Papillomavirus-Specific CD4\(^+\) T Cells Exhibit Reduced STAT-5 Signaling and Altered Cytokine Profiles in Patients with Recurrent Respiratory Papillomatosis

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Recurrent respiratory papillomatosis (RRP) is caused by human papillomavirus type 6 (HPV-6) or HPV-11. Specific HLA-DR haplotypes DRB1*01:02 and DRB1*03:01 are associated with the development of RRP, disease severity, and Th2-like responses to HPV early proteins. Th1-like responses to HPV proteins have been shown to be protective in animal models. Therefore, we investigated the hypothesis that RRP patients have dysfunctional Th1-like, HPV-specific T cell responses. Using MHC class II tetramers, we identified immunogenic peptides within HPV-11 early proteins. Two distinct peptides (E6_113–132 and E2_19–20) contained DRB1*01:02- or DRB1*03:01-restricted epitopes, respectively. An additional peptide (E2_281–300) contained an epitope presented by both alleles. Peptide binding, tetramer, and proliferation assays identified minimal epitopes within these peptides. These epitopes elicited E2/E6-specific CD4⁺ T cell responses in RRP patients and healthy control subjects, allowing the isolation of HPV-specific T cell lines using tetramers. The cytokine profiles and STAT signaling of these tetramer-positive T cells were measured to compare the polarization and responsiveness of HPV-specific T cells from patients with RRP and healthy subjects. HPV-specific IFN-γ secretion was substantially lower in T cells from RRP patients. HPV-specific IL-13 secretion was seen at modest levels in T cells from RRP patients and was absent in T cells from healthy control subjects. HPV-specific T cells from RRP patients exhibited reduced STAT-5 phosphorylation and reduced IL-2 secretion, suggesting anergy. Levels of STAT-5 phosphorylation and IFN-γ secretion could be improved through addition of IL-2 to HPV-specific T cell lines from RRP patients. Therapeutic vaccination or interventions aimed at restoring Th1-like cytokine responses to HPV proteins and reversing anergy could improve clinical outcomes for RRP patients.

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H uman papillomaviruses (HPVs) are ubiquitous viruses that are typically held in check by a competent immune system, as evidenced by the presence of HPV-specific memory T cells in most individuals (1, 2). Although the majority of HPV infections are short-lived and asymptomatic, persistent infections may progress to precancerous lesions or invasive cancers (3). In addition, a small subset of HPV-exposed individuals develop recurrent respiratory papillomatosis (RRP), a condition in which benign tumors of the larynx and upper respiratory tract result in significant morbidity and occasional mortality (4, 5). RRP is caused by HPV-6 and -11 infection (6), is more common in children than adults (4.3 and 1.8 cases per 100,000, respectively), and in children is typically associated with vertical transmission from an HPV-6/11–infected mother during vaginal delivery (7). There is significant clinical variability between patients, with some requiring surgical procedures as often as every 2 weeks to maintain a patent airway, whereas others have no recurrence after their first presentation (8).

HPV-induced diseases, including RRP, are characterized by the absence of HPV-specific, cytotoxic T lymphocytes and the absence of CD4⁺ Th1 cells that secrete IFN-γ, IL-2, and TNF-α (9–11). These cell types are critical in generating effective antiviral T cell adaptive immunity (12). In particular, Th1-like responses to E2 and E6 protein were clearly protective in animal models (13–15). Responses to E2 and E6 proteins may also contribute to effective HPV-specific immunity in human subjects (16), but currently this has not been directly shown. The specific immunologic mechanisms that predispose to RRP and result in the development of disease are not well characterized; however, select class II haplotypes (DRB1*01:02, DRB1*03:01, and DQB1*02:01) are associated with the development of RRP and the expression of IL-10 and IL-4 by effector T cells (17). Based on other disease models, the absence of effective CD4⁺ T cell immunity against HPV could be a consequence of functional exhaustion, caused by the persistent presence of Ag, or by anergy, caused by incomplete T cell activation in an environment deficient in costimulation or high in coinhibition (18, 19).

The objective of this study was to investigate mechanisms of immune dysfunction in RRP patients by comparing the HPV-specific CD4⁺ T cell responses of RRP patients and healthy
subjects. After identifying prevalent DRA1/B1*01:02 (DR0102)- and DRA1/B1*03:01 (DR0301)-restricted epitopes within the HPV E2 and E6 proteins, HPV-specific polyclonal T cells (T cell lines) were isolated using tetramers and characterized on the basis of their cytokine profiles and STAT signaling. Our findings support a paradigm in which persistent HPV infection leads to increased Th2 polarization, altered signaling, and impaired CD4+ Th1-like T cell immunity.

Materials and Methods

Human subjects

Samples for this study were obtained from healthy volunteers at Benaroya Research Institute and from individuals with RRP at Long Island Jewish Medical Center. All subjects were recruited with informed consent obtained after approval by the North Shore-Long Island Jewish or the Benaroya Research Institute Institutional Review Board. RRP subjects had mild-to-moderate or severe disease, defined by a disease severity score ≤0.06 (mild-to-moderate) or >0.06 (severe) at the time of direct endoscopic surgical removal, or having tracheal, bronchial, or pulmonary extension of disease (severe) (17). All subjects were confirmed by high-resolution HLA class II genotyping to have HLA DRB1*01:02 or HLA DRB1*03:01 haplotypes (17).

Peptides, MHC class II protein, and tetramer assembly

Panels of overlapping 20-mer peptides with sequences based on HPV E2 and E6 protein sequences and positive control peptides, influenza A hemagglutinin (HA), and sperm whale myoglobin (MW 137,148), were synthesized on polystyrene pins with 9-fluorenylmethoxycarbonyl chemistry by Mimotopes (Clayton, Australia) with a 2-aa overlap. Each peptide was dissolved in DMSO at 20 mg/ml and subsequently diluted as needed for various assays. To produce recombinant DR0102 and DR0301 protein, we purified soluble protein from insect cell culture supernatants by affinity chromatography and dialyzed against phosphate storage buffer, pH 6.0, followed by affinity chromatography (22). The peptide sets were subsequently conjugated as tetramers using R-PE streptavidin (BioSource International, Camarillo, CA) at a molar ratio of 8:1 (20).

Peptide binding competition assay

Various concentrations of each test peptide were incubated in competition with 0.01 mM biotinylated reference peptide, influenza A hemagglutinin 306–318 (PKYVKQNLTKLAI) for DR0102, and myoD17–148 (LRFRKDIAYKYE) for DR0301, in wells coated with DR0102 or DR0301 (eProteins, Inc., previously described) (20). After washing, the remaining biotin-peptide was labeled using europium-conjugated streptavidin (Perkin Elmer) and quantified using a Victor2 D time resolved fluorometer (Perkin Elmer). Peptide binding curves were simulated by nonlinear regression with Prism software (version 4.03, GraphPad Software) using a sigmoidal dose–response curve. IC50 binding values were calculated from the resulting curves as the peptide concentration needed for 50% inhibition of peptide binding.

Tetramer-based T cell assays

Tetramer-guided epitope mapping was conducted as previously described to define epitopes within the E2 and E6 proteins restricted by DR0102 and DR0301 (22, 23). PBMCs were isolated from the blood of healthy subjects and individuals with RRP by Ficoll (GE Healthcare) underlay, and CD4+ T cells were isolated using the Miltenyi CD4+ T cell isolation kit. Cells were restimulated in T cell medium with 10 μg/ml irradiated PBMCs plus 40 U/ml IL-2 (20). Expanded cells were stained with tetramers and analyzed on a FACS Calibur (Becton Dickinson). To assess proliferation, we plated 104 T cells/well in T cell medium with 10 μg/ml irradiated PBMCs from an HLA-matched donor (with DRB1*01:02 or DRB1*03:01 haplotype) and 0, 1, or 10 μg/ml peptide (in triplicate), incubated them at 37°C for 48 h, pulsed them with [3H]thymidine (1 μCi/well) and harvested them 18 h later, and measured [3H]thymidine incorporation with a scintillation counter.

Production of IL-2 and IL-13 by HPV-specific T cell lines was characterized using cytokine capture kits (Miltenyi Biotec) according to manufacturer’s instructions. In brief, T cells were activated by incubating with 10 μg/ml tetramer, 10 μg/ml anti-CD28, and 2 μg/ml anti-CD49d. After 4 h of incubation at 37°C, cells were washed twice in PBS and labeled in 100 μl medium on ice for 10 min with a bispecific Ab—Ab ligand directed against both CD45 and cytokine (INF-γ, TNF-α, IL-5, or IL-10). Prelabeled wash medium was then added to a final volume of 2 ml, and cells were incubated at 37°C for 45 min under gentle rotation to allow cell surface capture of secreted cytokines. Cells were washed once in PBS and then stained for 15 min on ice with an FITC- or allophycocyanin-conjugated Ab directed against the cytokine of interest, washed, and analyzed on a FACS Calibur instrument (BD Biosciences). For IL-2 rescue experiments, HPV-specific T cell lines were incubated overnight in T cell medium with or without exogenous IL-2 (40 U/ml) before cytokine analysis.

Statistical analysis

For tetramer, cytokine capture, and STAT signaling data experimental results were analyzed for statistical differences using the Student t test with Welch’s correction for unequal variances. ICS data were log10 transformed, determined to be normally distributed with comparable standard deviations between groups, and analyzed by a two-tailed unpaired t test and reported as means and SDs after back transformation. All analyses were performed using Prism software (version 4.03, GraphPad Software).

Results

Identifying DR0102- and DR0301-restricted E2/E6 epitopes

CD4+ T cells from RRP patients and healthy subjects were stimulated with peptide pools spanning the HPV-11 E2 and E6 protein sequences. The design of these peptide sets is shown in
Supplemental Table I. After 14 d, cells were stained with pooled peptide tetramers (Supplemental Fig. 1) and again using single peptide tetramers (Fig. 1). As shown in Fig. 1A, cells from a healthy subject with a DRB1*03:01 haplotype that were stained with E21–20, E2281–300, and the corresponding pools resulted in positive staining. All remaining pools were negative. B, CD4+ T cells from a healthy subject with a DRB1*01:02 haplotype were stimulated with peptide pools spanning the E2/E6 protein sequences. After 14 d, cells were stained with DR0102 tetramers. Cells that were stained with E2281–300, E6113–132, and the corresponding pools gave positive staining. Staining with E681–100 was subsequently shown to be background staining because staining irrelevant cells with this tetramer gave similar results (not shown). All remaining pools were negative (Supplemental Fig. 1B).

Defining minimal DR0102- and DR0301-restricted HPV epitopes

Complementary proliferation, tetramer staining, and peptide binding experiments were conducted using truncated versions of...
T cell expansion in certain healthy subjects, none of these differed toward increased IFN-γ in their cytokine responses. Among RRP subjects, there was a trend for increased cytokine secretion in subjects with mild disease, whereas another had enhanced TNF-α secretion, whereas another had enhanced TNF-α secretion. The deficit in TNF-α secretion appeared to be an HPV-specific phenomenon, because nonspecific induction of STAT-5 using IL-2 (rather than tetramer stimulation) elicited similar signaling in RRP patients compared with healthy subjects. The observed difference was not statistically significant. B, CD4+ T cell lines from RRP patients (upper panels) and healthy subjects (lower panels) were activated using HPV peptide tetramers (plus CD28/CD49d) and assayed using a multiplex cytokine capture assay. As shown in these representative plots (gated for tetramer-positive cells), T cell lines isolated from RRP subjects were typically deficient in their capacity to secrete cytokines such as IFN-γ. C, Cytokine secretion by DR0102/E6113–132 (gray symbols), DR0102/E2281–300 (open symbols), DR0301/E21–20 (half-filled symbols), and DR0301/E2281–300-specific (black symbols) T cell lines measured by cytokine capture for 16 cell lines isolated from RRP patients (triangles) and 14 cell lines isolated from HLA-matched healthy subjects (circles). Data indicate a significant deficit in IFN-γ secretion by RRP patients as compared with healthy subjects (p < 0.01). One subset of patients was deficient in TNF-α secretion, whereas another had enhanced TNF-α secretion. The asterisk indicates a significant difference between patients and healthy subjects (p < 0.05).

Table I. Summary of E2/E6 epitopes

<table>
<thead>
<tr>
<th>Residues</th>
<th>Amino Acid Sequence</th>
<th>No. of Patients</th>
<th>No. of Healthy Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR0102</td>
<td>E6113–132</td>
<td>2/2</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>E2281–300</td>
<td>2/2</td>
<td>3/3</td>
</tr>
<tr>
<td>DR0301</td>
<td>E21–20</td>
<td>4/4</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td>E2281–300</td>
<td>4/4</td>
<td>7/7</td>
</tr>
</tbody>
</table>

*Minimal stimulatory epitopes, as determined by proliferation and tetramer assays, are underlined. The most likely core binding residues, based on assay results and binding predictions, are in boldface.

Responsiveness of healthy subjects and RRP patients to HPV E2/E6 peptides

Using the peptides identified through initial epitope mapping experiments, we assayed T cell responses to E2/E6 in healthy subjects and RRP patients by stimulating purified CD4+ T cells with HPV peptide and staining with tetramers after 2 wk of in vitro culture (Fig. 3A). Although the data suggested stronger T cell expansion in certain healthy subjects, none of these differences was statistically significant. To assess functional responses to E2/E6 peptides, we isolated CD4+ T cell lines from healthy subjects and RRP patients, activated using HPV peptide tetramers, and assayed for cytokine release using a capture assay. As shown in these representative plots (gated for tetramer-positive cells), T cell lines isolated from RRP patients were typically deficient in their capacity to secrete cytokines such as IFN-γ. As shown in Fig. 3C, the deficit in IFN-γ secretion by RRP patients as compared with healthy subjects was statistically significant (p < 0.01), whereas levels of secreted IL-5 and IL-10 were not significantly different. One subset of patients was deficient in TNF-α secretion, whereas another had enhanced TNF-α secretion. These TNF-α-producing cell lines had the highest IFN-γ secretion. Table II lists a summary of clinical and immune response data for each individual included in the study, including their cytokine responses. Among RRP subjects, there was a trend toward increased IFN-γ secretion in subjects with mild disease, but this did not reach statistical significance (p = 0.1). Both patients with significant TNF-α and IFN-γ secretion had mild disease, and one had improved clinically in response to an experimental immunomodulator.

STAT signaling of HPV-specific T cell lines

To address the underlying mechanism of deficient cytokine production in RRP, we measured STAT-4, STAT-5, and STAT-6 signaling in multiple CD4+ T cell lines isolated from RRP patients and HLA-matched healthy subjects. For these experiments, tetramer-stimulated E2/E6-specific T cell lines were stained with phospho-specific STAT-4, STAT-5, and STAT-6 Abs and analyzed by flow cytometry. As shown in Fig. 4A, STAT-4 and STAT-6 signaling was comparable in RRP patients and healthy subjects. In contrast, STAT-5 signaling was significantly lower (p = 0.017) in RRP patients than in healthy subjects. The observed difference in STAT-5 signaling appeared to be an HPV-specific phenomenon, because nonspecific induction of STAT-5 using IL-2 (rather than tetramer stimulation) elicited similar signaling in RRP patients and healthy subjects (Fig. 4B). STAT-5 signaling was reduced in patients regardless of specificity in that T cell lines with all three
of the HLA/peptide restrictions tested showed low levels of phosphorylation.

**IL-2 and IL-13 production by HPV-specific T cells**

The observation of reduced STAT-5 accompanied by unaltered STAT-4 suggests a possible decrease in autocrine levels of STAT-5 signaling cytokines such as IL-2. Therefore, we measured the IL-2 production of tetramer-stimulated E2/E6-specific T cells by intracellular cytokine staining in multiple CD4+ T cell lines isolated from RRP patients and HLA-matched healthy subjects. As shown in Fig. 5A, IL-2 production was significantly decreased \((p = 0.0034)\) in RRP subjects as compared with healthy control subjects. These results demonstrate a lack of IL-2 production by HPV-specific T cells. Interestingly, these HPV-specific CD4+ T cells did not express PD-1 or CTLA-4 (data not shown). As shown in Fig. 5B, IL-13 production by HPV-specific T cells was modest, but significantly increased \((p = 0.0009)\) in RRP subjects as compared with healthy control subjects, suggesting a Th2-like polarization. However, these HPV-specific cells rarely produced IL-5 (Fig. 3) and did not secrete appreciable amounts of IL-4 (data not shown).

**Table II. Summary of clinical and immune response data**

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>HLA</th>
<th>Age (y)</th>
<th>Onset</th>
<th>Disease Score</th>
<th>IFN-γ (^a)</th>
<th>STAT-5 (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRP 1</td>
<td>DR0102</td>
<td>63</td>
<td>Adult</td>
<td>Mild/Moderate</td>
<td>(0.018)</td>
<td>0.2</td>
</tr>
<tr>
<td>RRP 2</td>
<td>DR0301</td>
<td>53</td>
<td>Adult</td>
<td>Mild/Moderate</td>
<td>(0.0)</td>
<td>4.0</td>
</tr>
<tr>
<td>RRP 3</td>
<td>DR0301</td>
<td>30</td>
<td>Adult</td>
<td>Severe</td>
<td>(0.075)</td>
<td>0.6</td>
</tr>
<tr>
<td>RRP 4</td>
<td>DR0301</td>
<td>49</td>
<td>Adult</td>
<td>Severe</td>
<td>(0.068)</td>
<td>1.6</td>
</tr>
<tr>
<td>RRP 5</td>
<td>DR0102</td>
<td>46</td>
<td>Adult</td>
<td>Severe</td>
<td>(0.088)</td>
<td>0.2</td>
</tr>
<tr>
<td>RRP 6</td>
<td>DR0301</td>
<td>26</td>
<td>Juvenile</td>
<td>Mild/Moderate</td>
<td>(0.005)</td>
<td>6.0</td>
</tr>
<tr>
<td>Cont 1</td>
<td>DR0102</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>49.4</td>
</tr>
<tr>
<td>Cont 2</td>
<td>DR0102</td>
<td>47</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>43.2</td>
</tr>
<tr>
<td>Cont 3</td>
<td>DR0301</td>
<td>38</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>5.5</td>
</tr>
<tr>
<td>Cont 4</td>
<td>DR0301</td>
<td>55</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cont 5</td>
<td>DR0301</td>
<td>25</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>5.6</td>
</tr>
<tr>
<td>Cont 6</td>
<td>DR0301</td>
<td>28</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>10.2</td>
</tr>
<tr>
<td>Cont 7</td>
<td>DR0301</td>
<td>48</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>3.4</td>
</tr>
<tr>
<td>Cont 8</td>
<td>DR0301</td>
<td>33</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\(^a\)Average percentage of positive cells in IFN-γ capture assay.

\(^b\)Average percentage of positive cells in phospho-specific STAT-5 assay.

\(^\dagger\)Surgical debulking performed.

\(^\ddagger\)Subject improved clinically (severe to mild/moderate) in response to an experimental immunomodulator as part of a preclinical trial that concluded immediately before these data were obtained.

NA, not available.

**FIGURE 4.** STAT-5 signaling of E2/E6-specific T cells is altered in patients with RRP and is reversible with IL-2. **A,** STAT phosphorylation of tetramer-stimulated DR0102/E6\(_{113–132}\) (gray symbols), DR0102/E2\(_{281–300}\) (open symbols), DR0301/E2\(_{1–20}\) (half-filled symbols), and DR0301/E2\(_{281–300}\)-specific (black symbols) T cells as measured by phospho-specific flow cytometry in 10 cell lines isolated from RRP patients (triangles) and 7 cell lines isolated from HLA-matched healthy subjects (circles). STAT-4 signaling was elevated in one RRP patient, but was virtually absent in all other subjects. No significant differences were observed between the two groups. STAT-5 signaling was significantly decreased in RRP subjects. STAT-6 signaling was unaltered between the two groups. The asterisk indicates a significant difference between patients and healthy subjects \((^* p < 0.05)\). **B,** STAT-5 phosphorylation of IL-2–stimulated T cells as measured by phospho-specific flow cytometry in RRP patients and HLA-matched healthy subjects. Signaling was comparable in the two groups.

**FIGURE 5.** E2/E6-specific T cells show decreased IL-2 and enhanced IL-13 production in patients with RRP. **A,** IL-2 production of tetramer-stimulated DR0102/E6\(_{113–132}\) (gray symbols), DR0102/E2\(_{281–300}\) (open symbols), DR0301/E2\(_{281–300}\)-specific (black symbols) T cells as measured by intracellular cytokine staining for five cell lines isolated from RRP patients (triangles) and five cell lines isolated from HLA-matched healthy subjects (circles). IL-2 production was significantly decreased in RRP subjects. **B,** IL-13 production of tetramer-stimulated E2/E6-specific T cells as measured by intracellular cytokine staining in RRP patients and HLA-matched healthy subjects. IL-13 production was increased in RRP subjects. The asterisk indicates a significant difference between patients and healthy subjects \((^* p < 0.05)\).
Effect of IL-2 pretreatment on HPV-specific cytokine secretion

Given the lack of IL-2 production by HPV-specific T cells in RRP patients, it seemed plausible that addition of exogenous IL-2 could restore the cytokine production of these cells. To test this possibility, we measured the cytokine secretion of HPV-specific T cell lines with or without preincubation with exogenous IL-2. As shown in Fig. 6, IL-2 treatment caused a significant (p = 0.01) increase in IFN-γ secretion for cell lines from RRP patients, raising them near the normal range. In contrast, IL-2 treatment caused no change in IFN-γ secretion for cell lines from healthy subjects.

Discussion

RRP is a rare but serious disease that can significantly reduce the quality of life and cause mortality because chronic HPV infection in these patients can lead to the formation of papillomas that occlude the upper airway. HPV exposure is ubiquitous (virtually all individuals can be expected to have had exposure to at least one HPV strain), and memory T cell responses to HPV early proteins are commonly seen in the healthy population; however, symptomatic infections are uncommon (1, 2). Approximately 5% of all individuals have evidence of HPV infection in the larynx without evidence of disease, but only a small fraction of these individuals develop RRP (27). In addition, only a subset of RRP patients develops an unrelenting and severe disease (8). RRP patients exhibit chronic infection with HPV-6 or -11 and can express serum Abs (28). A study of the genetic background of RRP patients identified an enrichment of select HLA class II genes that correlated with reduced IFN-γ expression (17). These observations suggest that the immune mechanism that underlies RRP susceptibility involves a deficiency in protective CD4+ Th1-like T cell responses against HPV. Indeed, previous studies have observed deficits in Th1 cytokine production and CD4+ T cell immunity against HPV E2 and E6 proteins in the context of cervical cancer (29, 30).

In this study, we investigated CD4+ T cell responses directed against the HPV-11 E2 and E6 proteins. Responses to these proteins have been previously implicated as being important in effective papillomavirus-specific immune responses (1, 9, 14–16). Using MHC class II tetramers, we identified multiple antigenic peptides within E2/E6 proteins that could be detected in RRP patients and healthy control subjects with DRB1*01:02 and DRB1*03:01 haplotypes. One of these peptides, E2281–300, could be presented by both DR0301 and DR0102. Notably, a closely related HPV-16 peptide was identified in a previous study as a DR1-restricted epitope (31). As summarized in Supplemental Table II, that antigenic region of the E2 protein is highly conserved among HPVs. The N-terminal epitope within the leader sequence is much less conserved, but still is present in several strains. Therefore, these epitopes are likely to be relevant for studying various HPV-induced diseases.

For several reasons, it is likely that RRP patients exhibit HPV-specific immune dysregulation rather than global immune defects. Patients with RRP do not manifest other chronic infections, and their peripheral CD4+ T cell frequencies are comparable with those of unaffected control subjects (32, 33). One possible mechanism for HPV-specific immune dysregulation is the induction of regulatory T cells (Tregs) within papillomas or other HPV-infected tissues. In preliminary studies, we reported a 2- to 7-fold enrichment of functional Tregs in papillomas compared with autologous blood from patients with RRP (33), whereas the frequency of Tregs in the peripheral blood of RRP patients was comparable with that found in control subjects. Enriched and functional Tregs in papillomas would likely suppress Th1-like responses to HPV in papilloma tissues, whereas having a lesser impact on responses in the periphery. In agreement with this notion, CD4+ T cells specific for these E2/E6 epitopes were detectable in all of the subjects tested in our study. No statistically significant differences were observed between the E2/E6-specific T cell responses of RRP patients and healthy subjects. However, it is possible that the Th2-like response polarization generated by immunosuppressive immunocytes responding to these HPVs would be more likely to be involved in the inability of RRP patients to clear this infection (33).

Some evidence suggests that HPV-specific immune dysregulation may be directly modulated by viral proteins and/or by the tumor microenvironment (11, 36). It was previously demonstrated that allospecific CTL activity was reduced in PBMCs that had been pretreated with recombinant HPV-11 E6 protein (33). It is plausible that the functional responses of CD4+ T cells would also be reduced by similar mechanisms. These effects would likely impact responses in the periphery. To directly address the question of immune dysregulation, we isolated T cell lines from multiple RRP and healthy subjects and assessed their functional responses to E2/E6 peptides. Assaying these for cytokine release revealed robust IFN-γ secretion from healthy subjects and significantly reduced IFN-γ secretion from RRP subjects compared with healthy control subjects. With the exception of two patients (three cell lines), RRP subjects also tended to exhibit reduced TNF-α secretion. These patients with significant TNF-α secretion also secreted appreciable levels of IFN-γ and had mild disease. Interestingly, one of these subjects had improved clinically in response to an experimental immunomodulator, anecdotally suggesting a connection between immune phenotype and prognosis. Although levels of secreted IL-5 were modest and unaltered, RRP subjects produced modest levels of IL-13 protein in response to tetramer stimulation, suggesting a Th2-like response polarization.
We previously observed polarized expression of a Th2-like repertoire of cytokines by tumor-infiltrating lymphocytes in studies of the same patient cohort (11, 37). Taken together with our current data, this suggests that deficient protective Th1-like CD4+ T cell immunity to HPV can be detected both in the relevant tissue and in peripheral blood.

In addition to results suggesting that there are differences in response polarization, RRP subjects exhibited a clear reduction in STAT-5 signaling in response to immunodominant HPV E2/E6 peptides. This reduction was HPV specific and also reversible, because the deficiency in STAT-5 signaling in RRP patients was comparable with healthy subjects after the administration of IL-2. These results indicate an intrinsic defect within HPV-specific CD4+ T cells in RRP. Taken together, these data suggest a lack of autocrine IL-2 production, directly resulting in reduced STAT-5 and, consequently, dampening cytokine responses. Loss of IL-2 production has been described as one of the first functional defects exhibited by exhausted T cells, followed by the loss of other effector functions and, in some cases, deletion (38). Progressive T cell exhaustion is commonly observed in settings of chronic viral infection and is also a component of T cell dysfunction in cancer (18). Therefore, it would not be surprising to observe this phenomenon in association with a chronic condition such as RRP. However, although exhaustion is associated with persistently high antigenic loads, HPV16 generally use nonpreferred codons, resulting in decreased levels of viral protein synthesis and subsequent antigenic function (39). In addition, peripheral HPV-specific CD4+ T cells from RRP subjects did not express PD-1, a marker that has been strongly associated with exhaustion (40).

These factors appear to preclude exhaustion as the best explanation for reduced Th1-like CD4+ T cell effector function in RRP. An alternative explanation for this functional deficit would be anergy. Anergy, which is induced by TCR ligation in the absence of adequate costimulation, is associated with impaired proliferative capacity and lack of IL-2 production (19). In the anergic state, phosphorylation and activation of selected signaling molecules in response to TCR stimulation are significantly impaired (41). Unlike the exhausted state, which is rescued by decreasing antigenic load, sustained levels of Ag are not required to maintain clonal anergy and it is reversed by stimulation with IL-2 (41). We observed improvement in HPV-specific CD4+ T cell responses from RRP subjects in an assay that included the addition of exogenous IL-2. Specifically, addition of exogenous IL-2 resulted in a significant increase in IFN-γ secretion by T cell lines isolated from RRP subjects. These cells lacked CTLA-4 expression, which has been shown to be important in some models of anergy induction (42). However, it has also been shown in CTLA-4-deficient mice that T cells can persist in a hyporesponsive state in the absence of CTLA-4 (43). Furthermore, induction of anergy has been shown to occur in the presence of Abs that block the CTLA-4/B7 interaction (44). This concept that HPV-specific, CD4+ T cells from RRP patients may be anergic and require exogenous IL-2 replacement to restore function is supported by previous reports of the reduction of RRP severity after IFN-γ therapy (45–47). This suggests that restoration of a Th1-like microenvironment in HPV-infected tissues can restore HPV-specific T cell function in RRP.

In summary, our results demonstrate that RRP patients have only marginally reduced numbers of HPV-specific T cells, but significantly reduced functional CD4+ T cell responses to E2/E6 epitopes. These findings support a paradigm in which persistent HPV infection leads to increased Th2-like polarization, caused by tissue and HPV-specific events, and induction of clonal anergy through inadequate costimulation in respiratory papillomas. Anergized T cell populations have been shown to regain functional attributes through the effects of IL-2 and other common γ-chain cytokines.

Therefore, further investigation should be aimed at therapeutic interventions designed to reverse clonal anergy and restore HPV-specific CD4+ T cell effector function by the development of a therapeutic vaccine that enhances Th1-like function and prevents/blocks Th2-like responses to HPV proteins, thereby improving clinical outcomes for RRP patients. To this end, the T cell assays described in this study would be useful for evaluating responsiveness to therapeutic strategies that can restore the Th1-/Th2-like balance to these HPVs in patients with RRP.

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Disclosures
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