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MAJOR ARTICLE

Differences in the Immune Microenvironment of Anal Cancer Precursors by HIV Status and Association With Ablation Outcomes

Yuxin Liu,[1] Michael M. Gaisa,[2] Xiaofei Wang,[5] Talia H. Swartz,[2] Yotam Arens,[3] Karen A. Dresser,[5] Carlie Sigel,[4] and Keith Sigel[3]

[1]Department of Pathology, [2]Division of Infectious Diseases, and [3]Division of General Internal Medicine, Icahn School of Medicine at Mount Sinai, and [4]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York; and [5]Department of Pathology, University of Massachusetts Medical School, Worcester

Background. Anal high-grade squamous intraepithelial lesions (HSILs) are the precursors to anal cancer and frequently persist or recur following electrocautery ablation (EA). Impaired mucosal immunity may facilitate anal carcinogenesis. We characterized the immune microenvironment of anal HSILs in correlation with human immunodeficiency virus (HIV) serostatus and ablation outcomes.

Methods. Using immunohistochemistry, mucosa-infiltrating $CD4^+$ and $CD8^+$ lymphocytes were quantified in HSILs and benign mucosa from 70 HIV+ and 45 HIV− patients. Clinicopathological parameters were compared.

Results. Anal HSILs harbored more T lymphocytes than benign mucosa regardless of HIV status ($P \leq .03$). Total T lymphocyte count and $CD8^+$ subset were significantly higher in HIV+ HSILs versus HIV− HSILs (median cell count, 71 vs 47; 47 vs 22/high power field [HPF]; $P < .001$), whereas the $CD4^+$ subset was comparable between groups (median, 24 vs. 25; $P = .40$). Post EA, HSILs persisted in 41% of HIV+ and 19% of HIV− patients ($P = .04$). Unadjusted analysis showed trends toward EA failures associated with HIV seropositivity (incidence rate ratio [IRR], 2.0; 95% CI, .8–4.9) and increased $CD8^+$ cells (IRR, 2.3; 95% CI, .9–5.3).

Conclusions. Human immunodeficiency virus is associated with alterations of the immune microenvironment of anal HSILs manifested by increased local lymphocytic infiltrates, predominately $CD8^+$. Human immunodeficiency virus seropositivity and excess mucosa-infiltrating $CD8^+$ cells may be associated with ablation resistance.

Keywords. human immunodeficiency virus (HIV); anal cancer precursors; immune microenviroment; mucosa-infiltrating lymphocytes.

The incidence of squamous cell carcinoma of the anus (SCCA) has increased at a rate of 2.2% per year in the United States over the past decade [1, 2]. Anal high-grade squamous intraepithelial lesions (HSILs) are the immediate precursors for SCCA and are caused by persistent infection of high-risk human papillomavirus (HR-HPV) in the anal canal [3, 4]. Men who have sex with men (MSM) have a high prevalence of HPV infection in the anal canal regardless of human immunodeficiency virus (HIV) status [5, 6]. However, HIV-infected (HIV+) MSM are more likely to develop HSILs than their uninfected (HIV−) counterparts: the 4-year incidence of anal HSILs is estimated to be 49% in HIV+ and 17% in HIV− MSM [7]. Although anal HSILs are histologically identical among HIV− and HIV+ patients, they are more likely to persist and to resist treatment in the latter group. Even among those undergoing antiretroviral therapy, HIV+ MSM develop SCCA at a significantly higher rate than their HIV− counterparts [8, 9]. This suggests that despite systemic HIV virological suppression, disturbances in the local microenvironment may play a role in the progression of anal cancer precursors [10, 11].

The anal canal, part of the mucosa-associated lymphoid tissue system (MALT), is populated by immune cells such as Langerhans cells, $CD4^+$ T-helper cells, and $CD8^+$ T-cytotoxic cells [12]. The few existing studies pertaining to the anal MALT system have shown abnormal immune responses in HIV+ individuals, including the depletion of Langerhans cells and $CD3^+$ lymphocytes and increases in $Foxp3^+$ T-regulatory cells, along with multiple regulatory cytokines such as interleukin 8, interleukin 23, tumor necrosis factor α, interleukin 17A, and interferon γ [13–16]. Little is known about the immune microenvironment of anal cancer precursors or its alterations in the setting of HIV coinfection [17]. Similarly, only scant evidence is available regarding the influence of mucosa-infiltrating lymphocytes on the natural history and treatment response of anal HSILs.

This study aims to characterize the subpopulations of mucosa-infiltrating lymphocytes in anal HSILs, correlating them with HIV serostatus and responsiveness to electrocautery ablation (EA). Our results should expand current understanding of cell-mediated mucosal immunity in anal carcinogenesis and thereby facilitate the further development of diagnostic tests and targeted immunotherapeutic strategies.

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Correspondence: K. Sigel, MD, PhD, Division of General Internal Medicine, Mount Sinai School of Medicine, One Gustave Levy Place, Box 1087, New York, NY 10029 (keith.sigel@mssm.edu).

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METHODS

Patient Selection

After institutional review board approval was obtained from the Icahn School of Medicine at Mount Sinai, we searched the Mount Sinai high-resolution anoscopy (HRA) database from January 2013 to January 2016 for patients with their first episode of incident biopsy-proven anal HSILs. We included male patients self-reporting as MSM with known HIV status and available anal biopsy tissue. Information regarding age, race/ethnicity, smoking status, HPV vaccination history, HIV status, $CD4^+$ T-cell count, and HIV-1 plasma viral load (VL) level was abstracted from the electronic medical record.

High-Resolution Anoscopy and Pathology Review

All patients underwent HRA and biopsy performed by a single provider (M. G.) following previously described techniques [18]. Anal mucosa with gross appearance suspicious for HSILs or cancer was biopsied, and the anatomic location within the anal canal was recorded. One pathologist (Y. L.) examined all hematoxylin and eosin–stained slides from the biopsies and diagnosed all biopsies using the terminology and morphological criteria published in the Lower Anogenital Squamous Terminology (LAST) project: benign, low-grade squamous intraepithelial lesion (LSIL), and HSIL [19]. P16 immunohistochemistry was used in a subset of cases, where strong/diffuse positive immunoreactivity supported the diagnosis of HSILs [20]. Benign mucosal samples and HSILs with sufficient tissue for further immunohistochemistry study were selected. Anorectal cytology (ARC) samples were collected within 3 months of or concomitantly with HRA, and results were reported using the 2001 Bethesda System terminology: negative for intraepithelial lesions or malignancy; atypical squamous cells of undetermined significance (ASC-US); LSIL; atypical squamous cells, cannot exclude HSIL (ASC-H); and HSIL [21]. Oncogenic HPV subtype analysis was performed from liquid cytology fluid using the Roche Cobas HPV kit capable of detecting 14 types of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

Electrocautery Ablation and Follow-up

Anal HSILs were treated with EA using a hyfrecator (ConMed Corporation) by a single provider (M. G.). Target lesions were fulgurated and debrided with blunt and sharp dissection to healthy tissue, and submucosal vessels were coagulated. Following EA, patients underwent surveillance HRA at an average interval of 8.5 months (range, 1–20 mo). If HRA revealed suspicious lesions, a biopsy was performed. Random biopsies of healthy-appearing tissue were not pursued during this study.

Immunohistochemistry and Quantification of T-Lymphocyte Subsets

Immunohistochemistry was performed on 5-μm sections of formalin-fixed, paraffin-embedded tissue subjected to monoclonal antibodies against CD4 (VECTOR VP-C319, mouse clone 4B12, 1:200 dilution) and CD8 (DAKO M7103, mouse clone C8/144, 1:80 dilution) using the Dako EnVision+ horseradish peroxidase kit (Carpinteria, CA). Sections of tonsil tissue were used as positive controls. Mononuclear cells with dark brown cytoplasmic signal were recorded as positive cells. All cell counts were performed by a single pathologist (Y. L.) without knowledge of the subject's HIV status. Lymphocytes within the squamous epithelium (ie, intraepithelial T cell) and underlying stroma (ie, stromal T cell) were counted separately using light microscopy at 400× magnification. Three independent high-power fields were assessed for each case, avoiding lymphoid follicles.

Statistical Analysis

Baseline characteristics were compared by HIV status using the Wilcoxon test for nonnormally distributed continuous variables (age, $CD4^+$ T-cell count) and the χ^2 test and Fisher's exact test for categorical variables where appropriate. We then calculated median values for infiltrating $CD4^+$ and $CD8^+$ cells by location (intraepithelial, stromal, and total), testing for differences by HIV, HR-HPV status, and HPV vaccine history using the Wilcoxon test. In HIV+ subjects, we evaluated for an association between peripheral CD4 cell counts and mucosa-infiltrating cell counts by calculating correlation coefficients. For all subjects with follow-up HRA, we then calculated incidence rates for recurrent (at different anatomic sites of the anal canal) or persistent (at the site of the previously treated lesion) HSILs after ablation therapy and compared these rates by HIV status, smoking history, HPV vaccination history, and tertiles of mucosal CD4, CD8, and CD4/CD8 ratio infiltration using Poisson methods. All analyses were performed in STATA version 13 (Stata Corp, College Station, TX).

RESULTS

Patient Baseline Characteristics According to Human Immunodeficiency Virus Status

A total of 115 MSM (70 HIV+ and 45 HIV−) with biopsy-proven anal HSILs were studied. Table 1 illustrates patient baseline characteristics according to HIV status. The 2 groups shared a similar distribution for age range and smoking status (both $P > .05$). The HIV+ group included a higher proportion of black (20%) and Hispanic (27%) patients, whereas the HIV− group was predominantly white (71%; $P = .01$). Regarding HPV genotypes, both groups revealed similar rates of HPV-16 and HPV-18 infections (49% vs 47%). The HIV+ group had a higher rate of infection by other HR-HPV strains than the HIV− group (40% vs 24%; $P = .007$). The HIV+ group had significantly higher rates of ASC-H and HSIL cytology (24% vs 11%; $P = .001$) as well as multifocal HSILs (71% vs. 36%, $P < .001$) than their HIV− counterparts. All HIV+ individuals were undergoing antiretroviral therapy at the time of visit and had a median serum CD4+ cell count of 677 cells/mL (interquartile range, 469–865 cells/mL). HIV RNA was <50 copies/mL in 40 patients (57%), 50–1000 copies/mL in 18 (26%), and >1000 in 12 (17%).

Quantitation of Mucosal CD4+ and CD8+ T Lymphocytes

As shown in Table 2 and Figure 1, CD4+ and CD8+ T lymphocytes were quantified in 115 anal HSILs (70 HIV+, 45 HIV−) and 23 benign anal mucosa samples (11 HIV+, 12 HIV−). Benign anal mucosa from HIV+ and HIV− subjects showed similarly scant total mucosa-infiltrating CD4+ and CD8+ lymphocytes (17 vs 14, $P = .50$; 11 vs 10, $P = .90$), including the intraepithelial as well as stromal compartments.

Anal HSILs harbored a significantly higher number of lymphocytes than benign mucosa in both HIV+ and HIV− groups ($P \leq .03$ and $P \leq .01$). The total CD8+ T-cell count was significantly higher in HIV+ HSILs than in HIV− HSILs (median 47 vs 22; $P < .001$). The total CD4+ T-cell count was similar between the 2 groups (median, 24 vs 25; $P = .40$). Within the dysplastic epithelial layer (ie, intraepithelial T cell), both CD4+ and CD8+ T-cell counts were higher in HIV+ HSILs than HIV− HSILs (3 vs 1; 15 vs 8; $P \leq .001$). Within the stromal compartment, a significant increase of CD8+ T cells was observed in the HIV+ HSILs compared with the HIV− HSILs (32 vs 14; $P < .001$), whereas CD4+ T-cell counts were similar between the 2 groups (20 vs 25; $P = .80$). We also found a significant difference in stromal CD8 infiltration in subjects with HR-HPV infection compared with those without HR-HPV on concurrent cytology specimens (23 vs 14; $P = .03$), but otherwise there were no differences in infiltrating cell counts in any other tissue compartments by HPV status (all $P > .08$; results not otherwise shown). Similar analyses comparing lymphocyte infiltration amounts by HPV vaccination history also did not demonstrate any statistically significant differences (all $P > .08$; results not otherwise shown). There was no correlation noted between recent serum CD4 cell counts and mucosal T-lymphocyte counts (all R values ≤ 0.10, with corresponding P values $> .40$). An example of HIV+ anal HSILs with corresponding CD4+ and CD8+ mucosa-infiltrating lymphocytes is illustrated in Figure 2.

Efficacy of Electrocautery Ablation

A total of 78 patients (51 HIV+ and 27 HIV−) with biopsy-proven HSILs were treated with EA and underwent surveillance HRA. The average interval between diagnosis and ablation was 2 months (range, 1–10 mo). After EA, 21 HIV+ and 5 HIV− patients had biopsy-proven HSILs at the previously treated site (41% vs 19%; $P = .04$) (Table 3), with mean time-to-recurrence of 9 (range, 5–22) months and 9 (range, 6–16) months, respectively. The remaining 30 HIV+ and 22 HIV− patients had normal mucosa or LSILs, with mean follow-up time of 14 (range, 3–36) months and 10 (range, 4–23) months, respectively. The success of treatment was 67% (n = 52/78) overall, 59% (n = 30/51) for the HIV+ group, and 81% (n = 22/27) for the HIV− group.

There was no statistical difference in postablation HSIL incidence between the HIV+ and HIV− group (3.8 vs.2.0 per 100 person-months; $P = .20$). In the unadjusted analysis, none of

Table 1. Patient Baseline Characteristics According to Human Immunodeficiency Virus Status

Characteristic	HIV+ (n = 70)	HIV− (n = 45)	*P* value
Age, median (IQR)	41 (23–64)	42 (32–52)	.70
Race/ethnicity, no. (%)			
White	31 (44)	32 (71)	.01
Black	14 (20)	3 (7)	
Hispanic	19 (27)	5 (11)	
Other	6 (9)	5 (11)	
Smoking status, no. (%)			
Current smoker	17 (24)	5 (11)	.10
Former smoker	16 (23)	15 (33)	
Never smoker	37 (53)	25 (56)	
CD4 Count, median cells/mm^3 (IQR)	677 (469–865)	N/A	
HIV RNA (copies/mL), no. (%)			
<50	40 (57)	N/A	
50–1000	18 (26)		
≥1000	12 (17)	N/A	
High-risk HPV type, no. (%)			
16,18, or both	34 (49)	21 (47)	.007
Others	28 (40)	11 (24)	
Negative	3 (4)	0 (0)	
Unknown	5 (7)	13 (29)	
Anorectal cytology, no. (%)			
LSIL or less	49 (70)	26 (58)	.001
HSIL or ASC-H	17 (24)	5 (11)	
Unknown	4 (6)	14 (31)	
HPV vaccination, no. (%)	15 (21)	16 (36)	.20
Number of biopsy-proven HSILs, no. (%)			
Unifocal	20 (29)	29 (64)	<.001
Multifocal	50 (71)	16 (36)	

Abbreviations: ASC-H, atypical squamous cells, cannot rule out high-grade lesion; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; IQR, intraquartile range; LSIL, low-grade squamous intraepithelial lesion.

Table 2. Quantity of Mucosa-Infiltrating T Lymphocytes in Anal High-Grade Squamous Intraepithelial Lesions and Benign Anal Mucosa According to HIV Status and Compartment

	Anal HSIL			Benign anal mucosa		
T-cell subset	HIV+ (n = 70)	HIV− (n = 45)	P value	HIV+ (n = 11)	HIV− (n = 12)	*P* value
Total[a]						
CD4+	24[b]	25	.40	17[b]	14	.50
CD8+	47[b]	22[c]	<.001	11[b]	10[c]	.90
Intraepithelial compartment[a]						
CD4+	3[b]	1	.001	1[b]	1	.90
CD8+	15[b]	8[c]	<.001	2[b]	3[c]	.90
Stromal compartment[a]						
CD4+	20[b]	25	.80	12[b]	13	.40
CD8+	32[b]	14	<.001	7[b]	6	.60

Abbreviations: HIV, human immunodeficiency virus; HSIL, high-grade squamous intraepithelial lesion.

[a]Median cell count detected in 3 high-power fields (400×).

[b]Statistically significant comparisons between HIV+ HSILs and HIV+ benign mucosa ($P \leq .03$).

[c]Statistically significant comparisons between HIV− HSILs and HIV− benign mucosa ($P \leq .01$).

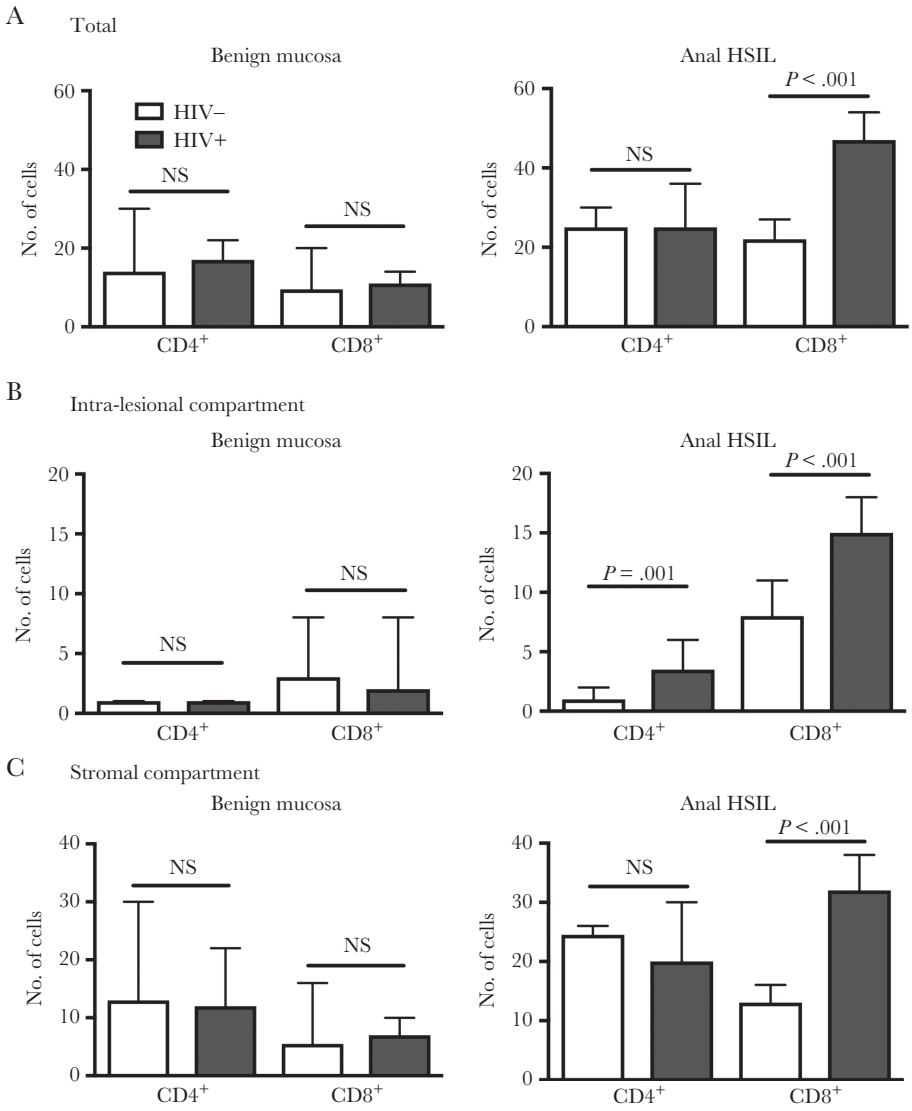

Figure 1. Quantity of mucosa-infiltrating T lymphocytes in anal high-grade squamous intraepithelial lesions and benign anal mucosa according to human immunodeficiency virus status and compartment. Abbreviations: HSIL, high-grade squamous intraepithelial lesions; NS, not significant.

the predictors (HIV status, current smoking, HPV vaccination, total CD4+ or CD8+ T cells, and CD4/CD8 ratio) (Table 4) revealed any significant associations with persistence or incidence of postablation HSILs. However, we observed a trend toward more ablation failures among HIV+ subjects (incidence rate ratio (IRR), 2.0; 95% confidence interval (CI), .8–4.9), as well as increased total CD8+ T cells (IRR, 2.3; 95% CI, .9–5.3).

DISCUSSION

Anal HSILs, the HPV-associated anal cancer precursors, follow a more virulent course in HIV+ MSM than in their HIV– counterparts [22, 23]. The underlying mechanisms for the progression of anal HSIL to cancer are yet to be fully established. Growing evidence indicates that impaired local, rather than systemic, immunity plays a critical role in anal carcinogenesis [24, 25].

In our cohort of 115 MSM, we found that anal HSIL immune microenvironments differed significantly by HIV status. The total number of mucosa-infiltrating lymphocytes, predominately CD8+ T cells, was more than double in HIV+ anal HSIL cases. The excess CD8+ T cells were distributed unevenly, tending to scatter in the basal portion of the dysplastic squamous epithelium and to concentrate in the mucosal lamina propria immediately underneath. In contrast, CD4+ T-cell density was comparable between the 2 groups and not associated with HIV status. Our findings suggest there is a distinct cell-mediated immune response potentially affecting anal precancerous lesions in the setting of HIV infection.

Human immunodeficiency virus infection is believed to create a more conducive microenvironment for HPV-induced precancerous lesions to persist and progress [26, 27]. It is puzzling why HIV selectively increases mucosal CD8+ cytotoxic T

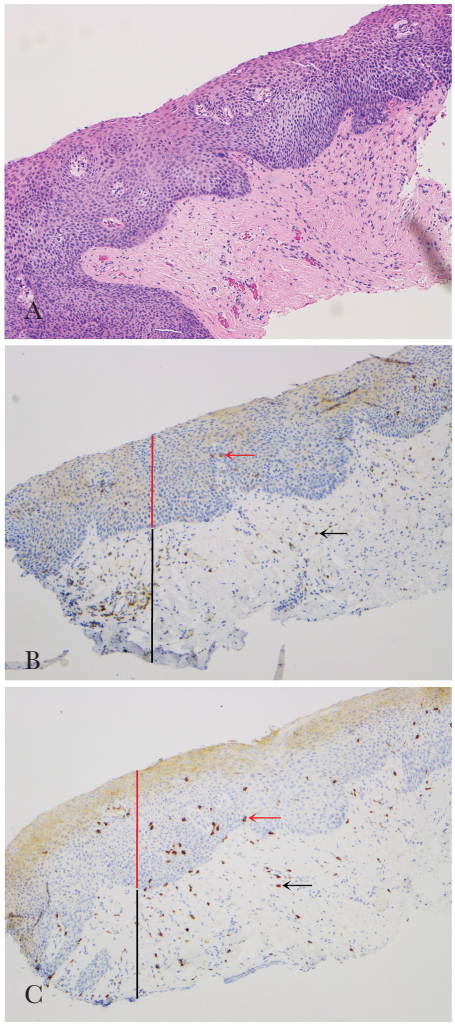

Figure 2. Example of human immunodeficiency virus–positive anal high-grade squamous intraepithelial lesions (HSILs) and corresponding mucosa-infiltrating T lymphocytes. *(A)*, The lesion shows severe cytological atypia and numerous mitoses in the squamous epithelium, consistent with HSILs. Hematoxylin and eosin, original magnification 100×. Scattered $CD4^+$ T lymphocytes (red and black arrows) (*B*) and numerous $CD8^+$ T lymphocytes (red and black arrows) (*C*) are present in the dysplastic epithelium (intraepithelial compartment, red line) and underlying lamina propria (stromal compartment, black line). Immunohistochemistry using antibodies against CD4 and CD8, original magnification 100×.

cells, major players in cellular immune defense and the clearance of viral infection. Several studies on cervical immunity have described similar phenomena, noting a marked increase of primarily $CD8^+$ infiltrates in HIV+ cervical mucosa, particularly in cervical HSILs [28, 29]. Furthermore, $CD8^+$ T cells predominate in cervical HSILs that progress to invasive cancer, whereas $CD4^+$ T cells tend to occupy lesions that spontaneously regress [30]. Taken together, increased mucosal $CD8^+$ infiltration appears to be a consistent finding; further investigation is needed to explain this counterintuitive phenomenon.

Treatment options for anal HSIL include local destruction of individual lesions using various ablative techniques [31]. Reported success rates of local ablation range widely from

Table 3. Recurrence or Persistence of Anal High-Grade Squamous Intraepithelial Lesions After Ablation According to Human Immunodeficiency Virus Status

Outcome	HIV+ HSIL (n = 51)	HIV− HSIL (n = 27)	*P* value
Persistence/recurrence of HSILs, no. (%)	21 (41)	5 (19)	.04
HSIL incidence rate after ablation, per 100 person-months (95% CI)	3.8 (2.4–5.9)	2.0 (.6–4.6)	.20

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; HSIL, high-grade squamous intraepithelial lesion.

35% to 88% depending on patient cohort, provider experience, and surveillance method and duration [32–34]. Nevertheless, postablation persistence and/or recurrence are common and significant limitations that require patients to undergo repeat treatment and long-term follow-up [35, 36]. In our cohort, postablation surveillance was pursued with HRA and biopsy of the previously treated site. A single, experienced provider (M. G.) performed all HRA and ablative procedures. The overall EA success rate was 67% (mean, 12-month follow-up; range, 3–36): 59% for the HIV+ group and 81% for the HIV− group. In unadjusted analyses, the HIV+ group trended toward a 2-fold higher risk for postablation persistence or recurrence. Although the associated risk is notable, it did not reach statistical significance, presumably because of our small sample size.

These results are similar to the findings Goldstone et al published in previous studies using infrared coagulation for the treatment of anal HSILs [37, 38]. The success rate in their series for HIV+ MSM was 65% versus 81% for HIV− MSM (median, 575 days follow-up). Analogous to our cohort, the risk of persistent HSIL among HIV+ MSM was twice as high as among HIV− MSM, but it reached statistical significance in their cohort. The authors speculated that the increased risk was due to a combination of impaired host immune function, HIV infection increasing the oncogenic potential of HPV, and/or a greater burden of disease.

Among the potential predictors in our study (HIV status, smoking history, total $CD4^+$ and $CD8^+$ T cells, CD4/CD8 ratio), longitudinal analysis showed that the quantity of mucosal $CD8^+$ infiltration was associated with the greatest increased risk for HSIL persistence following ablation, although this increase was not statistically significant. It is possible that these excess $CD8^+$ cells are a local manifestation of the dysfunctional immune activation that has been associated with adverse complications of HIV, including non-AIDS cancer, chronic obstructive pulmonary disease, and overall mortality [39, 40]. Contrary to the current view that tumor-infiltrating lymphocytes are associated with favorable outcomes in malignancies, Grabenbauer et al reported that large numbers of granzyme B^+ $CD8^+$ T cells had a significantly negative prognostic effect on the clinical outcomes of anal carcinoma, possibly attributable to the selection of therapy-resistant tumor cell clones [41–44].

Table 4. Unadjusted Poisson Regression Models Evaluating Predictors of High-Grade Squamous Intraepithelial Lesions Recurrence or Persistence Rates After Ablation

Predictor	Unadjusted incidence rate ratio (95% CI)
HIV status	2.0 (.80–4.9)
Current smoking	0.9 (.4–2.3)
HPV vaccination	0.9 (.4–2.1)
Total CD4 T cell	
<19 cells/HPF	Referent
19–29 cells/HPF	0.2 (.07–.8)
>29 cells/HPF	0.9 (.5–1.9)
Total CD8 T cell	
<26 cells/HPF	Referent
26–45 cells/HPF	1.5 (.6–4.2)
>45 cells/HPF	2.3 (.9–5.3)
CD4/CD8 ratio	
<0.46	Referent
0.46–0.96	1.2 (.6–2.5)
>0.96	0.5 (.2–1.3)

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; HPF, high-power field.

In summary, our study suggests that HIV alters the immune microenvironment of anal cancer precursors as demonstrated by the significant increase of local lymphocytic infiltrates, predominately $CD8^+$. Ablation resistance appears to be associated with HIV seropositivity and an excess of mucosa-infiltrating $CD8^+$ T lymphocytes. Further characterization of the function and interaction of these immune cells should assist in the development of preventive and immunotherapeutic treatments for this major source of morbidity in the HIV-infected population.

Notes

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