Immune Suppression in Premalignant Respiratory Papillomas: Enriched Functional CD4+ Foxp3+ Regulatory T Cells and PD-1/PD-L1/L2 Expression

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Abstract

**Purpose:** Respiratory papillomas, caused by human papillomaviruses types 6 and 11 (HPV6/11), are premalignant lesions with potential for malignant conversion. The cytokine and chemokine micromilieu of papillomas is T\(_{H2}\)-like with a marked absence of IFN-\(\gamma\) expression. To illuminate why patients with recurrent respiratory papillomatosis (RRP) fail to effectively control their disease, we further investigated the suppressive cellular microenvironment in papillomas.

**Experimental Design:** CD4\(^+\)CD25\(^{low}\)/Foxp3\(^+\) regulatory T cells (Treg) and CD4\(^+\)CD25\(^{low}\)/Foxp3\(^+\) T cells within papillomas were characterized and isolated. Their suppressor function was measured by inhibition of peripheral blood mononuclear cell (PBMC) proliferation. Expression of PD-1, CD69, and Helios was identified on these T cells. PD-L1, PD-L2, CCL17, and CCL22 mRNA was also identified in papillomas by quantitative PCR.

**Results:** Functional Tregs were markedly enriched in papillomas and strongly inhibited anti-CD3 and anti-CD28 antibody activated PBMC proliferation. The natural Treg marker Helios was reduced on Tregs from papillomas, indicating that the majority of Tregs in papillomas are adaptive. The majority of the papilloma-derived CD4\(^+\) T cells expressed the CD4\(^+\)CD25\(^{low}\)/Foxp3\(^+\)PD1\(^-\)CD69\(^-\) phenotype and failed to suppress PBMC proliferation, suggesting that they are chronically activated and exhausted. The Treg-attracting chemokine CCL22 was equally expressed by all laryngeal tissues examined. However, CCL17 was robustly expressed by papillomas compared with unaffected laryngeal tissues from RRP patients and individuals without RRP. PD-L1 was elevated in papillomas compared with control laryngeal tissues.

**Conclusions:** Papilloma CD4\(^+\) T cells are enriched with functional Tregs, and the adaptive Helios-Treg fraction was increased within the T\(_{H2}\)-like papilloma micromilieu. CD4\(^+\)CD25\(^{low}\)/Foxp3\(^+\) T-cells failed to suppress PBMC proliferation and may be exhausted. The PD-1/PDL-1 pathway may represent an additional immunosuppressive mechanism that contributes to defective HPV6/11 clearance in RRP. Clin Cancer Res; 18(7): 1925–35. ©2012 AACR.

Introduction

Recurrent respiratory papillomatosis (RRP) is characterized by the relentless recurrence of laryngeal papillomas that are induced by human papillomavirus (HPV) types 6 and 11. Laryngeal papillomas are premalignant lesions with potential for malignant conversion. This rare disease has an estimated incidence in the United States of 4.3/105 in children less than 14 years old, and of 1.8/105 in adults. The estimated cost of surgical treatment for RRP in the United States is greater than 100 million USD per year (1).

The strategic location of these lesions in the larynx and upper airway causes significant morbidity and, on occasion, mortality. Patients with severe disease can require more than 150 general surgeries under general anesthesia to maintain a patent airway. The interval between surgical interventions can vary between patients ranging from 3 weeks to several years. Laryngeal disease can spread to the lower respiratory tract, and on occasion RRP can progress to malignancy (1, 2).

The persistent recurrence of HPV6/11 infection in these lesions highlights the failure of RRP patients to generate
Translational Relevance

Respiratory papillomas induced by human papillomaviruses 6 and 11 are premalignant lesions with potential for malignant conversion and cause significant morbidity and, on occasion, mortality. Standard therapy for recurrent respiratory papillomatosis (RRP) is surgical extirpation, which significantly impacts on lifestyle (>150 surgeries per lifetime) and medical costs (>100 million US dollars yearly). The cytokine–chemokine micromilieu of papillomas is Th1-like with markedly reduced IFN-γ. We further investigated the microenvironment in papillomas toward ultimately developing a nonsurgical, medical treatment for RRP. Our studies show for the first time that papillomas contain an enrichment of functional CD4+ regulatory T cells (Treg), express Treg/Th1-like tropic chemokines (CCL17, CCL22), and contain T cells that are CD127 negative and express CD69 and PD-1, a phenotype of chronically activated and exhausted T cells. PD-L1, an inducer of effector T-cell dysfunction, is also expressed in papillomas. These studies provide potential new targets for future medical therapies that can reverse the immunosuppressive micromilieu in papillomas by restoring effective Th1-like anti-HPV responses.

effective HPV-specific, innate and/or adaptive immune responses, although they show normal immune homeostasis, and respond normally to other infectious microbes (1). Thus, a better understanding of the HPV-specific, T-cell dysfunction in RRP that supports chronic HPV infection of laryngeal keratinocytes could provide a rationale to develop nonsurgical strategies that would correct this defect.

Previously we described RRP as a Th1-like disease that is characterized by an imbalance in Th1/Th2-like cytokine and chemokine expression (1–3). We identified a correlation between RRP disease severity and the expression of select class II MHC genes that likely regulate this imbalance (1–4) and predispose HPV6/11-infected individuals to develop RRP (1, 4). Recently, we described differences in the repertoire of killer cell immunoglobulin-like receptor (KIR) gene haplotypes in RRP as compared with controls (1). In addition, we also identified natural killer cell dysfunction in RRP (1) and differential expression of Th1-like polarizing immune response genes in papillomas compared with autologous unaffected laryngeal tissue (1, 5).

The objective of this study was to further characterize the repertoire of T-cell subpopulations within tissue-infiltrating lymphocytes (TIL) from respiratory papillomas that support chronic HPV6/11 infection in these lesions.

In this article, we provide evidence for the first time that there is an enrichment of functional CD3+CD4+CD25−Foxp3− regulatory T cells (Treg; ref. 6) that infilitrate HPV6/11 induced, respiratory papillomas. We also identified the two chemokines (CCL17 and CCL22) that recruit Tregs into tissues where they are expressed (7). In addition, we show that the majority of CD4+Foxp3+ TILs (non-Tregs) in papillomas express a surface phenotype that is characteristic of exhausted T cells (PD1+CD69+CD25−CD127+; refs. 8–10), suggesting that this major T-cell fraction is chronically activated, quiescent, and unable to produce effector cytokines in response to these HPVs. Furthermore, we provide evidence that the PD-1/PD-L1 pathway (11) is likely active in RRP, given that papilloma-derived TILs express PD-1 strongly, and that papilloma tissue robustly expresses PD-L1, compared with control-derived laryngeal tissues. Taken together, these mechanisms may contribute to the immunosuppressive microenvironment in papillomas that prevent HPV-specific, effector Th1-like T-cell function in RRP.

Materials and Methods

Subjects and index of disease severity

Thirty-seven randomly selected patients with RRP with varying disease severity were studied. Before enrollment, all patients signed informed consent that was approved by the Institutional Review Board at the North Shore-LIJ Health System. Table 1 shows the demographics of all patients with RRP included in this study.

Heparinized blood and biopsies of autologous respiratory papillomas were obtained during surgical intervention. HPV typing was carried out as previously described in detail (3, 12). Disease severity is defined by a composite score that represents the extent of disease and is inversely proportional to the frequency of recurrence (1). The composite score was used to define the overall severity for an individual patient. Three or more surgeries in the previous 12 months with a

<table>
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<tr>
<th>Patients</th>
<th>All</th>
<th>Severity score</th>
<th>Male gender</th>
<th>Juvenile onset</th>
<th>Ethnicityb</th>
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<tr>
<td>Mild/moderate</td>
<td>20</td>
<td>0.004–0.048</td>
<td>13 (65)</td>
<td>7 (35)</td>
<td>W 3 B 1 A 1</td>
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<td>Severea</td>
<td>17</td>
<td>0.061–0.685</td>
<td>11 (65)</td>
<td>7 (41)</td>
<td>16 1 0</td>
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NOTE: Values in parenthesis are expressed in percentage.

aIncludes 3 patients with tracheal involvement.
bW, White/Caucasian; B, Black/African-American; A, Asian.
numeric score of 0.06 or more, or the extension of disease into the trachea, was defined as severe. A severity score of less than 0.06 was defined as mild/moderate disease (1). Severity scores for these study patients ranged from 0.004 to 0.685. Seventeen patients had mild/moderate RRP (score 0.004–0.048), and 17 had severe disease (score 0.061–0.685).

Isolation of PBMCs and CD4⁺ T regulatory cells from respiratory papillomas and peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus gradient centrifugation (GE Healthcare). These PBMCs were stained for surface and intracellular markers, or used as responders in the in vitro suppression assay described below. TILs were isolated from fresh papilloma biopsies by mechanical dissociation, and the resulting cell suspension was passed through a 70-micron mesh filter. Tregs were isolated from TILs using a CD4⁺ CD25⁺ Regulatory T-cell Isolation Kit (Miltenyi Biotech), as recommended by the manufacturer.

In addition to the isolation of cells by bead purification, effector memory CD3⁺ CD4⁺ CD122⁺low/CD25⁻ T cells (13) were isolated by cell sorting on a fluorescence-activated cell sorting (FACS) Aria IIu flow cytometer. Minced papilloma tissue was filtered, washed, resuspended in sorting buffer (0.25% bovine serum albumin/PBS with 1 mM EDTA) and surface stained with antibodies to CD3-Pacific Blue, CD4-FITC, CD127-PerCP-Cy5.5, and CD25-PE. Aliquots of the sort purified T-cell subpopulation were cocultured with autologous PBMCs in the suppression assay described below.

Antibody staining and flow cytometric analyses

PBMCs and TILs were washed in PBS containing 2% FBS and 0.1% sodium azide and surface stained for Tregs using the following combinations of directly conjugated antibodies: anti-CD4 PerCP, anti-CD25 FITC, and anti-CD127 PE (BD Biosciences). After washing, cells were stained for intracellular FoxP3 (anti-FoxP3-APC, clone PCH101, ebioscience) using the manufacturer’s recommended procedure. Stained and washed cell suspensions were resuspended in 1% formaldehyde. Additional specimens were stained for surface expression with anti-CD3-Pacific Blue, anti-CD28 (Pharmingen) monoclonal antibodies. Cell suspensions were incubated for 6 days in 96-well, round bottom plates in RPMI supplemented with 10% bovine serum. One μCi of ³H-thymidine was added to each well for the last 18 hours of incubation. Plates were harvested as previously described (14). The proliferative responses were expressed as mean ³H-thymidine incorporated counts per minute (CPM) of triplicate wells minus background.

mRNA expression of CCL17, CCL22, and programmed cell death ligands, PD-L1 and PD-L2

To determine whether papillomas expressed CCL17 and CCL22, known to be chemoattractants for Tregs (7), or expressed PD-L1 (CD274) and/or PD-L2 (CD273), the ligands for PD-1 (CD279; ref. 11), we determined the relative expression of these genes by quantitative real-time reverse transcriptase PCR (qRT-PCR). Relative levels of mRNA from papillomas (PAP, n = 9), clinically normal laryngeal tissues from RRP patients (normal adjacent, NA, n = 11), and controls without RRP (true normal, TN, n = 6) were determined. Briefly, total RNA was isolated using nucleic acid affinity spin columns (Qiagen) together with DNase-1 digestion. 1-Script One-step RT-PCR for Probes (BioRad) was done on an Applied Biosystems 7900HT thermocycler with gene-specific, intron-spanning, primers and either the appropriate FAM-labeled probe from the Universal Probe Library (Roche) or a FAM/TTMRA custom TaqMan probe (Supplementary Table). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the input RNA. mRNA expression of CCL17, normalized to GAPDH, was calculated relative to the mean NA tissue, using the ΔΔCt method (5). Similarly, mRNA expression of PD-L1 and PD-L2, normalized to GAPDH, was calculated relative to TN PD-L1 expression. The expression efficiency of the PD-L1 and PD-L2 probe/primer sets were linearly efficient among the range of cycles measured in this assay, allowing for cross-primer relative expression calculations. Data from each group had similar SDs, confirmed by the method of Bartlett, and were Gaussian distributed, confirmed by the method of Kolmogorov and Smirnov. Therefore ANOVA was used to analyze the 3 groups (TN, NA, and PAP), and a Turkey–Kramer
multiple comparison test and 2-tailed unpaired t tests were done to compare between 2 groups.

Statistical analysis
All statistical analysis employed a 2-tailed, unpaired t test done by InStat (GraphPad). When the data was not normally distributed, it was log-transformed, and if the SD was different between groups, a Welch correction was applied.

Results
The frequency of Foxp3⁺CD127low⁻ Tregs are increased in CD4⁺ T cells from papillomas compared with blood of patients with RRP

The flow cytometric strategy for identifying Tregs within TILs from a respiratory papilloma, and from the blood of the same patient, is shown in Fig. 1. CD4⁺ Tregs were identified by their expression of Foxp3⁺CD127low⁻, CD25⁺CD127low⁻, or the coexpression of both Foxp3 and CD25 on CD4⁺ T cells (Fig. 1A–E). The frequency of Tregs with bright Helios expression in papillomas was 3-fold greater than in the corresponding CD4⁺ T-cell fraction from the autologous PBMCs (Fig. 1C and D, top vs. bottom rows). Some Foxp3⁺CD127low⁻ CD4⁺ T cells in the papilloma were negative, or showed reduced expression of CD25, in contrast to the strong CD25 coexpression by the majority of Foxp3⁺CD127low⁻ CD4⁺ cells in PBMCs (Fig. 1E).

Figure 2A shows the composite results of 17 patients with RRP. Foxp3⁺CD127low⁻ Tregs were increased in CD4⁺ TILs obtained from respiratory papillomas (25.0% ± 8.0% of CD4⁺ T cells), compared with CD4⁺ T cells from autologous PBMCs (7.3% ± 1.8% of CD4⁺ T cells, P < 0.0001). Similar frequencies were observed for the CD25⁺CD127low⁻ subset of CD4⁺ cells in the TILs compared with PBMCs (19.5% ± 6.7% and 7.8% ± 1.8%, respectively, P < 0.0001). Tregs were increased in the tissue compared with blood of all patients studied, as shown by a tissue-to-blood ratio that ranged from 1.7 to 6.9 (mean ± SD = 3.5 ± 1.4). The frequency of Foxp3⁺ Tregs in CD4⁺ TILs from papillomas obtained from 5 patients with mild/moderate disease versus 12 patients with severe disease (27% ± 11% vs. 24% ± 7%, respectively) did not predict disease severity. In addition, the Foxp3⁺CD127low⁻/CD4⁺ cell fraction with reduced expression of CD25 (Fig. 2B) was prominent in TILs, but only a minor population in blood (46.7% ± 13.9% and 13.4% ± 5.4% of Foxp3⁺ CD4⁺ T cells, respectively (P < 0.0001), consistent with that reported in the blood of controls without RRP (15, 16).

The ratio of natural versus adaptive Tregs is altered in papillomas

The transcription factor Helios has recently been reported to distinguish nTregs from extrathymically derived adaptive Tregs (aTregs) and is expressed on CD3⁻CD16⁻CD56⁻CD8⁻CD127low⁻CD4⁺ Tregs identified by these surface markers in TILs from this papilloma was 3-fold greater than in the corresponding CD4⁺ T-cell fraction from the autologous PBMCs (Fig. 1C and D, top vs. bottom rows). Some Foxp3⁺CD127low⁻ CD4⁺ T cells in the papilloma were negative, or showed reduced expression of CD25, in contrast to the strong CD25 coexpression by the majority of Foxp3⁺CD127low⁻ CD4⁺ cells in PBMCs (Fig. 1E).

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The ratio of natural versus adaptive Tregs is altered in papillomas

The transcription factor Helios has recently been reported to distinguish nTregs from extrathymically derived adaptive Tregs (aTregs) and is expressed on 70% (on average) of Foxp3⁺ Tregs in human controls (17). Therefore we analyzed both the TILs and PBMCs of 6 patients for Helios expression on CD3⁻CD16⁻CD56⁻CD8⁻CD127low⁻ CD4⁺ T cells. The frequency of Tregs with bright Helios expression was significantly reduced in papilloma TILs compared with blood (31.2% ± 18.51% TILs, vs. 66.1% ± 6.5% blood, P < 0.005; Fig. 2C and D). Concomitantly, there was a significant increase in the Helioslow⁻ Tregs subpopulation in papilloma TILs, suggesting that within the papilloma microenvironment, there are more aTregs than in the blood.
The functional capacity of Tregs isolated from TILs was determined using an in vitro micro coculture suppression assay (Fig. 2E). CD25⁻CD4⁺ cells, enriched by magnetic bead selection from the papillomas of patients with RRP, were cocultured with anti-CD3 and anti-CD28 activated, autologous PBMCs. There was marked suppression of PBMC proliferation (66% ± 11.8%) when autologous Tregs, enriched from the papilloma tissue, were added. In contrast, when autologous additional blood-derived CD4⁺ T cells were added back as a control to similarly activated PBMCs, proliferation was enhanced. The mean ± SD suppression ratios were calculated by dividing the CPM of PBMCs alone (black bars), by the CPM of PBMCs with added TILs (light grey bars), or by the CPM of PBMCs with added autologous CD4 cells (grey bars; Fig. 3E). Using these ratios, the TILs markedly suppressed PBMC proliferation (−3.3 ± 1.3), whereas the autologous CD4 cells did not (1.5 ± 0.4; P = 0.0033).

Tregs in Premalignant RRP

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Treg-attracting chemokine CCL17 is differentially expressed by papillomas

To determine whether the increase of Tregs in papillomas was the result of the expression of chemokines known to attract Tregs into tissues (18), we compared CCL17 and CCL22 mRNA expression in 9 papillomas (PAP), 11 clinically normal laryngeal tissues from RRP patients (NA), and 6 control laryngeal tissues (TN) from patients without RRP (Fig. 3). All 3 types of tissues expressed equivalent amounts of CCL22 mRNA (data not shown). In contrast, the expression of CCL17 mRNA was upregulated in papillomas, compared with both types of control laryngeal tissues (Fig. 3). Thus, Tregs may be recruited to papillomas by CCR4 receptor engagement of an increasing gradient of CCL17 that is differentially
expressed by papillomas, together with normal constitutive robust expression of CCL22.

**CD4+ "non-Tregs" in TILs from papillomas show reduced CD127 expression and lack suppressor function**

We observed that the majority of the CD4+ T cells in TILs from papillomas were CD4+Foxp3−CD25−"non-Tregs"
and had low or absent expression of CD127 (49.2% ± 7.4%; Fig. 4). In comparison, the majority of CD4+ non-Tregs in the blood were CD127+, and only a minor subgroup of these cells lacked CD127 (22.2% ± 5.4%, P < 0.001). CD127 is constitutively expressed on naive T cells, is downregulated upon T-cell activation, and then reexpressed on a subset of effector T cells that ultimately differentiate into memory T cells (19). The absence of CD127 expression on T cells has been associated with T-cell exhaustion in patients with chronic infection or tumors (8, 19), whereas absent CD127, Foxp3, and CD25 expression is also a characteristic of effector Tr1-like cells (20, 21). Therefore we asked whether this major T-cell subpopulation in TILs (CD4+CD25low/CD127low/) could also suppress PBMC proliferation. This purified
cell fraction from TILs was cocultured with anti-CD3 and anti-CD28 antibody activated autologous PBMCs to determine whether they could suppress PBMC proliferation. CD4^+CD25^{low}−CD127^{low}− T cells from papilloma-derived TILs from 2 different RRP patients did not suppress PBMC proliferation (data not shown). However, in a parallel experiment, CD4^+CD25^{low}−CD127^{low}− T cells from the blood of a control without RRP showed 40% suppression of autologous PBMC proliferation. Thus, CD4^+CD25^{low}−CD127^{low}− T cells in control blood contain a subpopulation of functional Tr1-like effector cells, as described (6, 20, 21), whereas T cells with this phenotype in TILs from papillomas do not contain sufficient Tr1-like cells to show suppression in vitro.

**PD-1 and CD69 are highly expressed on TILs from papillomas**

To determine whether the PD-1/PD-L1/L2 pathway was relevant in the immunosuppressive micromilieu present in papillomas, TILs from 7 patients were stained with anti–PD-1 antibodies and analyzed by flow cytometry. PD-1 was expressed on 85% ± 7% of the CD3^+ T cells and on 92% ± 4% of CD4^+ T cells from these papillomas (Fig. 5A). In addition, the intensity of expression of PD-1 on TILs was much greater than that observed on T cells from the blood. The majority of TILs also expressed CD69, another marker of activation (9). These results suggested that the CD4^+ effector T cells within papilloma TILs are chronically activated as continuous engagement of the TCR is necessary for maintaining a high PD-1 expression (9). The majority of TILs did not express CD25 and therefore they may be in an exhausted state with reduced capacity to proliferate and produce cytokines, as has been shown for CD8^+PD-1^+ T cells during chronic infection (10). Thus, in addition to the decreased CD127 expression that was observed on the majority of CD4^+ TILs, the papilloma microenvironment also contributes to effector dysfunction by upregulating PD-1 expression on T cells.

**PD-L1 and PD-L2 are expressed by papillomas**

To determine whether the engagement of PD-1 that is highly expressed on the majority of CD3^+ TILs could represent a mechanism that induces T-cell exhaustion in papillomas, we analyzed PD-L1 and PD-L2 mRNA extracted from papilloma tissue, clinically normal laryngeal tissue, and control laryngeal tissues from individuals without RRP. PD-L1 mRNA in papillomas (PAP, n = 9) was relatively expressed 145 times more than laryngeal tissues from individuals without RRP (TN, n = 6) and 93 times more than clinically normal laryngeal tissues from RRP patients (NA, n = 11; P < 0.0001 ANOVA and P < 0.0001 unpaired 2-tailed t test for TN vs. NA, and TN vs. PAP; Fig. 5B). In contrast, all 3 types of laryngeal tissues expressed PD-L2 mRNA equivalently and at relative expression levels of 15 to 32 times that of PD-L1 in TNs. Thus, PD-1 ligation on CD3^+ TILs by PD-L1 expressed by papillomas may represent an additional inhibitory pathway that blocks effector T-cell function in these lesions as previously reported in other systems (8).

**Discussion**

There has been intense interest in the role of Tregs and their respective subpopulations in both murine and human disease. Increased Treg numbers and function have been...
linked to chronic bacterial, parasitic, and viral (HIV, HCV, and EBV) infection, whereas a low frequency and/or reduced Treg function have been linked to the resolution of acute infection and the reestablishment of tumor immunity in various cancers, as recently reviewed (22). Most relevant to our studies are recent descriptions of Tregs in chronic HPV infection of the human cervix, specifically cervical intraepithelial neoplasia and cervical cancer caused by HPV16 and 18. These studies showed that the frequency of Tregs increased with HPV-induced cervical disease (23, 24).

In this article, we provide evidence for the first time that there is an increased frequency of functional CD4⁺ CD127low/⁻ Tregs within the total CD4⁺ TIL population in HPV6/11-induced respiratory papillomas. The frequency of these Tregs within respiratory papillomas did not predict disease severity, unlike that reported in cervical neoplasia (23, 24). Thus, an increased frequency of functional Tregs likely contributes to the chronicity of HPV-induced RRP, but may not greatly influence the severity of this disease.

The increased frequency of Tregs has been shown to contribute to the suppression of infiltrating cytotoxic T cells in human cancerous lesions allowing these tumors to escape immunosurveillance (7). Several types of CD4⁺ Tregs have been described, including CD4⁺ natural Tregs (nTregs) that express the transcription factor Forkhead box P3 (Foxp3) and the high-affinity interleukin (IL)-2 receptor α-subunit (CD25), but fail to express the α-subunit of the high-affinity receptor for IL-7 (CD127). They are phenotypically defined as CD4⁺ CD25⁺Foxp3⁺ CD127low/⁻ T cells (6). nTregs develop within the thymus and exert their suppressive activity by several mechanisms as recently reviewed (25). Adaptive Tregs (aTregs) share the same phenotype as nTregs (26); however they may also show the number of Tregs in papilloma CD4⁺ T cells in papilloma lesions (1). The involvement of these cytokines in generating, activating, and expanding Tregs from naive CD4⁺ T cells that differentiate into Foxp3 expressing aTreg following engagement of their T-cell receptors has been documented (7, 30). Of interest, recent studies have shown that prostaglandin E₂ (PGE₂) can induce Foxp3 expression and enhance the suppressive activity of CD4⁺ CD25⁺ Tregs and that these responses could be reversed by COX-2 inhibition (31, 32). Respiratory papilloma and their surrounding uninfected laryngeal tissues from patients with RRP have elevated COX-2 expression (33). Thus, the laryngeal microenvironment in RRP is a rich source of PGE₂ that can further support Treg function.

Our studies also showed that there are a substantial number of Tregs in papilloma CD4⁺ TILs that have reduced or negative CD25 expression. These Tregs have been reported to function similarly to their CD25high Treg counterparts, although less efficiently (6). In studies using Foxp3-GFP knock in mice, the expression of Foxp3-GFP correlated with suppressor activity irrespective of CD25 expression (34). It has also been suggested that CD4⁺ Foxp3⁺ CD25⁻ Tregs can show functional plasticity and can loose Foxp3 expression, whereas CD4⁺ Foxp3⁺ CD25high T cells represent a more stable Foxp3⁺ Treg population (35, 36). Stable Foxp3 expression depends on the DNA methylation status of the Treg-specific determining region of the Foxp3 gene (37). Demethylation of the Foxp3 locus and high expression of the Ikaros transcription factor Helios have recently been reported to be a specific “signature” of thymic-derived nTregs (37, 38). From our studies, the CD4⁺ Foxp3⁺ CD127low/⁻ T cells in papilloma TILs include both Helioshigh, nTregs, and Helioslow/⁻, aTregs (Fig. 2C and D). Importantly, the frequency of Helioshigh Tregs in papillomas was lower than in the blood of the same RRP patient (Fig. 2C and D). This suggests that the papilloma microenvironment is conducive to de novo generation of aTregs from T₄₀-0-like T cells. Novel therapies aimed at destabilization of Foxp3 expression on Tregs in papillomas may benefit RRP patients by blocking Treg function and thereby restoring effective T₁₁-like responses in these premalignant lesions.

Our studies also showed that a large population of CD4⁺ Foxp3⁺ cells (non-Tregs) in papilloma TILs lacked, or had reduced CD127 and CD25 expression. Expression of IL-7 and IL-2 receptors regulate naive and memory T-cell...
homoeostasis, proliferation, and differentiation (39). The function of CD3+CD4−CD25−CD127−Foxp3− T cells is varied. This population can include Tr1-like T cells and previously activated, but exhausted, effector memory T cells (6, 8, 10, 20, 21). The expansion of this population has been shown to be an index of dysfunction in CD4+ cells from HIV-infected individuals (13) and was linked to enhanced, tumor-specific, CD4+ T-cell apoptosis (19). The CD3+CD4+CD25+CD127− fraction isolated from papiloma TIL did not suppress autologous PBMC proliferation. In addition, in a single experiment, this TIL fraction failed to express IL-10, TGF-β, or IFN-γ mRNA after anti-CD3 and anti-CD28 antibody stimulation (data not shown). Thus, we suspect that this TIL subpopulation contains chronically activated T cells that express CD69+ and PD-1high and represent dysfunctional effector T cells, as described in other diseases (9, 19).

Our observations that CD3+ T cells isolated from papillomas expressed PD-1 brightly (Fig. 5A), but not PD-L1, identified by flow cytometry (data not shown), and that papillomas express both PD-1 ligands (PD-L1 and PD-L2; Fig. 5B), suggest that the PD-1/PD-L1/PD-L2 pathway may also contribute to the immunosuppressive milieu in papillomas. PD-L1 is expressed on a wide variety of tissues and tumors, and both PD-L1 and PD-L2 are expressed on professional antigen-presenting cells (11). High levels of PD-L1 expressed by tumor cells have been correlated with an unfavorable prognosis in cancer (41). PD-1 is a major regulator of CD8+ T-cell exhaustion and viral control in chronic infection and tumor progression, and it has become an important potential therapeutic target in these diseases (41, 42). However, the PD-1/PD-L1 pathway in CD4+ effector T-cell and Treg function is more controversial, and the mechanisms that regulate exhaustion in PD1+CD4+ and PD1+CD8+ T cells may differ between the these 2 cell types in adaptive cellular immunity (9, 19, 43–45). In addition, conflicting reports of the role of PD-1 on CD4+ Treg function suggested that PD-1/PD-L1 ligation on CD4+ Tregs supported Treg stability and expansion (40, 46–48), whereas others suggested that this pathway inhibited Treg expansion and suppressive function (49, 50). Further studies are needed to determine how nTreg or aTreg stability, expansion, and function, are modulated by interactions between PD-1 and its ligands in RRP and in various tumors at different anatomic sites.

In our studies, CD4+ non-Treg T cells from HPV6/11-induced papillomas showed upregulated PD-1 expression along with expression or absence of other markers of chronic activation and terminal differentiation (CD25+CD127−CD69+). Continued interaction with PD-1 ligands within papillomas may therefore block effector T-cell function and/or induce their apoptosis. As a consequence, these T cells within papillomas are likely unable to expand into polyfunctional T helper cells that could control HPV6/11 infection.

In summary, we show that the overwhelming majority of CD4+ cells in papillomas are either actively involved in immune suppression or are chronically stimulated and likely exhausted effector T cells that fail to respond to HPV/tumor antigen exposure. Better understanding of PD-1 expressing TILs, and the identification of the antigen(s) that induce T-cell exhaustion in papillomas, could help develop novel strategies to reverse T-cell exhaustion and restore T1H1-like function in RRP. Clinical trials using PD-1/PD-L1 blockade in cancer and infectious diseases should be monitored closely for their applicability in RRP. Additional characterization of the interactive cycle of cells and antigens within papillomas that polarize immune responses toward HPV6/11 tolerance may provide the rationale to develop novel immune-based therapies that can restore effective T-cell clearance of these viruses.

Disclosure of Potential Conflicts of Interest
V.R. Bonagura has received honoraria as consultant to Baxter and is a consultant and is on the advisory board of CSL-Behring. A.I. Abramson and B.M. Steinberg have received commercial research grant from Pfizer, Inc. The authors disclosed no potential conflicts of interest.

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