Papillomavirus-Specific CD4+ T-cells Exhibit Reduced STAT-5 signaling and Altered Cytokine Profiles in Patients with Recurrent Respiratory Papillomatosis

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Abstract

Recurrent Respiratory Papillomatosis (RRP) is caused by HPV-6 or -11. Specific HLA-DR haplotypes, DRB1*01:02 and DRB1*03:01 are associated with the development of RRP, disease severity, and T_H2-like responses to HPV early proteins. T_H1-like responses to HPV proteins have been shown to be protective in animal models. Therefore, we investigated the hypothesis that RRP patients have dysfunctional T_H1-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 $E2_{1-20}$) 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 controls, 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 absent in T-cells from healthy controls. 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 T_H1-like cytokine responses to HPV proteins and reversing anergy could improve clinical outcomes for RRP patients.

Keywords

Human; MHC; T-cells; Antigens/Peptides/Epitopes; human papillomavirus

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Introduction

Human papillomaviruses (HPV[2]) are ubiquitous viruses which 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). While the majority of HPV infections are short-lived and asymptomatic, persistent infections may progress to pre-cancerous lesions or invasive cancers (3). In addition, a small subset of HPV-exposed individuals develop recurrent respiratory papillomatosis (RRP[3]), a condition in which benign tumors of the larynx and upper respiratory tract result in significant morbidity, and in 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, while 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 $CD4^+$ T_H1 cells that secrete IFN-γ, IL-2, and TNF-α (9–11). These cell types are critical in generating effective anti-viral T-cell adaptive immunity (12). In particular, T_H1-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 at present this has not been directly shown. The specific immunologic mechanisms which 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 antigen, or by anergy, caused by incomplete T-cell activation in an environment deficient in co-stimulation or high in co-inhibition (18–19).

The objective of the present 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 DR0102[4] and DR0301[5] 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 T_H2 polarization, altered signaling, and impaired $CD4^+$ T_H1-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 following approval by the North Shore-LIJ Institutional Review Board. RRP subjects had mild-moderate or severe disease, defined by a disease severity score ≤0.06 (mild-moderate) or >0.06 (severe) at the time of direct endoscopic surgical removal, or having tracheal, bronchial, or pulmonary extension of

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[2]HPV: human papillomavirus
[3]RRP: Recurrent Respiratory Papillomatosis
[4]DR0102: DRA1/B1*01:02 protein
[5]DR0301: DRA1/B1*03:01 protein

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 (HA6) 306–318 and sperm whale myoglobin (myo7) 137–148 – were synthesized on polyethylene pins with 9-fluorenylmethoxycarbonyl chemistry by MIMOTOPES (Clayton, Australia) with a 12 amino acid 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, soluble protein was purified from insect cell culture supernatants by affinity chromatography and dialyzed against phosphate storage buffer, pH 6.0 as previously described (20). To assemble MHC class II tetramers, DR0102 or DR0301 protein was biotinylated at a sequence-specific site using biotin ligase (Avidity, Denver, CO) prior to dialysis into phosphate storage buffer. The biotinylated monomer was loaded with 0.2 mg/ml of peptide by incubating at 37°C for 72 hours in the presence of 2.5 mg/ml n-octyl-β-D-glucopyranoside and 1 mM Pefabloc SC (Sigma-Aldrich, St. Louis, MO). Peptide loaded monomers were subsequently conjugated as tetramers using R-PE streptavidin (Biosource International, Camarillo, CA) at a molar ratio of 8 to 1 (20).

Peptide binding competition assay

Various concentrations of each test peptide were incubated in competition with 0.01 mM biotinylated reference peptide – $HA_{306-318}$ (PKYVKQNTLKLAT) for DR0102 and $myo_{137-148}$ (LFRKDIAAKYKE) for DR0301 – in wells coated with DR0102 or DR0301 protein as previously described (21). After washing, the residual 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 non-linear regression with Prism software (Version 4.03, GraphPad Software Inc.) using a sigmoidal dose response curve. IC50 binding values were calculated from the resulting curves as the peptide concentration needed for 50% inhibition of reference 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). Peripheral Blood Mononuclear Cells (PBMC) were isolated from the blood of healthy subjects and individuals with RRP by Ficoll® (GE healthcare) underlay and CD4^{+} T-cells isolated using the Miltenyi CD4+ T-cell isolation kit. Cells from the CD4-negative fraction were incubated in 48 well plates (2.5×10^6 cells per well) for 1 hour and then washed, leaving adherent cells as antigen presenting cells. After adding ~2 million CD4^{+} T-cells per well, each well was stimulated with a pool of five consecutive HPV peptides (20 amino acids long with a 12 residue overlap). After 14 days, 75–100 μl of resuspended cells were stained with pooled peptide PE-conjugated tetramers for 60 min at 37°C. Subsequently, cells were stained with CD4-APC, CD3-PerCP and CD25-FITC mAbs (eBioscience) and analyzed by flow cytometry. Cells from pools that gave positive staining were analyzed again using the corresponding individual peptide tetramers. T-cell responses to single peptides were assayed in a similar fashion except that each well was stimulated with a single HPV peptide and stained after 14 days using a single peptide-loaded tetramer.

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[6]HA: influenza A hemagglutinin
[7]Myo: sperm whale myoglobin

T-cell sorting and proliferation assays

CD4$^+$ tetramer-positive cells were isolated as previously described using a FACS Vantage (Becton Dickinson) into 96-well plates containing T-cell medium (RPMI 1640 with 10% human serum, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin) and expanded by adding 2 μg/ml phytohemagglutinin and 200,000 irradiated PBMCs plus 40 U/ml IL-2 (20). Expanded cells were stained with tetramers and analyzed on a FACSCalibur (Becton Dickinson). To assess proliferation, 10^4 T-cells/well were plated in T-cell medium with 10^5 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 at 37°C for 48 hours, pulsed with [^{3}H]thymidine (1 μCi/well) and harvested 18 hours later, and [^{3}H]thymidine incorporation measured with a scintillation counter. For blocking experiments, anti-DR antibody (protein A purified from L243 supernatant) or anti-DQ antibody (protein A purified from SPVL3 supernatant) was added at 20 μg/ml.

Cytokine Assays

The secretion of IFN-γ, TNF-α, IL-5 and IL-10 by HPV specific T-cell lines was characterized using cytokine capture kits (Miltenyi Biotec) according to manufacturer's instructions. Briefly, T-cells were activated by incubating with 10 μg/ml tetramers, 10 μg/ml anti-CD28, and 2 μg/ml anti-CD49d. After 4 hours of incubation at 37°C, cells were washed twice in PBS and labeled in 100 μl of medium on ice for 10 min with a bi-specific Ab-Ab conjugate directed against both CD45 and cytokine (IFN-γ, TNF-α, IL-5 or IL-10). Pre-warmed 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 a FITC or APC-conjugated antibody directed against the cytokine of interest, washed, and analyzed on a FACSCalibur instrument (BD Biosciences). For IL-2 rescue experiments, HPV specific cell lines were incubated overnight in T cell medium with or without exogenous IL-2 (40 U/ml) prior to cytokine analysis.

Production of IL-2 and IL-13 by HPV specific T-cell lines was characterized by intracellular cytokine staining. Briefly, T-cells were activated by incubating with 10 μg/ml tetramers, 10 μg/ml anti-CD28, and 2 μg/ml anti-CD49d at 37°C for 2 hours and then an additional 4 hours in the presence of 10 μg/ml monensin. Cells were then harvested, re-suspended in Fixation/Permeabilization solution (BD Biosciences), washed in Perm/Wash buffer (BD Biosciences), and stained with anti-IL2 APC (Miltenyi) or anti-IL-13 PE (BD biosciences) according to manufacturer's instructions. Cells were then washed and immediately analyzed by flow cytometry.

Assessing STAT Signaling

CD4$^+$ tetramer-positive T-cell lines were activated with 10 μg/ml tetramers, 10 μg/ml anti-CD28, and 2 μg/ml anti-CD49d for various time intervals and then characterized by phospho-specific flow as previously described (24). In brief, T-cells were fixed with BD Biosciences Phosflow Buffer I, permeabilized using BD Phosflow Buffer III, and then divided to allow for analysis of multiple phosphoproteins (anti-pSTAT4, anti-pSTAT5, and anti-pSTAT6) using the respective phospho-specific antibodies from BD Biosciences. Phosphospecific Abs were added simultaneously for 20–30 min, washed, resuspended in staining buffer and kept at 4°C before analysis on a FACSCalibur instrument (BD Biosciences).

Statistical Analysis

For tetramer, cytokine capture, and STAT signaling data experimental means were analyzed for statistical differences using the Student's t test with Welch's correction for unequal variances. ICS data was $\log_{10}$ transformed, determined to be normally distributed with comparable standard deviations between groups, and analyzed by a 2-tailed unpaired t-test and reported as means and standard deviations after back transformation. All analyses were performed using Prism software (Version 4.03, GraphPad Software Inc.)

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 Supplementary Table I. After fourteen days, cells were stained with pooled peptide tetramers (Supplementary Figure 1) and again using single peptide tetramers (Figure 1). As shown in Figure 1A, cells from a healthy subject with a DRB1*03:01 haplotype that were stained with $E2_{1-20}$, $E2_{281-300}$ and the corresponding pools resulted in positive staining. All remaining pools were negative (Supplementary Figure 1A). As shown in Figure 1B, cells from a healthy subject with a DRB1*01:02 haplotype that were stained with $E2_{281-300}$, $E6_{113-132}$ and the corresponding pools gave positive staining. Staining with $E6_{81-100}$ was subsequently shown to be elevated background staining because staining irrelevant cells with this tetramer gave similar results (not shown). All remaining pools were negative (Supplementary Figure 1B).

Defining minimal DR0102 and DR0301-restricted HPV epitopes

Complimentary proliferation, tetramer staining, and peptide binding experiments were conducted using truncated versions of each antigenic E2/E6 peptide to identify the core residues required for peptide presentation and T-cell activation. The sequences of these peptides are summarized in the bottom portion of Supplementary Table I. Some representative examples of these experiments are shown in Figure 2. As shown in Figure 2A, a DR0301-restricted $E2_{281-300}$-specific T-cell line proliferated in response to $E2_{285-300}$ but not $E2_{284-296}$. This proliferative response was shown to depend on peptide presentation by HLA-DR and not HLA-DQ through the use of blocking antibodies (data not shown). The same T-cell line could be stained with tetramers loaded with $E2_{285-300}$ but not $E2_{284-296}$ (Fig 2B). While both peptides were able to bind to recombinant DR0301, $E2_{285-300}$ bound with higher affinity (Fig 2C). Experiments using a DR0102-restricted $E2_{281-300}$-specific T-cell line indicated that the same minimal epitope is presented and recognized in the context of DR0102. Similar experiments implicated $E2_{5-17}$ as the minimal epitope within $E2_{1-20}$ and $E6_{115-130}$ as the minimal epitope within $E6_{113-132}$. These minimal epitopes are summarized in the first two columns of Table I. In general these core antigenic regions are in agreement with the published binding motifs for DR0102 and DR0301 (25–26).

Responsiveness of Healthy Subjects and RRP patients to HPV E2/E6 Peptides

Using the peptides identified through initial epitope mapping experiments, T-cell responses to E2/E6 were assayed in healthy subjects and RRP patients by stimulating purified CD4$^+$ T-cells with HPV peptide and staining with tetramers after two weeks of *in vitro* culture (Figure 3A). While the data suggested stronger T-cell expansion in certain healthy subjects, none of these differences were statistically significant. To assess functional responses to E2/E6 peptides, CD4$^+$ T-cell lines were isolated from healthy subjects and RRP patients, activated using HPV peptide tetramers, and assayed for cytokine release using a capture assay. T-cell lines that were isolated from RRP subjects (Figure 3B) were typically deficient

in their capacity to secrete cytokines, while most healthy subjects exhibited robust secretion of T_H1 cytokines such as IFN-γ. As shown in Figure 3C, the deficit in IFN-γ secretion by RRP patients as compared to healthy subjects was statistically significant (p<0.01), while levels of secreted IL-5 and IL-10 were not significantly different. One subset of patients was deficient in TNF-α secretion while 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, STAT-4, STAT-5, and STAT-6 signaling was measured in multiple $CD4^+$ T-cell lines isolated from RRP patients and HLA matched healthy subjects. For these experiments tetramer stimulated E2/E6 specific T-cells lines were stained with phospho-specific STAT-4, STAT-5, and STAT-6 antibodies and analyzed by flow cytometry. As shown in Figure 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, since non-specific induction of STAT-5 using IL-2 (rather than tetramer stimulation) elicited similar signaling in RRP patients and healthy subjects (Figure 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 STAT5-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 Figure 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 Figure 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 T_H2-like polarization. However, these HPV specific cells rarely produced IL-5 (Figure 3) and did not secrete appreciable amounts of IL-4 (data not shown).

Effect of IL-2 Pretreatment on HPV Specific Cytokine Secretion

Given the lack of IL-2 production by HPV specific T-cells in RRP subjects, 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 pre-incubation with exogenous IL-2. As shown in Figure 6, IL-2 treatment caused a significant (p=0.01) increase in IFN-γ secretion for cell lines from RRP patients. In contrast, IL-2 treatment caused no change in IFN-γ secretion for cell lines from healthy subjects.

Discussion

Recurrent respiratory papillomatosis (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 leads to the formation of papillomas in 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 percent 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 subjects exhibit chronic infection with HPV-6 or HPV-11 and can express serum antibodies (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 (17). These observations suggest that the immune mechanism that underlies RRP susceptibility involves a deficiency in protective $CD4^+$ T_H1-like T-cell responses against HPV. Indeed, previous studies have observed deficits in T_H1 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 controls with DRB1*01:02 and DRB1*03:01 haplotypes. One of these peptides, $E2_{281-300}$, 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 Supplementary Table II, that antigenic region of the E2 protein is highly conserved among HPV strains. 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 to those of unaffected controls (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 two to seven fold enrichment of functional Tregs in papillomas compared to autologous blood from patients with RRP (33), while the frequency of Tregs in the peripheral blood of RRP patients was comparable to that found in control subjects. Enriched and functional Tregs in papillomas would likely suppress T_H1-like responses to HPV in papilloma tissues, while 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 in vitro culture methodology used could minimize differences in responsiveness between the two groups. In any case, these observations confirm that HPV-6/-11 infection is recognized by patients and controls and thus, is not a subliminal infection below the threshold of immune recognition as once thought (33–34). Thus, clonal ignorance (as suggested for certain $CD8^+$ T-cell specificities) is not a viable explanation for why patients with RRP do not clear HPV-6/-11 infection (35), rather "low level immune tolerance" 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 PBMC that had been pre-treated 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 be likely to 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 to healthy control subjects. With the exception of two patients (three cell lines) RPP 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. While 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 T_H2-like response polarization. We previously observed polarized expression of a T_H2-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 T_H1-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, as the deficiency in STAT-5 signaling in RRP patients was comparable to healthy subjects after the administration of IL-2. These results suggest 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, while exhaustion is associated with persistently high antigenic loads, HPVs generally use non-preferred 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 T_H1-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 co-stimulation, 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 antigen 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 hypo-responsive state in the absence of CTLA-4 (43). Furthermore, induction of anergy has been shown to occur in the presence of antibodies 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 following IFN-γ therapy (45–47). This suggests that restoration of a T_H1-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 T_H2-like polarization, due to 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 gamma 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 T_H1-like, and prevents/blocks T_H2-like responses to HPV proteins, and thereby improves 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 T_H1-/T_H2-like balance to these HPVs in patients with RRP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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replication and associated with T cell dysfunction. J Immunol. 2007; 179:1979–1987. [PubMed: 17641065]

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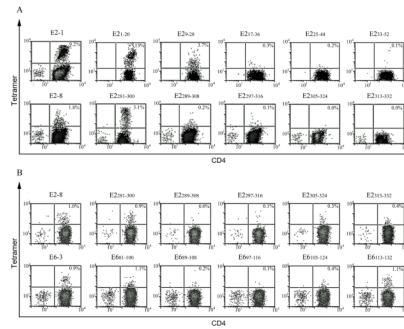
Figure 1. Identifying E2/E6 CD4+ T-cell epitopes
(A) CD4^{+} T-cells from a healthy subject with DRB1*03:01 haplotype were stimulated with peptide pools spanning the E2/E6 protein sequences. After fourteen days, cells were stained with DR0301 tetramers. Cells that were stained with $E2_{1-20}$, $E2_{281-300}$ and the corresponding pools gave positive staining. All remaining pools were negative. (B) CD4^{+} T-cells from a healthy subject with DRB1*01:02 haplotype were stimulated with peptide pools spanning the E2/E6 protein sequences. After fourteen days, cells were stained with DR0102 tetramers. Cells that were stained with $E2_{281-300}$, $E6_{113-132}$ and the corresponding pools gave positive staining. Staining with $E6_{81-100}$ was subsequently shown to be background staining.

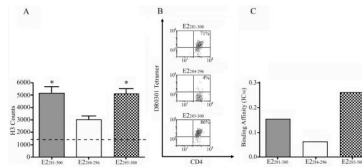**Figure 2. Identifying minimal epitopes within HPV E2/E6 peptides**

(A) Proliferation of an HPV-specific T-cell line in response to $E2_{281-300}$, $E2_{284-296}$, or $E2_{285-300}$ peptide (dashed line indicates background proliferation). Asterisks denote proliferation that is at least 3-fold above background. (B) Staining of a T-cell line with DR0301 tetramer loaded with $E2_{281-300}$, $E2_{284-296}$, or $E2_{285-300}$ peptide (quadrant boundary set based on the staining of an unloaded tetramer). (C) Relative binding affinity of $E2_{281-300}$, $E2_{284-296}$, or $E2_{285-300}$ peptide to recombinant DR0301 protein in competition with the $myo_{137-148}$ reference peptide.
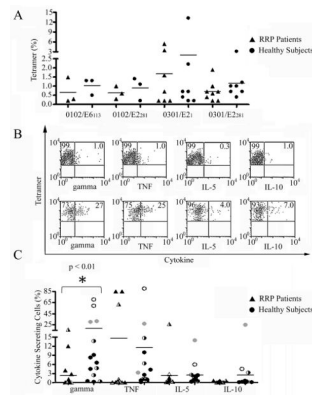
Figure 3. E2/E6 Specific T-cell responses and Cytokine Secretion
(A) Percentage of E2/E6 tetramer positive T-cells after *in vitro* culture as measured in RRP patients and HLA matched healthy subjects. While many of the stronger responses were observed in healthy subjects, these differences were not 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/E6$_{113-132}$ (gray symbols), DR0102/E2$_{281-300}$ (open symbols), DR0301/E2$_{1-20}$ (half-filled symbols), and DR0301/E2$_{281-300}$ (black symbols) specific 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). The data indicates a significant deficit in IFN-γ secretion by RRP patients as compared to healthy subjects (p<0.01). One subset of patients was deficient in TNF-α secretion while another had enhanced TNF-α secretion.
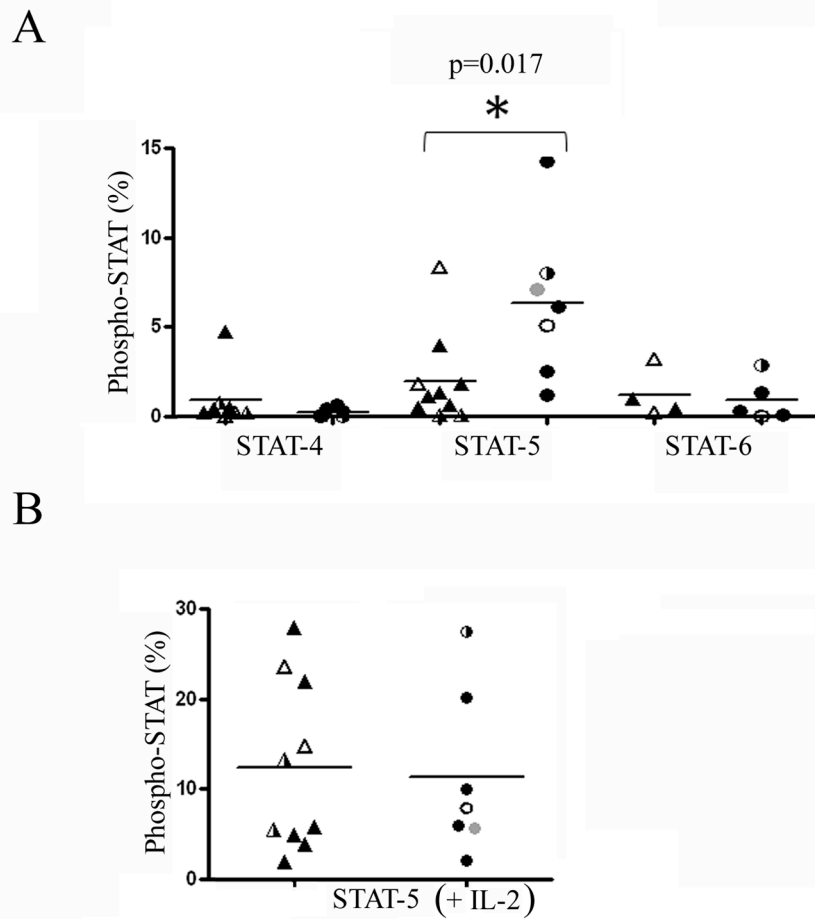
Figure 4. STAT-5 Signaling of E2/E6 Specific T-cells is Altered in Patients with RRP and is Reversible with IL-2

(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 $DR0301/E2_{281-300}$ (black symbols) specific 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. STAT-4 signaling was elevated in one RRP patient, but 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. (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.
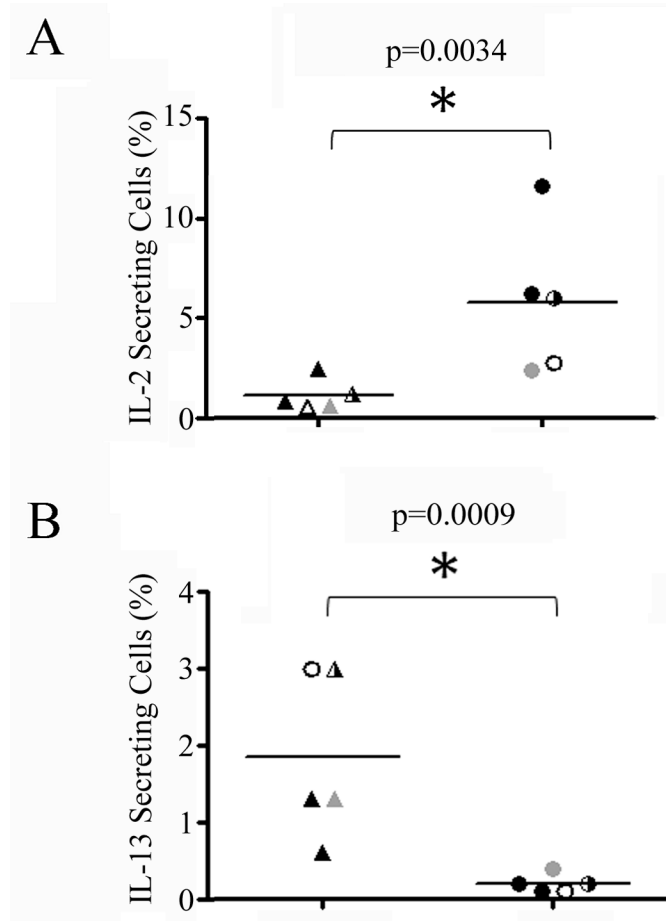
Figure 5. E2/E6 Specific T-cells show Decreased IL-2 and Enhanced IL-13 Production in Patients with RRP

A) IL-2 production of tetramer stimulated DR0102/E6$_{113-132}$ (gray symbols), DR0102/E2$_{281-300}$ (open symbols), DR0301/E2$_{1-20}$ (half-filled symbols), and DR0301/E2$_{281-300}$ (black symbols) specific T-cells as measured by intracellular cytokine staining for 5 cell lines isolated from RRP patients (triangles) and 5 cell lines isolated from HLA matched healthy subjects. 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.
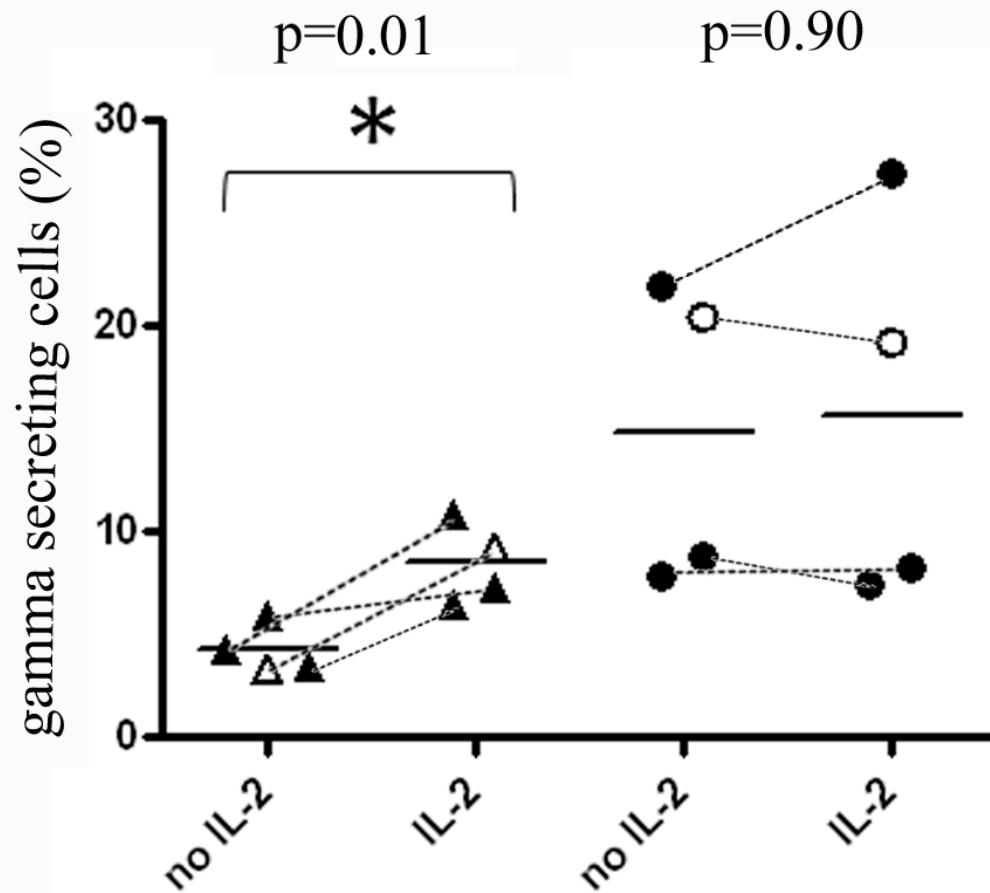
Figure 6. Cytokine Secretion of HPV Specific T-cells is Increased in Patients with RRP after Pretreatment with IL-2

(A) IFN-γ secretion by DR0102/E2$_{281-300}$ (open symbols) and DR0301/E2$_{281-300}$ (black symbols) specific T-cell lines measured by cytokine capture for 4 cell lines isolated from RRP patients (triangles) and 4 cell lines isolated from HLA matched healthy subjects (circles) with or without pretreatment with exogenous IL-2 (identical cell lines connected by dashed lines). The data indicates a significant improvement in IFN-γ secretion for RRP patients (p=0.01) but not for healthy subjects (p=0.90). (B) Fold increase in IFN-γ secretion by HPV specific T cell lines from RRP patients and healthy subjects after pretreatment with exogenous IL-2. An average 1-fold increase (no change) was observed for healthy subjects and a 2-fold increase was observed for RRP patients, which represents a statistically significant difference (p=0.035).

Table I

Summary of E2/E6 Epitopes

E2/E6 residues	Amino Acid Sequence[*]	# of Patients	# of Healthy
DR0102			
E6$_{113-132}$	EIE<u>KLKHI**LGKARFIKL**</u>NNQ	2 of 2	2 of 3
E2$_{281-300}$	SAAT<u>**PIVQLQGDSN**</u>CLKCFR	2 of 2	3 of 3
DR0301			
E2$_{1-20}$	MEAIA<u>**KRLDACQDQ**</u>LLELYE	4 of 4	5 of 7
E2$_{281-300}$	SAAT<u>PIVQ**LQGDSNCLK**</u>CFR	4 of 4	7 of 7

[*] 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 bolded.

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Table II

Summary of Clinical and Immune Response Data

Subject ID	HLA	Age	Onset	Disease Score	IFN-$\gamma^{\ddagger}$	STAT-5§
RRP 1	DR0102	63	Adult	Mild/Moderate (0.018)†	0.2	1.8
RRP 2	DR0301	53	Adult	Mild/Moderate (0.0) *	4.0	1.2
RRP 3	DR0301	30	Adult	Severe (0.075)†	0.6	3.9
RRP 4	DR0301	49	Adult	Severe (0.068)†	1.6	0.1
RRP 5	DR0102	46	Adult	Severe (0.088)†	0.2	8.3
RRP 6	DR0301	26	Juvenile	Mild/Moderate (0.005)†	6.0	0.6
Cont 1	DR0102	NA	None	None	49.4	5.6
Cont 2	DR0102	47	None	None	43.2	7.0
Cont 3	DR0301	38	None	None	ND	2.5
Cont 4	DR0301	55	None	None	ND	ND
Cont 5	DR0301	25	None	None	5.6	4.6
Cont 6	DR0301	28	None	None	10.2	14.1
Cont 7	DR0301	48	None	None	3.4	ND
Cont 8	DR0301	33	None	None	2.5	ND

*Subject improved clinically (severe to mild/moderate) in response to an experimental immunomodulator as part of a pre-clinical trial which concluded immediately before these data were obtained.

†Surgical de-bulking performed

‡Average percentage of positive cells in interferon-γ capture assay

§Average percentage of positive cells in phospho-specific STAT-5 assay

NA= Not available; ND= Not Done