

The PD-1 and PD-L1 Pathway in Recurrent Respiratory Papillomatosis

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Objectives/Hypothesis: Generation of an immunosuppressive microenvironment may enable a persistent human papillomavirus infection in the setting of an otherwise normal immune system. We hypothesized that expression of the T-lymphocyte co-inhibitory receptor programmed death 1 (PD-1) and its ligand PD-L1 would be increased in the recurrent respiratory papillomatosis (RRP) microenvironment compared to normal controls.

Study Design: Case-control study.

Methods: Formalin-fixed paraffin-embedded respiratory papilloma and normal controls were obtained under institutional review board approval, stained for CD4, CD8, FoxP3, and PD-1, and scored by automated cell count. PD-L1 staining was scored by a blinded pathologist using an adjusted inflammation score that accounted for epithelial and immune infiltrate.

Results: Thirty-nine RRP cases and seven controls were studied. All immunologic markers demonstrated significantly increased staining in RRP specimens compared to normal controls (all $P < .01$). PD-1 correlated with both CD4 ($P < .0001$) and CD8 ($P < .001$) cell counts. Epithelial staining for PD-L1 (68%) and PD-L1+ infiltrating immune cells (76%) were observed in the majority of papilloma samples. The strongest staining for PD-L1 was usually observed in the basal papilloma layer adjacent to the immunologic infiltrate in the vascular core. Disease severity inversely correlated with CD8 cell counts ($P = .01$). A correlation between disease severity and other immunologic markers was not observed.

Conclusions: Most RRP specimens demonstrate PD-1 T-lymphocyte infiltration and PD-L1 expression on both papilloma and infiltrating immune cells. This study suggests that this checkpoint pathway may be contributing to local immunosuppression in RRP, and opens the door for clinical trials utilizing PD-blocking monoclonal antibodies.

Key Words: Recurrent respiratory papillomatosis, laryngeal papilloma, human papillomavirus, programmed death.

Level of Evidence: NA

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INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a chronic viral infection of the aerodigestive tract that causes a debilitating, chronic disease in both pediatric and adult populations.¹ Several lines of evidence highlight the importance of the immune system in controlling human papillomavirus (HPV) infection and its associated neoplasms, including RRP.² Despite the fact that 5% of the population show evidence of laryngeal HPV infection,³ only a very small percentage develop RRP. Patients with RRP have a normally functioning systemic immune system by overall

measures of immune function,⁴ and do appear to mount a peripheral antibody response to the viral proteins.⁵ However, the local cytokine and immune cell environment established by the papilloma is T helper cell (Th)-2 biased,⁶ polarizing effector T lymphocytes away from a Th1-type cytotoxic CD8+ T-cell response (CTL) that could clear virally infected cells.

One mechanism of CTL inhibition within papillomas is inadequate interleukin (IL)-2/interferon- γ expression and high IL-4/IL-10 expression that maintains maturation failure of CD8+ T cells.⁶ Cytokine barriers prevent ingress of CTLs,⁷ and there is an enriched population of immune suppressive FoxP3+ T cells.⁸ Expression of programmed cell death-1 (PD-1) and its ligand programmed cell death ligand-1 (PD-L1) is another hypothesized immunosuppressive pathway in RRP.⁸ PD-1 is a T-cell receptor that when activated provides a negative feedback signal for T-cell function⁹ and is a marker for CD8+ T-cell exhaustion.¹⁰ PD-L1 is more highly expressed in active papilloma than clinically normal epithelium in RRP patients, and a large population of PD-1+CD4+ T cells may function to stabilize the regulatory T-cell (Treg) population.¹¹ However the cell type(s) expressing PD-L1 and their location are not currently known in RRP, colocalization of PD-1 and PD-L1 has not been demonstrated, and there is no information on the relationship between PD-1 pathway component expression and clinical outcomes or patient demographics in RRP.

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TABLE I.
Clinical Demographics.

Variable	Patients, n = 39
Age, yr	
Mean (SD)	36 (22)
Range	3–84
Gender, no. (%)	
Male	22 (56%)
Female	17 (44%)
Disease onset, no. (%)	
Juvenile	26 (67%)
Adult	13 (33%)
Disease severity, no. (%)	
Mild/moderate	26 (67%)
Severe	13 (33%)

SD = standard deviation.

We therefore sought to identify PD-1 and PD-L1 expression in papilloma samples using immunohistochemistry, describe differences in their expression compared to normal controls, and correlate their expression to patient demographics and disease severity.

MATERIALS AND METHODS

Case Materials

Formalin-fixed paraffin-embedded papilloma tissue was obtained through the archives of surgical pathology at the Johns Hopkins Hospital according to institutional review board (IRB)-approved protocols. Supraglottic mucosa collected under IRB approval from patients undergoing phonosurgery for benign vocal fold pathology served as normal controls, and all normal controls were nonsmokers. Demographic and clinicopathologic data were obtained from patient electronic medical records. Disease severity was scored as a linear variable by taking the number of laryngeal subsites involved with papilloma,¹² divided by the number of days between surgeries, and averaged over the previous five surgeries to minimize variability. Subsites affected by papilloma were extracted from operative notes and archived clinical images from clinical examinations prior to surgery. This score incorporates disease extent as well as recurrence, and replicates retrospectively the prospective severity scoring system of Abramson et al.,¹³ which has been used for many studies of immune function in RRP.

Immunohistochemistry

The CD4, CD8, and FoxP3 immunohistochemistry (IHC) was performed by the pathology laboratories of the Johns Hopkins Hospital. PD-1 and PD-L1 staining was performed manually, with tissue sections deparaffinized and heat-induced antigen retrieval performed in Tris-ethylenediaminetetraacetic acid buffer. After blocking, the sections were incubated with PD-1 (315M-96; Cell Marque, Rocklin, CA) or PD-L1 (SP142; Spring Bioscience, Pleasanton, CA) anti-human polyclonal antibodies followed by a secondary biotinylated anti-goat immunoglobulin (Ig)G (Jackson ImmunoResearch Laboratories, West Grove, PA). For detection, ABC-HRP (Vector Laboratories, Burlingame, CA) was used, and sections were visualized with the substrate diaminobenzidine (DAB) (Vector Laboratories) and counterstained with hematoxylin.

Immunohistochemistry Scoring

To minimize scoring variability and allow for quantitative analysis, markers with intracellular expression (CD4, CD8, FoxP3, and PD-1) were scored using automated scanning (Aperio ImageScope; Leica Biosystems, Buffalo Grove, IL) to collect 10 random high-power fields (HPFs) per slide and count positive cells (ScanScope software; Leica Biosystems). As PD-L1 exhibited a more complex staining pattern, including membranous staining, these were scored by a blinded pathologist (J.A.B.). PD-L1 staining was scored using an adjusted inflammation score (AIS), which takes into account both membranous staining of tumor tissue and the degree of PD-L1 expression on infiltrating cells.¹⁴ The final AIS score is obtained by multiplying the percent of PD-L1 positive tumor cells (e.g., <5, 5, 10, 20, 30) by the intensity of intratumoral inflammation including tumor-infiltrating lymphocytes (TILs) and histiocytes (graded 0 to 3).

Statistical Analysis

Sample size was estimated for dichotomous variables of staining intensity and disease severity with a Fisher exact test and a 2 × 2 contingency table. Using a two-sided α of .05 and β of .2, and given equal distribution between severe and mild disease, we estimated it would require a sample size of 20 patients in each group to detect a 40% difference in expression of markers between the two groups, within the range previously reported for PD-1 expression as assessed by IHC. Clinical demographics were summarized using descriptive statistics. Mean values of positive cells/HPF were compared using the Student *t* test. Correlations between disease severity and immunologic markers was performed by calculating Pearson correlation coefficients. Significance was attributed to a $P < .05$.

RESULTS

Of the known cohort of 212 Johns Hopkins RRP patients, 39 RRP patients were selected for this study, and their demographic data are summarized in Table I. These patients were selected based upon the availability of tissue for analysis, in addition to surgery and follow-up at Johns Hopkins Hospital that allowed for estimation of disease severity. Attention was also paid to maintain a balance between age, male/female patients, juvenile/adult onset, and disease severity to ensure our selected cases were representative of RRP without a priori assumptions about expression patterns.

Representative photomicrographs for the IHC performed in this study are shown in Figure 1. TILs were variably positive for CD4, CD8, FoxP3, and PD-1. Consistent with recruitment of inflammatory cells, TIL markers were variably positive within papillomas and cumulatively demonstrated significantly greater TIL marker positivity than normal squamous epithelium (Fig. 2) ($P < .01$ for all markers). There is a range of positivity between the RRP samples, with some showing little TIL marker staining and others showing robust TIL marker positivity. Using PD-1 as an example, papilloma samples ranged in positivity from 0 to 73 (mean = 21) PD-1+ cells/HPF compared to 1 to 6 (mean = 4) PD-1+ cells /HPF in normal controls. There was a strong relationship between CD4+ and CD8+ staining ($r = 0.26$, $P < .001$), CD4+ and PD-1+ ($r = 0.37$, $P < .0001$), CD8+ and PD-1+ ($r = 0.27$, $P < .001$), and CD4+ and FoxP3+ ($r = 0.34$, $P < .001$). There was no significant

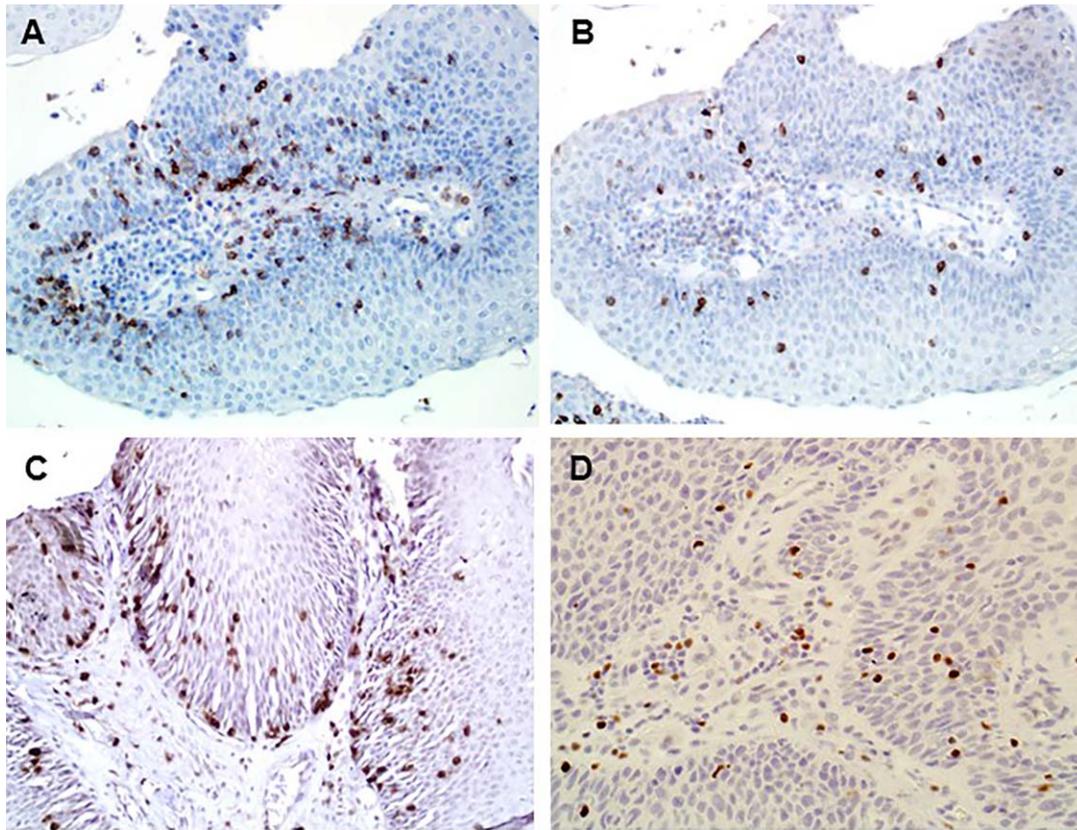


Fig. 1. Tumor-infiltrating lymphocytes in recurrent respiratory papillomatosis. Presence of tumor infiltrating lymphocytes confirmed in papilloma by immunohistochemistry for CD4 (A), CD8 (B), PD-1 (C), and FoxP3 (D). Magnification 200 \times .

correlation between CD8+ and FoxP3+ staining ($r = 0.02$, $P = .3$).

Thirty-four of the 39 cases had adequate tissue available for PD-L1 staining, and representative photomicrographs are shown in Figure 3. Two main types of staining patterns were seen—membranous staining on the squamous papilloma itself (Fig. 3A) and staining of infiltrating immune cells (Fig. 3B). This staining pattern is similar to that observed in melanoma¹⁴ and head and neck cancer.¹⁵ The strongest PD-L1 staining often occurred on the basal layer of the papilloma, directly adjacent to the vascular core that contains circulating immune cells (Fig. 3C,D). Using dual-color IHC, colocalization of PD-1 and PD-L1 staining was seen in the vascular core of the papilloma (Fig. 3E). Evaluated in a binary fashion as positive or negative staining, 68% (23/34) of papillomas exhibited some degree of positive epithelial cell staining, and 76% (26/34) demonstrated PD-L1+ infiltrating immune cells. Staining on less than 5% of papilloma cells was defined as negative (Fig. 3F) to maintain consistency with previous studies.^{14,16} No staining was seen on control samples. Papilloma PD-L1 positivity was further assessed via calculating of an AIS (Fig. 4) that accounts for both epithelial and infiltrating immune cell staining. This scoring system quantifies PD-L1 positivity as a linear variable, and demonstrates the wide range of PD-L1 expression that exists within RRP samples.

Finally, we correlated the disease severity score with TIL marker staining (Table II). Consistent with others studies, there was a negative correlation between CD8 staining and disease severity ($r = -0.35$, $P = .01$).⁶ CD4, FoxP3, PD-1, PD-L1 staining and AIS did not correlate with disease severity.

DISCUSSION

This study demonstrates at the protein level that the majority of RRP samples demonstrate infiltration of PD-1-positive immune cells and PD-L1 expression on both epithelial and infiltrating immune cells. Because anti-PD-1/PD-L1 monoclonal antibodies are now Food and Drug Administration (FDA)-approved for selected advanced and metastatic malignancies including head and neck squamous cell carcinoma,^{17,18} evidence suggesting that this pathway may be playing a role in local immune suppression within RRP samples has important translational research implications.

Our findings support the only previous study to demonstrate PD-1 expression in RRP samples.⁸ Because we evaluated inflammatory markers by IHC retrospectively in a large sample of patients rather than by flow cytometry, dual staining was not performed to specifically characterize the PD-1+ TIL population. Nevertheless, the strong correlation between CD4+ and PD-1+

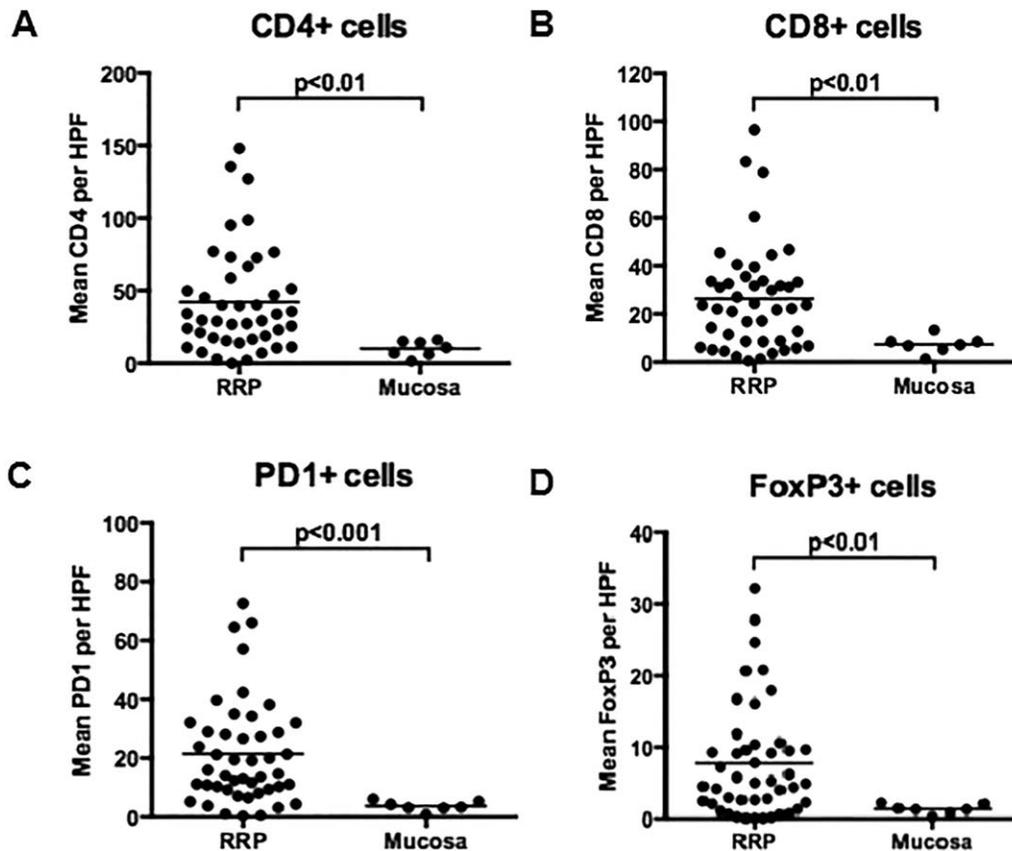


Fig. 2. Squamous epithelium in papilloma (n = 39) contains a significantly greater number of CD4 (A), CD8 (B), PD-1 (C), and FoxP3 (D) cells than normal laryngeal epithelium (n = 7). All $P < .01$. HPF = high-power field; RRP = recurrent respiratory papillomatosis.

infiltrating immune cells very likely represents the same CD4+PD-1+ T-lymphocyte population previously described. Similarly, we observed correlation between CD4+ and FoxP3+ staining, consistent with the known enhancement of a CD4+FoxP3+ Treg population in papilloma compared to blood from the same patients.⁸ A significant correlation between PD-1 and CD8 staining likely identifies an exhausted CD8+ T-lymphocyte population in RRP, often present in other solid tumors.^{8,19,20} The range of PD-1 positivity and variability amongst samples is interesting to note, including some samples that show no increased expression compared to normal controls. This suggests that the immunologic phenotype may vary considerably between patients, temporal changes may exist in expression patterns of PD-1 not captured on a single biopsy, or potential heterogeneity in the immune microenvironment even within a single patient's papilloma are all possibilities for future investigation.

Furthermore, we demonstrate that a high percentage of papilloma samples exhibit PD-L1 expression on the squamous papilloma itself, often in the basal layer of the epithelium, adjacent to where interferon-producing immune cells would encounter papilloma cells. This same pattern of colocalization between PD-1+ immune cells and PD-L1+ epithelial and immune cells has been described in HPV-associated oropharyngeal squamous cell carcinoma, at the tumor-host interface²¹ as well in

HPV-associated cervical cancer,²² and suggests that PD-pathway-mediated adaptive immune resistance may be playing a role in local immune suppression within the RRP tumor microenvironment.²³ Infiltrating immune cell types expressing PD-L1 in these RRP samples is poorly defined, but may include both T lymphocytes and antigen-presenting cells such as dendritic cells.²⁴

The correlation between PD-1 and PD-L1 expression and clinical outcomes is complicated by the numerous cell populations that can express these markers. Acknowledging the limitations of a retrospective analysis of disease severity using averaged intersurgical interval and disease distribution, we did not observe a significant correlation between our scoring of disease severity and PD-1 or PD-L1 expression, although we did identify an inverse relationship between higher CD8+ staining and decreased disease severity, a finding that collaborates the work of Bonagura et al.⁶ The presence of CD8+ infiltrating immune cells, likely representing CD8+ T lymphocytes, in the majority of RRP samples, suggests the presence of one or more mechanisms of suppression of T-lymphocyte function within these lesions. The strong correlation between CD8 and PD-1 staining suggests that a significant subset of RRP-infiltrating CD8+ T lymphocytes may be susceptible to PD-mediated inhibition in the presence of PD-L1. In HPV-associated oropharyngeal squamous cell carcinoma, high PD-1 (CD4+PD-1+ and CD8+PD-1+) infiltration in the

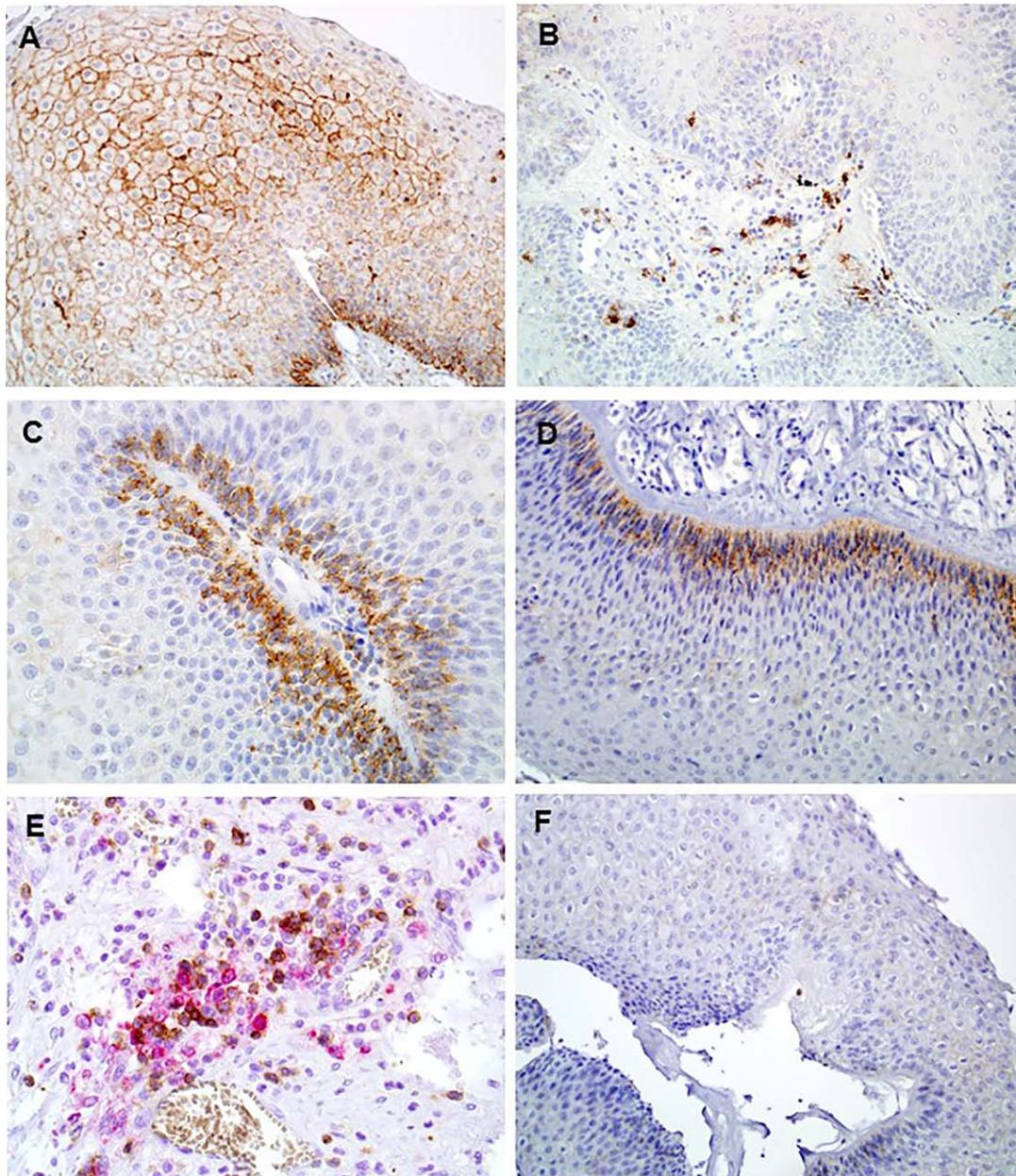


Fig. 3. PD-L1 expression in recurrent respiratory papillomatosis (RRP). Two main patterns of PD-L1 staining are seen, either membranous staining of squamous papilloma itself (A) or mononuclear inflammatory cells (B). In multiple cases, the basal layer of papilloma, adjacent to the vascular core, was the strongest site of membranous staining (C, D). Dual color immunohistochemistry (E) demonstrates that PD-1 (brown) and PD-L1 (red) expression colocalizes in the vascular core of RRP. Background staining in cases with no PD-L1 expression was minimal (F). Magnification 200 \times .

tumor was associated with improved overall survival.²³ The opposite effect is seen in a number of other diseases, such as renal cell carcinoma²⁵ or nasopharyngeal cancer,²⁶ where PD-1 expression is associated with a poor outcome. Tim-3 coexpression, which in combination with PD-1 is a marker of T-cell exhaustion, may differentiate between functionally exhausted or activated T lymphocytes²⁷ and could possibly clarify these discrepancies.

Taken together, these results provide evidence that the PD-1 and PD-L1 pathway may be playing a role in local immune suppression in RRP. The presence of PD-pathway receptors and ligands in the majority of

analyzed samples provides a strong rationale for investigating the role of PD-1 or PD-L1 checkpoint inhibitors in patients with RRP not well controlled with standard of care surgical interventions. Separate clinical trials investigating the clinical effect of the PD-1 mAb pembrolizumab and PD-L1 mAb avelumab are currently underway, and given the high rates of PD-1 and PD-L1 expression in the RRP population, many patients may be eligible for these trials. Furthermore, as expression of PD-L1 has been shown to be a predictor of disease response to checkpoint inhibitor therapies in certain tumor types,²⁸ correlation between baseline or induced

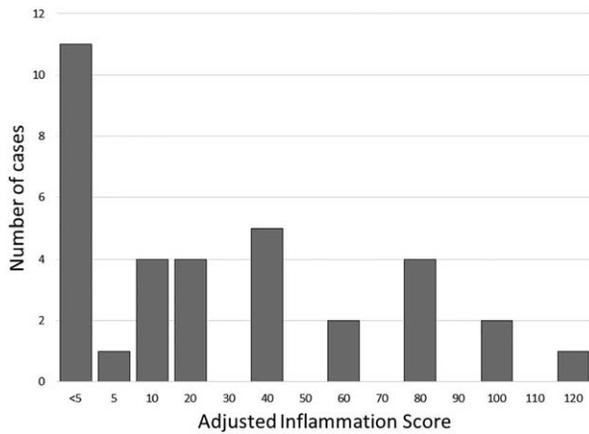


Fig. 4. Histogram of adjusted inflammation score for PD-L1 staining. This scoring scale incorporates both membranous staining of PD-L1 on the papilloma as well as the degree of PD-L1 positive infiltrating immune cells.

TABLE II.
Disease Severity Correlations.

Marker	Correlation With Disease Severity	P Value
CD4	0.16	.27
CD8	-0.35	.01
PD-1	0.15	.30
Fox P3	-0.01	.93
PD-L1	0.18	.26
AIS	0.10	.49

AIS = adjusted inflammation score.

PD-L1 expression and clinical response to checkpoint inhibitions may define the RRP patient population most likely to benefit from these immunotherapies.

CONCLUSION

The majority of RRP specimens demonstrate PD-1-positive infiltrating immune cells and PD-L1 expression on both squamous papilloma and infiltrating immune cells. This suggests that the PD-immune checkpoint pathway may be contributing to local immunosuppression in the RRP microenvironment, and provides a strong rationale for the execution of clinical trials utilizing PD-pathway blocking monoclonal antibodies in patients with aggressive RRP.

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