (4) the precise delineation of monocyte and cDC subpopulations yet unstudied during HIV-2 infection, (5) the correlation of their frequencies with plasmatic microbial translocation markers. Inversely, the limitations are the low numbers of the donors, particularly from the same sex, due to the changing recommendations of treatment during the inclusion period (2013–2015, protracted until the end of 2018), and the legal impossibility to know the origin of the healthy donors from the Parisian Blood Bank.

In conclusion, this study underlines that the choice of specific controls, although never perfect, helps refine studies in specific populations. It shows higher counts of inflammatory monocytes, and especially $slan^+$ monocytes, in healthy donors of West-African origin living in the Paris area, than in healthy donors from the local blood bank. In HIV-2-infected long term non progressor participants, and not in controls, these inflammatory monocyte counts correlated with plasmatic markers of microbial translocation, indicating a potential innate immune response to subclinical bacterial translocation, and therefore a potentially sensitive and early marker for inflammation linked to HIV-2 infection.

MATERIALS AND METHODS

Participant Blood Samples

Blood samples (**Table 1**) were collected from participants who reside in the Paris area. Twenty-nine participants were part of the ANRS IMMUNOVIR-2 study (part of the French ANRS HIV-2 CO5 cohort). These participants were adult, asyptomatic, treatment-naive individuals infected with HIV-2 alone. Nineteen were non-progressors (LTNP; 11 females, eight males, median age 50 years, range 21–66 years), with asymptomatic HIV-2 infection ≥8 years, with at least three CD4 cell counts or plasma viral load (pVL) measures during the 5 past years, stable CD4 cell counts (CD4) $\geq 500/mm^3$ since at least 5 years without a rapid decrase in the CD4 cell count slope (i.e., >50 cells/year) during the last 3 years). Among LTNP, 17 originated from West-Africa, i.e., either Ivory Coast, Republic of Guinea, Guinea Bissau, the Gambia, Ghana or Senegal. Ten were non-LTNP (P) (five females, five males, median age 54 years, range 41–70 years), also treatment-naive, except for mother-to-child HIV transmission preventive treatment. Among P, 9 originated from West-Africa. Exclusion criteria were: Ongoing opportunistic infection or malignant disease. Moreover, blood samples were collected from 30 age- and sex-matched healthy controls, seronegative for HIV-1 and HIV-2. Ten of these controls (GeoHD; six females, four males, median age 42 years, range 29–48 years) originated from a similar geographic area as most PLWHIV-2, i.e., West-Africa, based on the collection of the following data: birth country, nationality, presumed country where contamination occurred.

Sixteen other controls were also recruited by the blood bank (EFS-Saint-Antoine-Crozatier) and analyzed as controls, within an ethical convention with Inserm (seven females, nine males, median age 46 years). Exclusion criteria for all groups were: Age <18 years, Hb < 10g/dL in the past blood check less than a month before inclusion, known evolutive neoplasia, juridic incapacity. The study was approved by the ethics committee Comité de Protection des Personnes Ile de France XI. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Plasma were collected and kept frozen. Blood counts were performed by Coulter counting. Peripheral blood mononuclear cells were isolated by Ficoll density gradient and labeled immediately.

Flow Cytometry

Peripheral blood mononuclear cells were washed with cold PBS, stained with LiveDead (Life Technologies, 30 mn on ice), before adding 5% human serum (Sigma, 15 min), washed with Staining buffer (PBS, EDTA 2 mM, BSA 0.5%) and labeled in Staining buffer (20 min on ice), then washed, fixed in PBS 4% Paraformaldehyde. The following monoclonal antibodies were used: CD3-QDot605 (clone UCHT1 1/150) from Invitrogen; M-DC8-FITC (DD-1, 1/20), CD141(BDCA-3)-APC (AD5-14H12, 1/150) and CD303(BDCA-2)-PE (AC144, 1/10) from Miltenyi Biotec; CD1c(BDCA-1)-Pacific Blue (L161, 1/400; Biolegend); CD14-QDot655 (TüK4, 1/100; Invitrogen), CD19-ECD (J3-119, 1/10; Beckman Coulter), CD11c-AlexaFluor700 (3.9, 1/10; eBioscience); HLA-DR-PerCP (G46-6, 1/10), CD16-APC-H7 (3G8, 1/40), CD56-PE-Cy7 (NCAM 16.2 1/100), and CD45-Amcyan (2D1, 1/25) from BD Biosciences. Cells were washed with permeabilization buffer before flow cytometry and analysis (LSR II, BD; FlowJo v10.1, TreeStar, USA). Data acquisition and analysis were performed at the Cochin Cytometry and Immunobiology Facility. The absolute numbers of cells per blood microliter were calculated as before (13, 14, 21, 54, 55) by multiplying the Coulter blood count (performed independently on whole blood collected during the same blood sampling) of mononuclear cells (monocytes + lymphocytes), expressed as cells/μL, to the ratio [events for the population of interest/(lymphocyte + monocyte) events], expressed as a percentage, from flow cytometric event counts (**Supplementary Figure 1**).

Multiplex ELISA

sCD14, sCD163 LBP, and GM-CSF concentrations were measured in duplicates in the plasma using Human magnetic Luminex assays (Biotechne, R&D, Lille, France) at the Cochin Cytometry and Immunobiology Facility.

Statistics

The data from the different groups of donors were analyzed using the Kruskall-Wallis test with Dunn's post-tests correction for multiple testing, and the Mann-Whitney test when there were only two groups to compare. Chi square test was used to analyse proportions of female vs. male participants. Correlations were analyzed by Spearman tests. Differences were defined as statistically significant when $p < 0.05$. The Graphpad Prism v6.10 for Mac OS X was used.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Protection des Personnes Ile de France XI. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH, MI, JM, RC, and the IMMUNOVIR-2 group contributed to the conception and design of the study. NC and SM organized the database. MI, SI, MN, and AH performed the FlowJo and statistical analysis and drew the figures. MI and AH wrote the manuscript. MI, SI, JM, J-BG, KB, MA, SA, LVa, EH, SF, LVi, AH, and BC performed the labeling and FACS acquisition. KB performed the Luminex assays. SM is the PI of the ANRS-CO5 cohort. All authors contributed to IMMUNOVIR-2 brainstorming, manuscript revision, and they read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01658/full#supplementary-material

Supplementary Figure 1 | FACS gating strategy for DC and monocyte enumeration.

Supplementary Figure 2 | Correlations between monocyte counts and proviral loads.

Supplementary Figure 3 | Effect of sex on cell numeration.

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