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Brief Report

The Relation of Plasmacytoid Dendritic Cells (pDCs) and Regulatory T-Cells (Tregs) with HPV Persistence in HIV-Infected and HIV-Uninfected Women

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Abstract

Other than CD4+ count, the immunologic factors that underlie the relationship of HIV/AIDS with persistent oncogenic HPV (oncHPV) and cervical cancer are not well understood. Plasmacytoid dendritic cells (pDCs) and regulatory T-cells (Tregs) are of particular interest. pDCs have both effector and antigen presenting activity and, in HIV-positive patients, low pDC levels are associated with opportunistic infections. Tregs downregulate immune responses, and are present at high levels in HIV-positives. The current pilot study shows for the first time that low pDC and high Treg levels may be significantly associated with oncHPV persistence in both HIV-positive and HIV-negative women. Larger studies are now warranted.

Introduction

Plasmacytoid dendritic cells (pDC) and regulatory T-cells (Treg) may play an important role in HIV pathogenesis and disease progression. However, their role in the control of human papillomavirus (HPV), the viral cause of cervical cancer, an AIDS-defining malignancy, has not been carefully studied. pDCs are of particular interest, since diminished pDC count and function are strongly predictive of opportunistic infections in HIV-positive patients, independent of CD4+ T-cell count (19–21), and imiquimod, a topically applied toll-like receptor (TLR) agonist used to treat HPV-associated warts, may partly act through activation of local pDCs (6). pDCs are unique in that they bridge the innate and the adaptive immune systems (8,11). pDCs exist in the circulation and tissues as effector cells of the innate immune system that secrete interferon (IFN)-α and other type I IFNs upon stimulation by microbial pathogens. However, they are also precursor cells, and following antigen interaction. they are thought to mature and differentiate into dendritic cells (professional antigen presenting cells) that are critical in initiating the adaptive immune response through cognate interaction with CD4+ T-cells (7). *In vitro* data show that pDCs can respond to HPV antigens (10).

Tregs are involved in virtually all stages of the adaptive immune response (see Fazekas de St Groth and Landay, 2008, for review) (5). This includes downregulation of self-reactive immune cells, as well as the downregulation of T cell responses to foreign antigens at the expansion and effector stages. The mechanisms of action, though, remain somewhat uncertain. It has been suggested that Treg activity may play a role in the establishment of the HIV carrier state (3). The number of Tregs increases in the gut during HIV infection, and high Treg ratios have been reported in patients with low CD4+ and high HIV RNA levels (3,9). A small but growing literature suggests that high levels of Tregs and cytokines associated with Treg activity are more common in patients with HPV-related cervical lesions (13–15,18).

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To our knowledge, however, no studies of oncogenic HPV (oncHPV) infection and pDC levels have been reported, nor have the few prior studies of Tregs and HPV involved HIV-positive women despite the known abnormalities of Treg and pDC levels and function among HIV-positive patients. Therefore, we conducted a small pilot study to assess the relationship between pDC and Treg levels in HIV-positive women and oncHPV persistence (versus clearance). A smaller number of HIV-negative women were included as an additional comparison group and to obtain normal values.

Methods

Subjects and specimens

Data and specimens were obtained from the Women's Interagency HIV Study (WIHS), a large prospective cohort investigation. A detailed summary of the WIHS and its methods has been previously reported. Briefly, HIV-positive ($n=2543$) and at-risk HIV-negative ($n=895$) women were followed semiannually with clinical visits that involved a full gynecologic examination, including collection of a cervicovaginal lavage (CVL) for HPV DNA testing. Blood specimens were also collected and tested for total CD4+ T-cell count and HIV RNA levels in laboratories participating in the DAIDS Quality Assurance Program. Additional whole blood was collected in sodium heparin-coated tubes and used to separate and cryopreserve peripheral blood mononuclear cells (PBMCs) using standard, previously described methods (17).

For the current pilot study, we used stratified random sampling to select 19 ''oncHPV persisters'' and a comparison group of 19 ''oncHPV clearers.'' We defined an oncHPV persister as a woman who tested positive for at least one oncHPV type, whether a prevalent or incident infection, who then continued to test positive for one or more of the same oncHPV types for ≥ 1.5 years (four semi-annual visits). An oncHPV clearer was defined as a woman who tested positive for at least one oncHPV type at the time of blood collection but had no oncHPV at all detected at the next 6-month clinic visit. We limited the selection of HIV-positive women to those with a CD4+ $>350/\mu$L, since it was felt that the contribution of pDC and Treg levels to risk of persistent HPV might be difficult to discern in women with a very low CD4 count. All women had to have at least 2 years of follow-up (four visits plus baseline visit) with no more than one missing visit. PBMC specimens were available from 16 of the 19 oncHPV persisters (13 HIV-positive with a CD4+ $>350/\mu$L and 3 HIV-negative women) and 15 of the 19 oncHPV clearers (10 HIV-positive with a CD4+ $>350/\mu$L and 5 HIV-negative women).

HPV testing

HPV DNA was detected in CVL specimens using a well-established polymerase chain reaction (PCR) assay that employed MY09/MY11/HMB01 consensus primers (16,22). This assay has been shown to be highly sensitive and specific for the detection of >40 different individual HPV types (16). Amplification of a 268-base-pair fragment of the human β-globin gene was used as an internal control for specimen adequacy. The HPV types considered oncogenic were: 16/18/31/33/35/39/45/51/52/56/58/59/68, and all other HPV were considered non-oncogenic.

Flow cytometry

Flow cytometry for pDCs and Tregs was conducted twice for each subject, once using cryopreserved PBMC specimens obtained at the first visit when oncHPV was detected during this study, and again one year later. Briefly, the cryopreserved PBMCs were removed from liquid nitrogen (LN_2) storage, thawed rapidly in a 37°C water bath, washed, and then incubated overnight at 37°C 5% CO_2 in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (FBS). Following overnight incubation, the cells were washed with Dulbecco's PBS-($Ca^{++}Mg^{++}$ free) and stained with Aqua Live/Dead cell stain kit (Invitrogen) to assess cell viability. The PBMCs were then pre-treated with human Fc block (Miltenyi Biotec) and stained for cell surface markers. To assess pDCs, the PBMCs were incubated with the Lin1 FITC (BD), CD83 PE (Pharmingen), CD123 PerCP-Cy5.5 (Pharmingen), PDL-1 PE-Cy7 (eBioscience), CD11c AF700 (eBioscience), CD86 APC (Pharmingen), CCR7 eFluor780 (eBioscience), and HLA DR eFluor450 (eBioscience), washed and resuspended in 2% formaldehyde-DPBS. To assess Tregs, the PBMCs were stained with CD3 PacificBlue (Pharmingen), CD4 PE-Texas Red (CalTag:Invitrogen), CD25 APC (BD), CD45RO PE-Cy7 (BD), and PD-1 PE (eBioscience). Following surface staining, the Tregs were additionally washed, fix/permeabilized with the FoxP3 Staining Buffer Set (eBioscience), stained with FoxP3 FITC (eBioscience), and CTLA-4 PE-Cy5 (Pharmingen), washed, and resuspended in 2% formaldehyde-DPBS. All stained cells were held at 4°C until acquired and analyzed within 24 h on a LSR2 flow cytometer (BD) using FACS Diva™ 6.1.1 software (BD). The analysis of flow cytometry data was performed after gating on live (Aqua live dead) $Lin1^{-} \setminus HLA\ DR^{+} \setminus CD123^{+}$ or CD3+ \ CD4+ \ CD25+ \ FoxP3+ cells corresponding to pDC (% of PBMC) or Tregs (% of CD4), respectively.

Statistical methods

Analyses of these immunologic data were conducted using *t*-tests (for continuous variables), chi-square (for categorical or ordinal variables), as well as logistic regression models to conduct multivariate analyses. Both pDC% and Treg% were log transformed to normalize their distributions. All statistical tests were two-sided, and $p<0.05$ was used to define statistical significance.

Results

Table 1 shows selected patient characteristics and laboratory data in oncHPV persisters and clearers, stratified by HIV serostatus. Mean age was approximately 37 years in HIV-positive and HIV-negative oncHPV persisters, and HIV-positive clearers, but was 33 years in HIV-negative clearers; a moderate but statistically nonsignificant difference in this small study. HAART use in HIV-positive women did not significantly differ between persisters and clearers.

One of the selection criteria for HIV-positive women in this study was a CD4+ $>350/\mu$L, and neither the CD4+ count ($p=0.69$) nor HIV viral load ($p=0.27$) differed between oncHPV persisters and clearers in univariate analyses (Table 1). However, the percentage of CD4+ cells that were found to be Tregs (Treg%) was significantly greater in HIV-positive oncHPV persisters than clearers ($p=0.02$), and

Table 1. Selected Baseline Patient Characteristics and Laboratory Test Results in HIV-Positive and HIV-Negative Women who Tested Positive for Oncogenic HPV, Which then Either Persisted or Cleared

	HIV-Positive[a]			*HIV-negative*		
Characteristic	*oncHPV Persisters*[b] *(N = 13)*	*oncHPV Clearers*[c] *(N = 10)*	P *value*	*oncHPV Persisters*[b] *(N = 3)*	*oncHPV Clearers*[c] *(N = 5)*	P *value*
Age ± SD	37.6 ± 7.9	37.3 ± 5.2	0.91	37.2 ± 9.5	32.5 ± 15.8	0.66
Total CD4 count (SE)[a]	540 (42.40)*	579 (47.72)	0.81	1244 (250.12)*	1441 (652.50)	0.84
Mean Treg% (SE)	4.74% (0.38)	3.22% (0.26)	0.02	5.97% (1.41)	2.82% (0.18)	0.11
Mean pDC% (SE)	0.07% (0.02)	0.09% (0.01)	0.12	0.05% (0.02)	0.15% (0.03)	0.02
HIV RNA level						
Undetectable	3 (23%)	5 (50%)	0.22	NA	NA	
Detectable	10 (77%)	5 (50%)		NA	NA	
HAART use in past 6 mo., No. (%)						
No	6 (46.2)	3 (30.0)	0.67	NA	NA	
Yes	7 (53.9)	7 (70.0)		NA	NA	

HAART, highly active anti-retroviral therapy; oncHPV, oncogenic HPV; Pdc, plasmacytoid dendritic cell; SD, standard deviation; SE, standard error; Treg, regulatory T-cell.

*Significantly different between HIV-positive and HIV-negative persisters with $p < 0.05$.

[a]The selection of HIV-positive women was limited to those with a CD4 count > 350 since it was felt that the impact of pDC% and Treg% might be difficult to discern in women with a very low CD4 count. [b]An oncHPV Persister was a priori defined as a subject who tested positive for at least one oncHPV type at the time of blood collection and then continued to test positive for one or more of these exact oncHPV types for ≥ 1.5 years. [c]An oncHPV Clearer was a priori defined as a woman who tested positive for at least one oncHPV type at the time of blood collection but had no oncHPV detected 6 months later, at the next clinic visit.

similar but nonsignificant Treg% results were found in HIV-negative women ($p = 0.11$). Conversely, the percentage of PBMCs that were pDCs (pDC%) was nonsignificantly lower in HIV-positive oncHPV persisters than clearers ($p = 0.12$), and a similar but statistically significant difference was observed in HIV-negative women ($p = 0.02$).

Multivariate models controlling for age and HAART use were conducted in HIV-positive women. However, given the similarity of the univariate findings in HIV-positive and -negative subjects, we also conducted multivariate models that incorporated the data from both groups (Table 2). As in the univariate analysis, the positive Treg% association ($p = 0.05$) but not the negative pDC% association with oncHPV persistence ($p = 0.22$) was statistically significant in models limited to HIV-positive women. In the combined analysis of HIV-positive and HIV-negative subjects, however, the statistical significance of the Treg% ($p = 0.01$) and pDC% ($p = 0.03$) associations with oncHPV persistence was greater, while HIV-serostatus was not a significant factor ($p = 0.81$ in Treg% model, as well as in the pDC% model). The confidence intervals for the effects of pDC% and Treg% were wide, though, reflecting the small size of this pilot study, making it inappropriate to conduct models involving multiple immunologic covariates (e.g., incorporating both Treg% and pDC% in the same model).

Discussion

The results of this pilot study suggest that a high Treg% and low pDC% are each associated with increased risk of oncHPV persistence. These findings are consistent with growing understanding of the role of Tregs in downregulating host immune response to pathogens (14), and the dual roles of pDCs in the control of viral infection. pDCs are α-interferon-secreting effector cells of the innate immune system with a direct role in eliminating viral infection, but also act as antigen presenting cells of the adaptive immune system, important in the activation of T-cells (4,10). Furthermore, these associations were similar in both HIV-positive and HIV-negative women. That is, while prior studies by our group and others have shown that the risk of an oncHPV infection is several-fold higher in HIV-positive than HIV-negative women, the current data suggest that Treg% and pDC% are risk factors for the persistence of oncHPV in both groups (22).

Our data showing a positive association of Treg% with risk of oncHPV persistence are consistent with prior reports (13–15,18), though the current pilot study is, to our knowledge, the first to examine this relationship in HIV-positive women. Thus, while the number of studies is still small, the consistency of the findings regarding Tregs and oncHPV to date is provocative. Conversely, the current study is the only one in any population we are aware to examine the relation of pDC% and oncHPV persistence, and these data must therefore be considered preliminary and await confirmation.

The findings of this study have potential clinical significance since they suggest that modulating Tregs and PDCs may be a useful strategy to help resolve persistent oncHPV or oncHPV related neoplasia. It is noteworthy, therefore, that imiquimod, a topically applied toll-like receptor agonist used to treat HPV-related anogenital warts can activate pDCs (6). Imiquimod is not recommended for treatment of cervical lesions however, due to local cervical toxicity, likely attributed to the pro-inflammatory milieu induced by imiquimod. If confirmed, our results would suggest that additional efforts to target pDCs may be warranted. This may be especially relevant now that cervical oncHPV DNA testing is a routine part of cervical cancer screening, since physicians and their patients are often left with the dilemma

TABLE 2. ASSOCIATIONS OF ONCOGENIC HPV PERSISTENCE WITH PLASMACYTOID DENDRITIC CELL (PDC) AND REGULATORORY T-CELL (TREGS) PERCENTAGES ANALYZED IN MULTIVARIATE MODELS AMONG HIV-POSITIVE WOMEN (A, C) AS WELL AS USING A COMBINED DATASET OF BOTH HIV-POSITIVE AND HIV-NEGATIVE SUBJECTS (B, D)

(a) *pDCs in HIV-positive women*

Variable		*# of subjects*	*OR*[a]	*95% CI*		P *value*
				Lower	*Upper*	
log (pDC %)[b]		–	0.74	0.46	1.18	0.20
HAART	No	9	*ref*			
	Yes	14	0.72	0.11	4.69	0.73
Age	<35	9	*ref*			
	≥35	14	1.15	0.19	6.99	0.88

(b) *pDCs in HIV-positive and HIV-negative women*

Variable		*# of subjects*	*OR*[a]	*95% CI*		P *value*
				Lower	*Upper*	
log (pDC %)[b]		–	0.61	0.39	0.96	*0.03*
HIV Status[c]	HIV-	8	*ref*			
	HIV+	23	1.28	0.18	9.04	0.81
Age	<35	15	*ref*			
	≥35	16	1.13	0.21	6.11	0.89

(c) *Tregs in HIV-positive women*

Variable		*# of subjects*	*OR*[a]	*95% CI*		P *value*
				Lower	*Upper*	
log (Treg %)[b]		–	5.29	1.00	27.9	*0.05*
HAART	No	9	*ref*			
	Yes	14	1.90	0.16	22.8	0.61
Age	<35	9	*ref*			
	≥35	14	1.75	0.20	15.2	0.61

(d) *Tregs in HIV-positive and HIV-negative women*

Variable		*# of subjects*	*OR*[a]	*95% CI*		P *value*
				Lower	*Upper*	
log (Treg %)[b]		–	5.75	1.52	21.9	*0.01*
HIV Status[c]	HIV-	8	*ref*			
	HIV+	23	1.36	0.14	13.8	0.81
Age	<35	15	*ref*			
	≥35	16	1.37	0.19	9.98	0.89

95% CI, 95% confidence interval; HAART, highly active anti-retroviral therapy; pDC, plasmacytoid dendritic cell; OR, odds ratio; Treg, regulatory T-cell.

[a]pDC% and Treg% are both continuous variables and the *p* value is unaffected by the arbitrary choice of unit change chosen for presentation purposes in the table. Here we present ORs per 50% increase in pDC% or Treg% (i.e., 0.4 unit increase on log scale).

[b]Both pDC% and Treg% values were log transformed to normalize their distributions.

[c]While oncogenic HPV persistence did not vary by HIV-positivitity, it must be noted that all HIV-positive women in this small pilot had CD4 > 350.

of serial oncHPV positivity with no available methods to eradicate oncHPV infection. Furthermore, local Treg and pDC immunotherapy might be useful as adjuvants to reduce the risk of recurrent cervical neoplasia, especially in HIV-positive and other high risk populations. It is also possible that the relation of Tregs and pDCs with oncHPV persistence may play a role in the beneficial effects of HAART in reducing oncHPV persistence observed in prior studies (1,2,12). Larger and more comprehensive studies are therefore warranted to expand upon the current observations, and better understand the effects of pDCs and Tregs on HPV natural history.

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Author Disclosure Statement

Each of the authors declare that they have no commercial associations that might create a conflict of interest in connection with this submitted article.

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