Plasmacytoid Dendritic Cell Number and Responses to Toll-Like Receptor 7 and 9 Agonists Vary in HIV Type 1-Infected Individuals in Relation to Clinical State

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Abstract

In HIV-1 infection, plasmacytoid dendritic cell (PDC) numbers and function are decreased. No detailed comparisons of PDC responses to various stimuli in HIV-1-infected patients are available. Using for the first time purified PDCs, we compared PDC responses [interferon (IFN)- α production/cell] to various stimuli in a large number ($n=48$) of HIV-1-infected patients and healthy volunteers ($n=19$). Toll-like receptor (TLR)7- and TLR9-induced expression of PDC surface activation and maturation markers was also compared in the two populations. We have confirmed that PDC number coincides with CD4⁺ T cell counts and clinical state. Notably, we have shown that a direct association of PDC function in terms of IFN- α production/cell exists with PDC numbers and CD4⁺ cell counts when PDCs are exposed to a TLR9 ligand and HIV-infected cells, but not with a TLR7 ligand. Moreover, in the HIV-infected subjects but not the healthy controls, the magnitude of IFN- α release per PDC in response to the TLR7 ligand is significantly ($p<0.01$) lower than that to the TLR9 ligand. However, in both study populations, the TLR7 stimulation in comparison to TLR9 stimulation induced higher expression of PDC surface activation and maturation markers and significantly ($p<0.05$) decreased the expression of BDCA-2, a negative regulator of interferon. Furthermore, the cross-ligation of BDCA-2 significantly ($p<0.05$) inhibited TLR9- but not TLR7-induced IFN- α production by PDCs from both clinical groups. These findings suggest that differences exist in TLR7- and TLR9-induced IFN- α production by PDCs in HIV-infected individuals that are not directly related to BDCA-2 down-modulation.

Introduction

PLASMACYTOID DENDRITIC CELLS (PDCs) are the major type 1 interferon (IFN- α)-producing subset of hematopoietic cells. These cells rapidly produce IFN- α in response to virus infections such as HIV¹⁻⁴ reflecting constitutive expression of IFN regulatory factor (IRF-7).^{5,6} PDCs recognize pathogens via two Toll-like receptors (TLRs), TLR7 and TLR9.^{3,5} TLR7 senses single-stranded (ss) viral RNA as well as synthetic imidazoquinolines such as imiquimod, whereas TLR9 recognizes unmethylated CpG in viral or bacterial DNA or synthetic CpG oligodeoxynucleotides (ODNs).^{7,8}

TLR7 or TLR9 triggering, in addition to the production of IFN- α , activates the nuclear factor-kappa B (NF- κ B),^{5,9} which in turn enhances inflammatory cytokine responses and induces the up-regulation of dendritic cell activation and maturation markers (CD80, CD86, and CD83) and the chemokine receptor CCR7. The latter promotes PDC migration to the

secondary lymphoid organs.¹⁰ Hence, TLR triggering can switch the immature DC phenotype to an inflammatory (activated) DC that is capable of instructing the adaptive immune responses.^{4,5,9}

PDCs appear to play an important role in limiting HIV infection. Following exposure to HIV-1, they produce type 1 interferons that inhibit viral replication.¹¹⁻¹³ During advanced HIV-1 infection, a reduced blood PDC frequency correlates with high viral load, decreased CD4⁺ cell counts, and susceptibility to opportunistic infections.^{14,15} Along with their quantitative loss during HIV-1 infection, PDCs display phenotypic abnormalities such as an increased expression of the HIV-1 coreceptors, CXCR4 and CCR5, as well as costimulatory molecules, CD86 and CD40.¹⁶⁻¹⁸ PDCs from HIV-1-infected individuals also show functional abnormalities such as an impaired ability to stimulate T-lymphocyte proliferation.¹⁹ Diminished IFN- α production, presumably by PDCs, has also been observed when peripheral blood mononuclear cells

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(PBMCs) from HIV-infected people are exposed *in vitro* to TLR7 and TLR9 ligands or viruses such as HIV-1.^{15,20–22}

Induction of IFN- α by PDCs by various stimuli is directly related to the extent of down-regulation of the C-type lectin surface receptor BDCA-2 expressed on PDCs.²³ Thus, cross-linking or ligation of BDCA-2 with anti-BDCA-2 monoclonal antibodies (mAbs) has been reported to suppress IFN- α production by PDCs via TLR9 ligands such as HSV and CpG-ODNs^{23–26} and to a much lesser extent via a TLR7 ligand such as influenza virus.²³

Despite mounting evidence on reduced PDC number and function in HIV-1 infection, no detailed comparisons of the PDC responses to the various stimuli have been made. Moreover, whether the finding of reduced PDC function is observed with purified PDCs from HIV-infected individuals is not known. The current studies of HIV-infected individuals show differences in the responsiveness of purified PDCs to *in vitro* stimulation with TLR7 and TLR9 ligands as well as HIV-1. The data also suggest that BDCA-2 does not play a role in TLR7 induction of the IFN- α response by PDCs.

Materials and Methods

Patient characteristics

Forty-eight HIV-1-infected subjects (45 male, 3 female) were studied at different clinical states from the cohort in our laboratory at University of California, San Francisco (UCSF). The details of measurements such as CD4⁺ T cell counts, PDC numbers, and viral load [HIV-1 RNA (copies/ml)] for the studied HIV cohort are included in Table 1. Nine patients included in the study had CD4⁺ T cell counts below 200/ μ l and 24 of these subjects were on combination antiretroviral therapy (ART) consisting of anti-reverse transcriptase and anti-protease inhibitor drugs. The majority of patients ($n=20$) receiving ART had undetectable HIV-1 RNA levels (<75 copies/ml).

The 19 uninfected subjects (12 male, 7 female) were either volunteers from UCSF or blood donors at Blood Centers of the Pacific (San Francisco, CA). This study was approved by the Committee on Human Subjects at UCSF and written informed consent was obtained from the study participants.

Determination of PDC counts

Whole blood PDC determinations were performed on EDTA-anticoagulated blood samples within 4 h of collection. Briefly, aliquots of whole blood were preincubated with normal mouse IgG (CalTag Labs, Burlingame, CA) at a concentration of 50 μ g/ml to reduce nonspecific staining.

TABLE 1. DEMOGRAPHIC DETAILS OF THE HIV-INFECTED INDIVIDUALS INCLUDED IN THE STUDY

Number of subjects	48
Age; mean (range)	52 (34–72)
CD4 ⁺ T cell counts (cells/ μ l); mean (range)	503 (18–971)
Log ₁₀ HIV-1 RNA (copies/ml); mean (range)	2.6 $\times 10^4$ (<75–40.5 $\times 10^4$)
PDC counts (cells/ μ l); mean (range)	6.5 (0.9–19.3)

PDC, plasmacytoid dendritic cell.

Immunophenotyping was performed as described earlier²⁷ using a cocktail of FITC-conjugated lineage marker (CD3, CD14, CD16, CD20), CD4-PE (Becton Dickinson, San Jose, CA), and CD11c-APC (CalTag Labs, Burlingame, CA). A total of 100,000 peripheral blood mononuclear cells (PBMCs) were acquired and three-color flow cytometric acquisitions were performed on a FACSort (Becton Dickinson, San Jose, CA) and analyzed with CellQuest software (Becton Dickinson, San Jose, CA). PDCs were identified as lineage-negative and CD4⁺ cells without expression of CD11c.

PDC isolation and coculture

The PBMCs were obtained from heparin anticoagulated blood of HIV-1-infected patients or uninfected subjects using Ficoll-Hypaque gradients (Sigma Diagnostics Inc., St. Louis, MO). PDCs were then purified from the freshly isolated PBMCs using a BDCA-4 Cell Isolation Kit (Miltenyi Biotec, Auburn, CA).²⁸ Cross-linking of BDCA-4 has been reported to reduce PDC function.^{24,29} We have compared positive and negative PDC isolation kits and only detected a slight reduction in IFN- α production when PDCs were purified using positive selection (data not shown). After two steps with LS and MS columns, these cells were >95% pure as determined by flow cytometry.¹² The isolated PDCs were cultivated in RPMI 1640 medium containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin, supplemented with 20 ng/ml of interleukin (IL)-3 (R&D Systems, Minneapolis, MN).

The isolated PDCs were plated in 96-well flat-bottom plates at a density of 10⁴ cells/well in duplicate. They were then exposed to the TLR9 agonist CpG-A (ODN 2336; Coley Pharmaceutical Group, Wellesley, MA) or the TLR7 agonist S-27609 (3M Pharmaceuticals, St. Paul, MN). The optimal stimulatory concentrations of 1 and 5 μ M for the TLR9 and TLR7 agonists, respectively, as used in this study were obtained by prior titration studies of the two agonists (data not shown). In some experiments, an additional TLR9 agonist, HSV-1 (UV-irradiated), was used at 10⁶ PFU/ml concentrations. For *in vitro* PDC stimulation with HIV-1, phytohemagglutinin (PHA)-activated CD4⁺ T cells were acutely infected with HIV-1_{SE33} and cultured for 2 days. They were then cocultured (6 $\times 10^4$ cells) with PDCs as described previously.¹²

BDCA-2 cross-linking studies

The amount of anti-BDCA-2 mAb needed to suppress induction of IFN- α/β production has been reported. Titration studies of the anti-BDCA-2 (AC144) mAb (Miltenyi Biotec, Auburn, CA) have shown that concentrations of 78 ng/ml are sufficient for a 50% inhibition of the IFN- α response by PDCs.²³ An effective concentration of anti-BDCA-2 (AC144) and matched isotype IgG1 mAbs (Miltenyi Biotec, Auburn, CA) for a 60% inhibition of the IFN- α response was determined to be 250 ng/ml in our titration studies (data not shown). Therefore, in cross-linking experiments, PDCs were incubated for 30 min with anti-BDCA-2 (AC144) or matched isotype IgG1 mAbs at a 250 ng/ml concentration prior to addition of stimuli as described.³⁰

Cytokine assay

Supernatants of PDC cultures were harvested after 24 h and analyzed for total IFN- α activity using a sandwich

enzyme-linked immunosorbent assay (ELISA) utilizing IFN- α commercial kits (PBL Interferon Source, Piscataway, NJ) according to the manufacturer's instructions. In some cases, samples were analyzed in parallel with an IFN- α Multi-subtype ELISA kit from the same manufacturer as this kit measures IFN- α subtypes such as IFN- α B2, IFN- α H, and IFN- α I that are not quantified by the IFN- α A kit. All samples were diluted 1:1 with the dilution buffer provided in the ELISA kits and evaluated in duplicate. The results are expressed as IFN- α production per 10^4 PDCs.

Studies for expression of PDC surface markers

For PDC surface marker studies, an aliquot of PBMC was used to isolate PDCs for IFN- α production and the remaining PBMCs were plated at a density of 10^6 cells/well in 24-well flat bottom plates. Both PDC and PBMC cultures were stimulated with TLR7 and TLR9 agonists at optimal concentrations for 24 h. Supernatants of PDC cultures were harvested and analyzed for total IFN- α activity as described earlier. The stimulated PBMCs were collected, washed, and stained for markers of activation (CD80 and CD86), maturation (CD83), and migration (CCR-7). For this study, PDCs were identified as cells positive for BDCA-4 and negative for CD11c and CD14. All the monoclonal antibodies used were obtained from BD Biosciences, San Jose, CA except for BDCA-2 and BDCA-4 (Miltenyi Biotec). A total of 100,000 PBMCs were acquired and flow cytometry was performed on a FACSsort with CellQuest Software (BD Biosciences).

Statistical analysis

The Mann-Whitney test was utilized for comparisons between two independent groups and the Wilcoxon signed rank test was used for comparisons within subjects from the same clinical group. Correlations between continuous variables were described using the Spearman rank correlation coefficient. One-way ANOVA was used to compare means of groups within subjects from the same clinical population. All statistical analyses assumed a two-sided significance at p values ≤ 0.05 . Data analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Results

Correlation of PDC numbers with CD4⁺ T cell counts and viral load

In our study of 48 HIV-1-infected individuals at different clinical states, we observed that the PDC numbers correlate directly with absolute CD4⁺ T cell counts and percentages ($r=0.55$; $p<0.001$ and $r=0.36$; $p=0.01$, respectively) (Fig. 1A). PDC numbers correlated inversely with HIV-1 viral load ($r=-0.31$; $p=0.03$, Fig. 2A). These observations were similar (Figs. 1B and 2B) or reflected the trend (Figs. 1C and 2C) regardless of whether the study group received or did not receive ART. Our results are in agreement with previous studies^{14,20,31} and indicate further that individuals who advance to disease and subjects with AIDS have lower numbers of PDCs as compared to healthy infected subjects.

Relationship of PDC function (IFN- α production) with PDC numbers and CD4⁺ T cell counts

In evaluating PDC function, we observed that IFN- α release per PDC in response to the TLR9 agonist (CpG-A) correlated

FIG. 1. Correlation of CD4⁺ T cells with plasmacytoid dendritic cell (PDC) numbers. (A) CD4⁺ T cell counts as well as percentages correlate positively with PDC numbers in HIV-infected individuals ($n=48$). The trend in these associations remained true whether (B) the study group ($n=24$) did not receive antiretroviral treatment or (C) the study group ($n=24$) received antiretroviral treatment. Spearman's rank correlation coefficients (r) are shown where an asterisk (*) denotes level of significance ($p<0.05$).

positively with PDC numbers ($r=0.33$; $p=0.02$) (Fig. 3A). These observations were confirmed with HIV-infected CD4⁺ T cells, which showed a trend toward this association ($r=0.46$; $p=0.11$). A similar association was also found for HSV-1, another TLR9 agonist, which was used to stimulate PDCs from eight HIV-infected individuals (data not shown). Similar trends in the association of IFN- α production induced by these stimuli (CpG-A, HIV-infected CD4⁺ T cells, and HSV-1) were observed with CD4⁺ T cell counts ($r=0.24$; $p=0.10$, $r=0.38$; $p=0.19$, and $r=0.18$; $p=0.67$, respectively) (data not shown).

In contrast, this finding was not observed with the TLR7 agonist (S-27609)-induced IFN- α production and PDC numbers ($r=-0.22$; $p=0.13$, Fig. 3A) as well as CD4⁺ T cell counts ($r=-0.04$; $p=0.75$, data not shown). These observations for the TLR9 agonist (CpG-A) and TLR7 agonist (S-27609) remained uninfluenced when the HIV-infected subjects were divided into groups receiving or not receiving antiretroviral treatment (Fig. 3B and C).

PDCs from HIV-infected individuals differ in their responses to TLR7 and TLR9 agonists

In further studies of PDC function using purified cells, we observed that in HIV-infected subjects, CpG-A (the TLR9 agonist) was a significantly better inducer of IFN- α production than S-27609 (the TLR7 agonist) or HIV-infected CD4⁺ cells ($p<0.001$; Fig. 4). These observations remained the same whether the subjects were or were not on ART ($p<0.05$; data not shown). In addition, CD4⁺ cell numbers or PDC counts did not influence the observations that CpG-A was a significantly ($p<0.05$) better inducer of IFN- α production than S-27609 when the infected subjects were divided into groups based on median CD4⁺ cell numbers (515 cells/ μ l of blood) or median PDC counts (5.9 cells/ μ l of blood) (Fig. 5). CpG-A also remained a significantly ($p<0.05$) better inducer of IFN- α production as compared to HIV-infected CD4⁺ cells in some randomly selected HIV-infected individuals ($n=13$) from the study cohort when these subjects were divided into groups based on median CD4⁺ cell numbers and PDC counts ($p<0.05$; data not shown).

Within the control group, no such significant differences were noted for PDC responses to CpG-A as compared to S-27609 ($p=0.30$) or to HIV-infected CD4⁺ cells ($p=0.15$) (Fig. 4). Moreover, in a comparison of CpG-A-induced IFN- α production between HIV-infected individuals and control subjects, the differences in IFN- α production did not reach statistical significance ($p=0.46$). Similarly, the observations noted for IFN- α production induced by S-27609 and HIV-infected CD4⁺ cells ($p=0.25$ and $p=0.12$, respectively) were not significant. The somewhat higher median value for interferon production induced by CpG-A in HIV-infected subjects (Fig. 4) could be explained by the fact that several long-term asymptomatic subjects were part of the HIV-infected cohort. Their cells responded particularly well to TLR-9 stimulation.

IFN- α is composed of 13 subtypes that show relatively minor (78–98% homology between the subtypes) but highly conserved sequence differences.³² The antiproliferative and antiviral activities of different IFN- α subtypes seem to be well correlated, indicating large overlaps in the downstream signaling events regulating the distinct biological effects of IFN- α . To determine if the differences in IFN- α induced by the TLR7 and TLR9 agonists among HIV-1-infected individuals

FIG. 2. Correlation of viral load with PDC numbers. (A) HIV-1 RNA (copies/ml) correlates inversely with PDC numbers in HIV-infected individuals ($n=48$). The trend in this association remained true whether (B) the study group ($n=24$) did not receive antiretroviral treatment or (C) the study group ($n=24$) received antiretroviral treatment. Spearman's rank correlation coefficients (r) are shown where an asterisk (*) denotes level of significance ($p<0.05$).

FIG. 3. Correlation of PDC function with PDC numbers. (A) Interferon (IFN)- α (pg/ml) per 10^4 PDC induced by Toll-like receptor (TLR)9 agonist (CpG-A) but not by TLR7 agonist (S-27609) correlated positively with PDC counts in HIV-1-infected individuals ($n=48$). The trend in these associations remained the same whether (B) the study group ($n=24$) did not receive antiretroviral treatment or (C) the study group ($n=24$) received antiretroviral treatment. Spearman's rank correlation coefficients (r) are shown where an asterisk (*) denotes level of significance ($p < 0.05$).

were limited to any particular IFN- α subtype, we quantified IFN- α production by both IFN- α A and multi-subtype ELISA in five randomly selected HIV-infected subjects. Even though the magnitude of both TLR7 and TLR9 agonist-induced IFN- α responses was higher when quantified by multisubtype ELISA as compared to single IFN- α A ELISA, we did not observe any significant differences ($p=0.55$) in the ratios of TLR7- to TLR9-induced IFN- α as measured by both the ELISA kits (Fig. 6).

Expression of TLR7- and TLR9-induced PDC surface markers in HIV-infected individuals and healthy controls

TLR7 and TLR9 triggering induces the up-regulation of surface markers for activation (CD80, CD86), maturation (CD83), and the chemokine receptor (CCR7) on PDCs in addition to IFN- α production.^{12,13,33,34} Hence, we studied the above-mentioned PDC markers in healthy controls ($n=8$) and HIV-1-infected individuals ($n=10$) from our study population. These patients were selected based on their PDC counts that ranged from 2.4 to 14.8 per μ l of blood.

In a comparison of HIV-infected individuals and healthy controls, we did not observe any substantial differences in the relative expression of surface markers on unstimulated or stimulated PDCs in response to TLR7 and TLR9 agonists (Fig. 7). However, the TLR7-induced expression of the PDC activation markers (CD80, CD86), maturation marker (CD83), and chemokine receptor (CCR7) was higher than the TLR9-

FIG. 4. PDCs from HIV-infected individuals differ in their responses to TLR7 and TLR9 agonists. In HIV-1-infected individuals but not the healthy controls, IFN- α (pg/ml) production per 10^4 PDCs induced by the TLR9 agonist (CpG-A) was significantly ($p < 0.001$) higher than that observed with both the TLR7 agonist (S-27609) and HIV-infected CD4 $^+$ T cells. The Wilcoxon signed-rank test was used to determine the differences between IFN- α (pg/ml) induced by two agonists within each study population where an asterisk (*) denotes level of significance ($p < 0.01$). The bars represent the median values. In six healthy controls, the IFN- α (pg/ml) levels in response to TLR7 stimulation were low with two controls having no detectable IFN- α (pg/ml) production and four controls having less than 30 pg/ml IFN- α levels.

FIG. 5. Clinical state does not influence PDC responses. Differences in IFN- α (pg/ml) induced in 10^4 PDC by TLR7 and TLR9 agonists remained the same within the groups of HIV-infected individuals based on median CD4⁺ T cell counts (515 cells/ μ l of blood) or median PDC numbers (5.9 cells/ μ l of blood). The Wilcoxon signed-rank test was used to determine the differences between IFN- α (pg/ml) induced by two agonists where an asterisk (*) denotes level of significance ($p < 0.05$).

induced expression of these PDC surface markers in both study populations (Fig. 8). These differences were significant ($p < 0.05$) except for the expression of the PDC maturation marker (CD83) in the two clinical groups (Fig. 8).

Correlation of TLR7- and TLR9-induced expression of PDC surface markers with IFN- α production

Apart from assessing the expression of above-mentioned PDC surface markers in HIV-1-infected individuals ($n = 10$), we also evaluated the expression in parallel with IFN- α levels per PDC in response to TLR7 and TLR9 agonists. Positive correlations, however not significant ($p > 0.05$), were observed for TLR7- and TLR9-induced IFN- α levels per PDC with expression of the PDC activation markers (CD80, CD86), maturation marker (CD83), and chemokine receptor (CCR7) (Table 2). These correlations were comparable for TLR7 and TLR9 agonists.

FIG. 6. Differences in TLR7- and TLR9-induced IFN- α is not limited to a particular IFN- α subtype. The ratio of TLR7 (S-27609)-induced to TLR9 (CpG-A)-induced IFN- α in HIV-1-infected individuals is not significantly different ($p = 0.55$) when measured by single or multisubtype IFN- α ELISA.

FIG. 7. Expression of PDC surface markers in HIV-infected individuals and healthy controls. No significant differences were observed among HIV-infected individuals ($n = 10$) and healthy controls ($n = 8$) in the expression of surface markers on PDC stimulated with the (A) TLR9 agonist and (B) TLR7 agonist or (C) left unstimulated. The Mann-Whitney test was used to compare the medians of the expression of surface markers between both the study populations.

BDCA-2 expression on PDCs by TLR7 and TLR9 agonists

Previous studies have suggested that on activation by TLR7 and TLR9 ligands, PDCs lose the negative control exerted by BDCA-2 on IFN- α production.²³ Thus the decreased TLR7-

FIG. 8. Differences in expression of surface markers on PDC stimulated with TLR9 and TLR7 agonists. Significant differences were observed in the expression of PDC surface markers for activation (CD80, CD86), chemokine receptor (CCR7), but not maturation marker (CD83) on PDC stimulated with TLR9 and TLR7 agonists in both (A) healthy controls and (B) HIV-infected individuals. The Wilcoxon signed-rank test was used to compare the medians where an asterisk (*) denotes level of significance ($p < 0.05$).

induced IFN- α levels observed in HIV-infected individuals could be associated with the extent of BDCA-2 down-regulation. We therefore studied the effect of TLR7 and TLR9 stimuli on the PDC expression of BDCA-2 in the two clinical groups. We observed no significant difference in BDCA-2 expression on PDCs among HIV-infected individuals and healthy controls (Fig. 7). However, in contrast to the other PDC surface markers, TLR7 stimulation significantly ($p < 0.05$) reduced the BDCA-2 expression on PDCs to a greater extent than TLR9 stimulation in both study populations (Fig. 8). Notably, in HIV-infected individuals a positive correlation ($r = 0.05$) between BDCA-2 expression on PDC and IFN- α levels per PDC was observed with the TLR7 agonist as compared to the TLR9 agonist (Table 2).

TABLE 2. CORRELATION (SPEARMAN'S RANK) OF PLASMACYTOID DENDRITIC CELL SURFACE MARKERS WITH INTERFERON- α (pg/ml) INDUCED BY TLR7 AND TLR9 AGONIST IN HIV-INFECTED INDIVIDUALS ($n = 10$)

PDC surface markers	CpG-A (TLR9)		S-27609 (TLR7)	
	r	p value	r	p value
CD80	0.58	0.08 (ns)	0.56	0.09 (ns)
CD86	0.21	0.56 (ns)	0.21	0.56 (ns)
CD83	0.26	0.47 (ns)	0.15	0.68 (ns)
CCR7	0.52	0.13 (ns)	0.52	0.13 (ns)
BDCA-2	-0.44	0.20 (ns)	0.05	0.89 (ns)

PDC, plasmacytoid dendritic cells; r , Spearman rank correlation coefficient; ns, not significant.

To further assess the possible role of BDCA-2 down-regulation on the diminished TLR7-induced IFN- α production, we cross-linked BDCA-2 on PDCs from HIV-infected patients and healthy controls with anti-BDCA-2 or matched isotype mAbs prior to the addition of TLR7 and TLR9 agonists as previously described.²³ We observed that in both patients and healthy controls, BDCA-2 cross-linking significantly ($p < 0.05$) inhibited TLR9-induced IFN- α production but had no effect on TLR7-induced IFN- α responses (Fig. 9). These findings were the same when the multisubtype ELISA was used to measure IFN- α production induced by the two TLR agonists in HIV-infected subjects (Fig. 9).

Discussion

Progressive HIV infection has been associated with a decrease in the CD4⁺ cell and PDC numbers in the peripheral blood.^{14,20} In this study, with a large number of subjects, we confirmed the observation that during HIV infection, the PDC number mirrors the clinical state as reflected by CD4⁺ T cell counts (Fig. 1) and correlates inversely with HIV-1 viral load (Fig. 2). This association between PDC numbers and CD4⁺ cells remained the same in the presence or absence of antiviral treatment indicating the similar effect of ART on these two cell populations (Fig. 1).^{31,35}

Apart from a diminished frequency of PDCs in peripheral blood, a reduced PDC function as measured by IFN- α production by total PBMCs has also been reported during HIV-1 infection.^{14,20,36} In our study with purified PDCs from infected subjects we confirmed this direct correlation of PDC numbers with the level of IFN- α release per PDC (Fig. 3). Low PDC numbers correlated with less IFN- α production/cell. However, this finding was limited to IFN- α release per PDC in response to the TLR9 agonist (CpG-A) and HIV-infected CD4⁺ T cells. The TLR7 agonist did not show this correlation of PDC number with IFN- α production (Fig. 3). These observations were again independent of ART.

Our observations are in agreement with a recent study that shows that PDC responses to TLR7 stimulation were not associated with HIV-1 viral load and CD4⁺ T cell counts.³⁷ This lack of correlation between IFN- α production and PDC and CD4⁺ cell numbers in HIV-infected subjects when the TLR7 agonist was used (Fig. 3) cannot be readily explained. It does not reflect a reduced PDC number in peripheral blood and it is most likely not due to a low expression of TLR7. Studies have

FIG. 9. BDCA-2 down-modulation and IFN- α production by TLR9 and TLR7 agonists. Anti-BDCA-2 monoclonal antibodies (mAbs) significantly inhibit IFN- α production by the (A) TLR9 agonist but not the (B) TLR7 agonist in both HIV-infected individuals and healthy controls. The observations remained the same when single IFN- α A or multisubtype ELISA was used to measure IFN- α production. A one-way ANOVA test was used to compare the means of three groups within each study population where an asterisk (*) denotes level of significance ($p < 0.05$).

shown that TLR7 mRNA levels correlate with high viral loads and low CD4⁺ T cell numbers.³⁸ Additionally, TLR7 but not TLR9 expression is increased in both treated and untreated chronic HIV-1 infection.^{38,39}

Dendritic cells from HIV-1-infected individuals have also been reported to be less responsive to both TLR7 and TLR9 ligands as compared to uninfected individuals.^{21,36} However, we noted that the IFN- α release by PDC after exposure to TLR7 and TLR9 agonists was comparable between the infected and uninfected groups (Fig. 4). Our contrasting results could be due to methodological differences. We measured IFN- α release by purified PDCs. The other studies evaluated IFN- α release by PBMCs or used intracellular cytokine expression in total PBMCs to denote the frequency of IFN- α producing PDCs.^{21,36} Our results did suggest that in the HIV-infected individuals, but not healthy controls, there are differences in PDC responses to TLR7 vs. TLR9 stimuli (Figs. 4 and 5). The TLR7-induced IFN- α production per cell was significantly less than that of the TLR9-induced IFN- α production. This reduced PDC function in response to the TLR7

agonist was not limited to any particular IFN- α subtype as confirmed with the multisubtype IFN- α ELISA (Fig. 6). The decreased responsiveness of PDCs from HIV-1-infected individuals to *in vitro* stimulation with the TLR7 ligand could reflect prior *in vivo* TLR activation by the virus.⁴⁰ This possibility requires further study.

Moreover, the differences observed in the PDC production of IFN- α induced by TLR7 and TLR9 agonists in HIV-1-infected individuals but not in healthy controls (Fig. 4) do not reflect differences in the expression of PDC surface markers in the two clinical populations (Fig. 7); both gave the same results (Fig. 8). Additionally, no statistical difference was seen in TLR7- or TLR9-induced expression of the PDC maturation marker (CD83) in both HIV-infected individuals and healthy controls (Fig. 8). Thus, the decreased TLR7-mediated IFN- α production (Figs. 4 and 5) does not appear to be related to inhibition or reduced maturation of the PDCs in response to TLR7 stimuli. The correlations between TLR7- or TLR9-induced expression of the PDC surface markers and IFN- α levels were also comparable and, hence, do not reflect inefficient TLR7 triggering in the HIV-infected individuals (Table 2).

PDCs express immune inhibitory receptors, including BDCA-2, a C-type lectin that is a negative regulator of IFN- α production. TLR7 and TLR9 agonists down-regulate BDCA-2 on the PDC surface concomitant with IFN- α production.²³ We did not see any major difference in TLR7- and TLR9-induced BDCA-2 down-regulation on PDCs among HIV-infected individuals and healthy controls (Fig. 7). However, the TLR7 stimulation reduced BDCA-2 expression on PDCs significantly ($p < 0.05$) more than did TLR9 stimulation in both the study populations (Fig. 8). TLR7 stimulation also had a positive correlation with BDCA-2 expression on PDCs as compared to TLR9 (Table 2). These observations do not support the role of BDCA-2 down-regulation in TLR7-induced IFN- α production in HIV-infected individuals.

Previous studies have shown that cross-linking of BDCA-2 on the surface of PDCs inhibits IFN- α production in response to stimulation by TLR9 agonists such as HSV,²⁴ CpG-ODN,^{25,30} and sera from SLE patients.²³ Likewise, we observed that cross-linking of BDCA-2 on the PDCs from both HIV-infected and control populations significantly ($p < 0.05$) inhibited the IFN- α response to the TLR9 agonist presumably due to an inhibition of the BDCA-2-Fc ϵ R1c signaling receptor complex on the PDC cell surface.²⁶ However, this cross-linking did not block TLR7-induced IFN- α production (Fig. 9). These results confirm previous findings.^{12,23} The reason for this difference in the effect of BDCA-2 cross-linking on TLR7- and TLR9-induced IFN- α production is not evident. Nonetheless, our results suggest that the reduction in TLR7-induced IFN- α release by PDCs, in contrast to the response to TLR9, is not related to the extent of BDCA-2 down-modulation.

Our findings are supported by observations suggesting that HIV-1 gp120 inhibition of TLR9-mediated but not TLR7-mediated activation and IFN- α secretion by PDCs acts via BDCA-2 in a Ca²⁺-dependent manner.⁴¹ Similarly, other viruses such as HCV⁴² and HBV⁴³ have been shown to block stimulation via TLR9 most probably by interaction between the virus and BDCA-2 on the PDC surface followed by inhibition of signal transduction.⁴³ Our results suggest that another mechanism(s) or molecule(s), apart from the immune

inhibitory molecule BDCA-2, is involved in IFN- α induction through TLR7 even though both TLR7 and TLR9 use the MyD88-interferon regulatory factor (IRF)-7 pathway for IFN- α production.^{5,44} In this regard, other inhibitory PDC surface receptors aside from BDCA-2 such as ILT7-Fc ϵ RI gamma⁴⁵ could be associated with the TLR7-induced responses during HIV-1 infection.

In summary, our observations using purified PDCs from a large number of HIV-infected individuals confirm a direct relationship of PDC number and function with the clinical state as measured by CD4⁺ cell numbers. This relationship in terms of function was, however, limited to TLR9-induced IFN- α production by PDCs. We noted a deficit in IFN- α production upon TLR7 stimulation irrespective of the clinical and antiretroviral treatment state of the chronically HIV-infected patients.

Importantly, our studies showed that the diminished TLR7-induced IFN- α production by PDCs from HIV-infected individuals was not a consequence of abnormalities in TLR7-induced PDC surface marker expression and the extent of down-modulation of the immune inhibitory receptor, BDCA-2, on the PDCs. The findings notably indicated that BDCA-2 down-modulation does not affect TLR7-induced IFN- α production by PDCs. They suggest that a yet to be identified other mechanism(s) is responsible for TLR7-specific diminished PDC function in HIV-1 infection. Deciphering the mechanism that causes the TLR7 deficit may provide further insight into the immunopathogenesis of HIV-1 infection.

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Author Disclosure Statement

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