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Failure of Gamma Interferon but Not Interleukin-10 Expression in Response to Human Papillomavirus Type 11 E6 Protein in Respiratory Papillomatosis

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Recurrent respiratory papillomatosis (RRP) is a chronic, debilitating disease of the upper airway caused by human papillomavirus type 6 (HPV-6) or HPV-11. We describe responses of peripheral blood mononuclear cells (PBMC) and T cells from RRP patients and controls to the HPV-11 early proteins E6 and E7. PBMC were exposed in vitro to purified E6 or E7 proteins or transduced with fusion proteins containing the first 11 amino acids of the human immunodeficiency virus type 1 protein tat fused to E6 or E7 (tat-E6/tat-E7). T_H1-like (interleukin-2 [IL-2], gamma interferon [IFN-γ], IL-12, and IL-18), and T_H2-like (IL-4 and IL-10) cytokine mRNAs were identified by reverse transcription-PCR, and IFN-γ and IL-10 cytokine-producing cells were identified by enzyme-linked immunospot assay. These studies show that HPV-11 E6 skews IL-10–IFN-γ expression by patients with RRP toward greater expression of IL-10 than of IFN-γ. In addition, there is a general cytokine hyporesponsiveness to E6 that is more prominent for T_H1-like cytokine expression by patients with severe disease. Patients showed persistent IL-10 cytokine expression by the nonadherent fraction of PBMC when challenged with E6 and tat-E6, and, in contrast to controls, both T cells and non-T cells from patients expressed IL-10. However, E7/tat-E7 cytokine responses in patients with RRP were similar to those of the controls. In contrast, E6 inhibited IL-2 and IL-18 mRNA expression that would further contribute to a cytokine microenvironment unfavorable to HPV-specific, T-cell responses that should control persistent HPV infection. In summary, E6 is the dominant inducer of cytokine expression in RRP, and it induces a skewed expression of IL-10 compared to the expression of IFN-γ.

Recurrent laryngeal papillomatosis remains an immunologic enigma. Human papillomavirus type 6 (HPV-6) and HPV-11 are the predominant HPV viruses that cause papilloma development (21, 33), and it is unclear why only a very small faction (4 to 6/100,000) of HPV-6- and HPV-11-exposed individuals develop this disease (4). Furthermore, it is unknown why a still smaller group of individuals has a severe and unrelenting course of disease that can cause significant morbidity, and on occasion mortality, because of the strategic location of papillomas in the airway (2, 24). There is clinical variability between patients and, on occasion within a given patient (2, 9, 24, 48). However, the immunologic mechanism(s) that govern this variation in disease severity and predisposes only a small group of HPV-exposed individuals to develop recurrent respiratory papillomatosis (RRP) are unresolved. Some patients may require as many as 200 surgical procedures to maintain a patent airway (2, 24), and a small group of patients with RRP may progress to a more serious extension of their disease into the trachea and lungs (54). In contrast, some patients may show no signs of disease recurrence after their first clinical presentation.

The HPVs are ubiquitous viruses, and virtually all individuals have been exposed (10, 55, 58). Recent molecular epidemiologic observations suggest that HPVs are likely part of the commensal microflora of human epithelia, held in a subclinical state by a competent immune system but able to be reactivated under immunosuppressive conditions (55). Furthermore, because of the substantial homology of E6 and E7 proteins across HPV types (58), it is likely that most individuals have been exposed to common E6 and E7 epitopes. Thus, few individuals are likely to be HPV E6 and E7 immunologically naive.

Previously, we and others reported that the general immune responses made by patients with RRP to mitogens and specific antigens were comparable to those of normal, HPV-exposed controls without this disease (9, 19). Specifically, the mitogen proliferative responses to phytohemagglutinin, concanavalin A, and pokeweed mitogens, natural killer and lymphokine-activated killer cell cytotoxicity, and lymphocyte subset enumeration in patients with RRP were comparable to those of controls (9). Further investigation revealed an imbalance in the repertoire of T_H1- and T_H2-like cytokine responses made by peripheral blood mononuclear cells (PBMC) exposed to autologous papilloma tissue (8). To further illuminate the inherent HPV-specific immune dysregulation that occurs in RRP and favors chronic viral expression versus effective cellular immune responsiveness to HPV, we explored the cytokine repertoires expressed by PBMC and T cells from patients with RRP exposed to purified HPV early proteins E6 and E7. We

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cloned and expressed E6 and E7 from HPV-11 and used them as antigens in in vitro PBMC stimulation assays. In addition, we genetically engineered both E6 and E7 to contain at their N terminus, the transduction domain from the human immunodeficiency virus type 1 (HIV-1) tat protein to facilitate PBMC transduction with E6 or E7 (34, 43, 53). These fusion proteins should primarily result in class I major histocompatibility complex (MHC) expression of E6 or E7 peptides by PBMC (37).

We report here that PBMC from patients with severe disease failed to make cytokine responses at low concentrations of E6, and this diminished response correlated with severe disease. Furthermore, the gamma interferon (IFN-γ) mRNA response made to tat-E6 was disproportionally reduced, compared to IL-10 expression. In addition, tat-E6-transduced PBMC from patients with RRP enumerated by enzyme-linked immunospot (ELISPOT) assay contained cells from the nonadherent fraction of PBMC that produced interleukin-10 (IL-10). Both T cells and non-T cells from E6- or tat-E6-pulsed PBMC expressed IL-10 and IFN-γ mRNA. These responses provide insight into how HPV-11 E6 and E7 may affect the immunoregulatory cytokine milieu favoring chronic HPV-induced disease in patients with RRP.

TABLE 1. Clinical characteristics of patients with RRP

Patient	Age (yr) at assay	Sex	Disease severity	Score[a]	Total no. of surgeries
1	55	Male	Mild to moderate	0.01	7
2	28	Male	Mild to moderate	0.01	4
3	36	Female	Severe	0.14	2
4	29	Male	Severe	0.32	3
5	23	Female	Severe	0.18	12
6	44	Male	Severe	0.21	7
7	45	Male	Mild to moderate	0.03	6
8	11	Female	Severe	0.28	23
9	44	Male	Severe	0.10	8
10	30	Male	Severe	0.32	4
11	46	Male	Severe	0.06	24
12	7	Female	Severe	1.01	16
13	23	Female	Severe	0.08	13
14	5	Female	Severe	0.11	4
15	46	Male	Severe	0.08	26
16	55	Male	Severe	0.32	4
17	55	Male	Severe	0.10	5
18	42	Male	Severe	0.06	16
19	64	Female	Mild to moderate	0.01	12
20	31	Male	Severe	0.11	6
21	8	Female	Severe	NA[b]	21

[a] A disease severity score of ≥0.06 is defined as severe disease (see Materials and Methods).
[b] NA, score not available (severe stenosis and scarring).

MATERIALS AND METHODS

HPV typing. Patients with RRP infected with HPV-11 were identified by HPV typing. Briefly, total tissue DNA was extracted and HPV DNA was amplified by PCR and confirmed by Southern blot hybridization, essentially as previously described (29). Briefly, frozen tissue was reduced to a powder (Braun Dismembrator), DNA isolated with DNAzol (Gibco-BRL, Carlsbad, Calif.), precipitated with alcohol, and quantified by fluorometry (Hoeffer Scientific TKO fluorometer). HPV-11 primer sequences, nucleotides (nt) 7179 to 7196 and nt 77 to 99 (20) (GenBank accession number M14119), were used for PCR, with 50 ng of tissue DNA and 30 cycles of hot-start amplification with AmpliTaq Gold polymerase (Perkin-Elmer, Wellesley, Mass.). Southern blot hybridization was used to confirm HPV-11 patients by hybridizing 5 μl of PCR product with a cloned HPV-11 probe under stringent hybridization conditions ($T_m = -20$°C). As a positive control, 25 fg of cloned HPV DNA was mixed with 50 ng of HPV-negative human tonsil DNA and amplified and analyzed at the same time as the biopsy DNA in each experiment. Amplification without the addition of tissue DNA served as a negative control for each assay.

Study patients with RRP and controls. Thirteen HPV-11-infected patients with RRP and eight controls with no history of RRP were studied by using HPV-11 E6 and E7 as the activating antigens. In addition, eight other patients and eight additional controls were evaluated by using the transducing, fusion proteins tat-E6 and tat-E7 as the activating antigen as described below. Many of the controls should be considered as HPV exposed, given recent molecular epidemiologic observations that consider HPV, including HPV-11, as part of the commensal microflora of human epithelia, and the prevalence of memory T cells in a majority of HPV asymptomatic controls (10, 55). The immune responses of a subset of patients presented here were previously studied and were comparable to those of controls (9).

In all, 4 patients had mild-to-moderate disease, and 17 patients had severe disease. The disease severity score was determined as previously described (1, 25, 48). The clinical characteristics of these patients are shown in Table 1. The median age ± interquartile range of the patients was 36 ± 22.25 years, and the median age of the controls was 37.5 ± 7.0 years. Severe disease was defined as a growth rate of ≥0.06 (growth rate = disease severity score/number of days since last surgery; the range for patients with severe disease was 0.06 to 1.01) and ≥3 surgeries in the past 12 months. Mild-to-moderate disease was defined as a growth rate of <0.06 (the range for patients with patients with mild-to-moderate disease was 0.001 to 0.05).

Lymphocyte cultures with HPV-11 early proteins E6/E7 or tat-E6/tat-E7. A sample (20 ml) of blood was drawn by venipuncture after informed consent was obtained in accordance with the guidelines of our institutional review board. PBMC were obtained by Ficoll-Hypaque density centrifugation for in vitro stimulation with E6, E7, tat-E6, or tat-E7, followed by cytokine mRNA analysis. PBMC were counted and adjusted to 10^6 cells/ml in RPMI plus 10% fetal bovine serum and then exposed in vitro to E6 or E7 proteins in twofold dilutions, ranging from 50 to 0.2 ng/ml, in 1.5-ml conical centrifuge tubes for 4 h at 37°C in 5% CO_2-enriched air. Similar experiments were performed with tat-E6 or tat-E7 in the same medium for 16 h. PBMC were exposed to the tat-E6 or tat-E7 fusion proteins for various amounts of time to identify the maximum cytokine mRNA response made to these fusion proteins. Maximum IL-10 and IFN-γ mRNA were observed with the tat proteins at 16 h compared to 4-h exposure to E6 or E7 (data not shown). mRNA from each culture was analyzed for the expression of a panel of cytokines, including IL-2, IL-4, IL-10, IL-12, IL-18, and IFN-γ, by reverse transcription-PCR (RT-PCR).

Cloning and production of HPV-11 E6 and E7 proteins. HPV-11 E6 and E7 open reading frames were subcloned from wild-type HPV-11 (15) by PCR amplification with the following primers (HPV nucleotide numbers for each primer are indicated in boldface): E6 N terminus (*GACGACGACAAG***ATGGAAAGTA AAGATGCCTCC** [nt 102 to 123]), E6 C terminus (*GAGGAGAAGCCCGGT***T TAGGGTAACAAGTCTTCCAT** [nt 554 to 533]), E7 N terminus (*GACGACGA CAAG***ATGCATGGAAGACTTGTTACCCTA** [nt 529 to 553]), and E7 C terminus (*GAGGAGAAGCCCGGT***TTATGGTTTTGGTGCGCAGAT** [nt 826 to 805]). Nucleotides in boldface are the corresponding HPV-11 E6 and E7 sequences; nucleotides in italics allowed for the ligation-independent cloning of individual coding regions into pet 32-LIC (Novagen, Madison, Wis.). This resulted in the production of a fusion protein that also contained six contiguous histidine residues (His_6), present in the vector, to facilitate subsequent affinity purification as described below. All clones were sequenced to ensure the fidelity of the coding regions. Transformation of T7 transgenic strains of *Escherichia coli* resulted in the inducible expression of a single protein of the expected molecular weight identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Production of E6 and E7 fusion proteins containing the HIV-1 tat protein transducing domain and six histidine residues (tat-E6/E7). To generate tat fusion proteins of E6 and E7 capable of transducing PBMC, a two-step PCR-based cloning strategy was designed. Step 1 incorporated the corresponding nucleotides from the 11-amino-acid transduction domain (GGATACGGCCGT AAGAAGCGTCGTCAGCGTCGCCGT) of the HIV-1 tat protein (53) at the N terminus of HPV-11 E6 and E7 sequences shown above. A single glycine residue was incorporated following the tat sequence to permit free rotation between these domains.

Step 2 added six histidine residues $(CAT)_6$ to the N terminus of the tat-E6 and tat-E7 sequences. BamHI restriction sites were added at the end of the C-terminal primers and NcoI sites were added to the end of the N-terminal primers to allow for directional cloning into pET-11d (Novagen). This resulted in a single

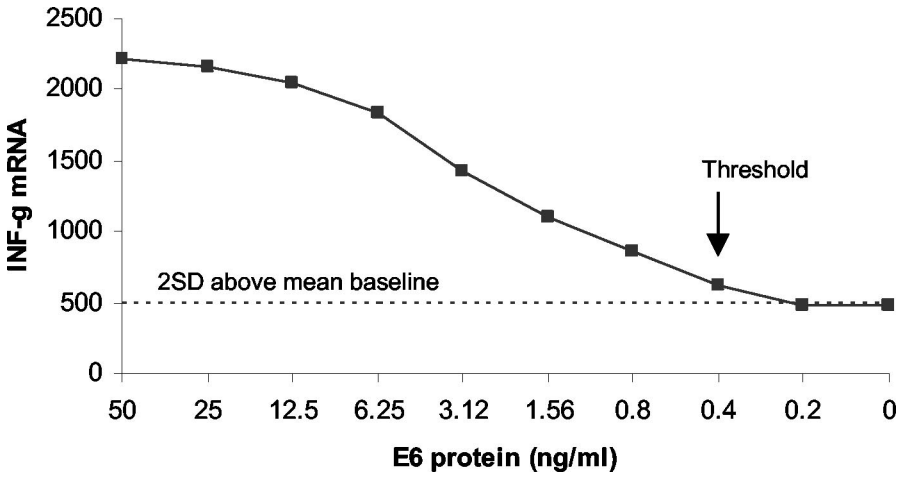


FIG. 1. Representative IFN-γ mRNA dose-response curve of a patient to E6 protein. The threshold equals the lowest concentration of E6 protein that induces a response that is ≥2 SDs above the mean response made by untreated cells (the baseline is indicated by a dashed line).

additional methionine and a single additional glycine residue at the N terminus of the tat-E6 and tat-E7 fusion proteins. All clones were sequenced to ensure the fidelity of the coding regions. Constructs were expressed in *E. coli* strain BL21(DE3) (Novagen).

E6, E7, tat-E6, and tat-E7 protein purification. *E. coli* transformed with the expression constructs were cultured in Luria Broth at 37°C to achieve log-phase growth, and then induced with 2.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. Cell pellets were extracted with 6 M guanidine in 1× binding buffer (10 mM Tris [pH 7.6], 1 mM NaCl) by sonication. Solubilized proteins were cleared of debris by centrifugation and subsequent filtration with a 0.45-μm-pore-size filter (Millipore, Bedford, Mass.). Cleared extracts were then purified by affinity chromatography over a His-Bind column (Novagen) and finally eluted with imidazole at a concentration of 150 mM for the native E6 and E7 proteins and at 250 mM for the transducing E7 protein tat-E7. Since the transducing form of HPV-11 E6, tat-E6, was insoluble in nonionic detergents, 8 M urea, and 6 M guanidine, a four-step extraction was performed to obtain purified tat-E6. Purification of tat-E6 started with 1% triton and 1% NP-40 in binding buffer, followed by two extractions with 6 M guanidine, and a final extraction with 0.025% SDS to yield a highly purified preparation of tat-E6 by SDS-PAGE. To ensure that the trace amounts of imidazole and SDS present from the elution buffers of the purified proteins did not affect the cytokine stimulation assay in the PBMC cultures, cells were treated with the buffers alone and processed as described above. There was no effect of the elution buffers alone on any of the cytokine mRNA levels that were measured (data not shown).

RT-PCR. Cytokine-specific RT-PCR was performed as described for IFN-γ, IL-2, IL-12, IL-18, IL-4, and IL-10 with commercially available primers specific for each of these cytokines (Clontech, Palo Alto, Calif.). Briefly, 0.5 μg of total RNA was primed with 10 μM oligo$(dT)_{16}$. RT was performed by using Omniscript R.T. (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. The resulting cDNA was diluted to a final volume of 100 μl, and 5 μl was used in a standard hotstart PCR (14). The RT-PCRs were in the linear range for detection at the antigen concentrations determined to be thresholds. PCR products were separated on a 2% agarose gel in 1× Tris-borate-EDTA for 20 min at 50 mV, and the gel was analyzed by using the IS-1000 digital imaging system and analysis software (Alpha Innotech Corp., San Leandro, Calif.) to obtain relative densitometry comparisons between bands. A CD3δ-specific PCR was simultaneously performed with all cytokine-specific amplifications to ensure that equal amounts of T-cell cDNA were being compared, as previously described (8). Cytokine-specific, dose-response curves were generated for each patient's PBMC exposed to each E6 or E7 protein. The threshold of each dose-response curve was identified as the minimal concentration of protein that induced an increase in mRNA level greater than two standard deviations (SDs) above the mean, untreated cell level (baseline) (Fig. 1). The thresholds for each cytokine response were collapsed to better illustrate these results as shown in Fig. 2, 3, 5, and 6.

FITC conjugation. A 200-μg portion of tat fusion protein was conjugated to 1 mg of activated fluorescein isothiocyanate (FITC; Pierce, Rockford, Ill.)/ml in conjugation buffer (100 mM carbonate-bicarbonate [pH 9.5]) for 1 h at room temperature in the dark. Uncoupled fluorochrome was separated from conjugated protein by using a D-Salt Dextran desalting column (Pierce). Labeled proteins were quantified and used to transduce 10^6 total PBMC at 1 μg/ml for 1 h at 37°C. Cells were washed three times in 1× phosphate-buffered saline (PBS; pH 7.2) and analyzed by flow cytometry, with gating on monocytes since they showed the greatest uptake of protein.

ELISPOT assay. To determine whether the changes in expression of cytokine-specific mRNA represented changes in secreted cytokine protein expression, a cytokine-specific ELISPOT assay was performed for both IFN-γ and IL-10 (23) with total or nonadherent fraction of PBMC from four patients and four controls by using human ELISPOT assay kits as specified by the manufacturer (BioSource International, Camarillo, Calif.). Nonadherent cells were obtained after a 72-h incubation with 100 μg of either E6 or tat-E6 in 12-well tissue culture plates. This concentration of E6 and E7 proteins was selected to ensure that all patients would express IFN-γ and IL-10, i.e., twice the highest concentration required to identify IFN-γ and IL-10 thresholds (Table 2). The nonadherent PBMC fractions were harvested by gentle aspiration with a pipette, washed, and studied by ELISPOT analysis according to the manufacturer's specifications. In addition, bovine serum albumin (BSA) was used as a negative control, and phorbol 12-myristate 13-acetate–ionomycin was included as a positive control.

Magnetic bead separation of T cells and non-T cells. $CD3^+$ T cells were isolated from non-T cells contained in E6- or tat-E6-pulsed PBMC by both positive and negative selection with microbead separation as previously described (38). Briefly, 2×10^6 PBMC pulsed with E6 or tat-E6 as described above were stained with either phycoerythrin (PE)-conjugated, anti-CD3 murine monoclonal antibodies (Becton Dickinson, San Jose, Calif.) as the positive selection or with a cocktail (anti-CD14, -CD16, -CD19, and -CD56) of PE-conjugated, murine monoclonal antibodies (Becton Dickinson) as the negative selection. Aliquots of these separately stained cells were then counterstained with murine immunoglobulin G1 anti-PE monoclonal antibodies conjugated to magnetic beads according to the manufacturer's specifications (Miltenyi, Auburn, Calif.). The stained cells were placed on MS minicolumns mounted on the OctoMACS magnet (Miltenyi). Unlabeled cells were eluted with three separate washes of 0.01 M PBS (pH 7.3) and 0.5% BSA. PE-labeled cells were obtained from the column by removing the column from the magnetic field and then flushing the column with PBS by using a sterile plunger. The total RNA was then extracted from these cell fractions, and a cytokine-specific (IFN-γ or IL-10) PCR was performed as described above.

Statistical analysis. A comparison of the cytokine mRNA responses made between patients with RRP and controls was performed by using a two-tailed, unpaired *t* test with a Welch correction for E6 (Fig. 2) and a Mann-Whitney test for tat-E6 (see Fig. 5). To determine whether differences in cytokine mRNA expression at a given threshold concentration of E6 protein could be identified between patients and controls (Fig. 3), a two-sided Fisher exact test was performed. A comparison of the number of cytokine-producing cells detected by ELISPOT assay was performed by using an unpaired *t* test with a Welch correction to calculate a two-tailed *P* value.

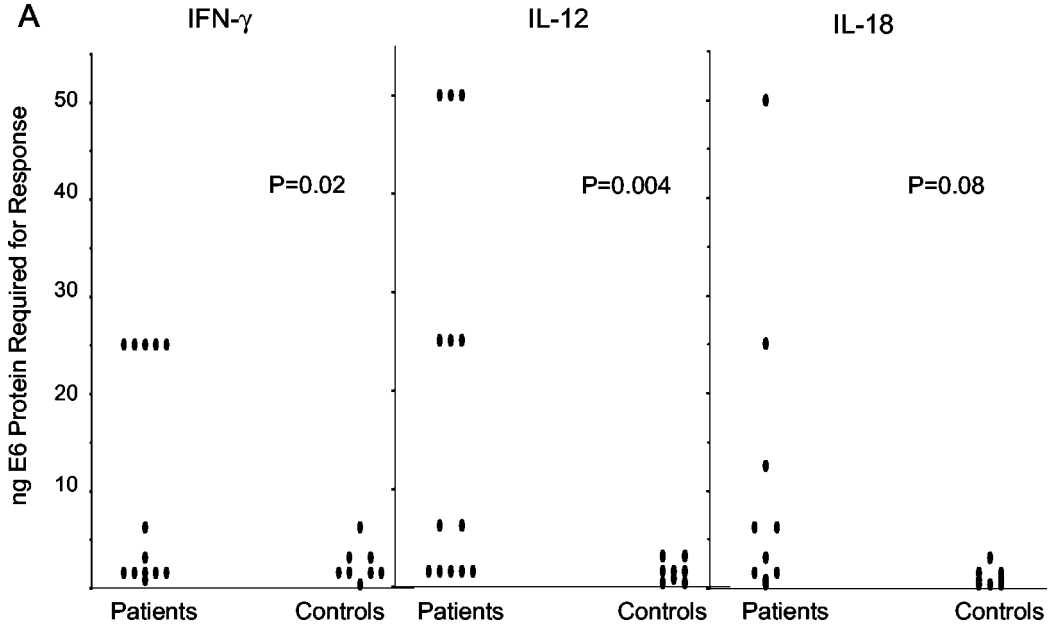
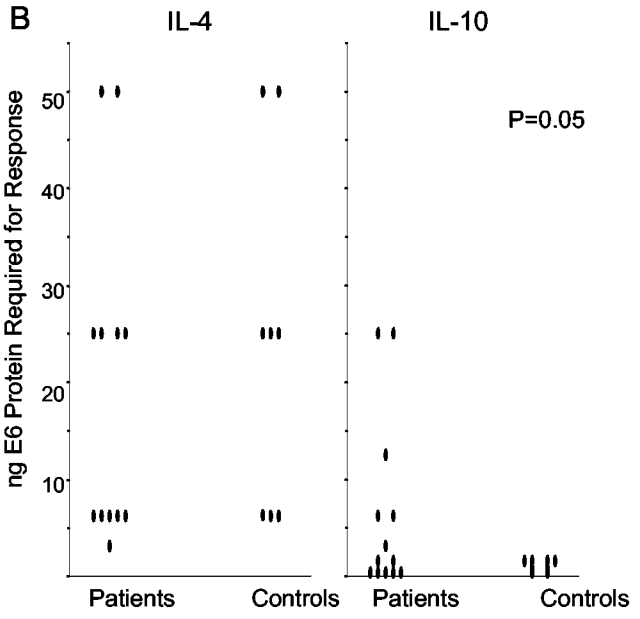


FIG. 2. Cytokine mRNA expression by PBMC in response to purified E6 protein. The minimal concentrations, or threshold values, of recombinant HPV-11 E6 required to induce an increase of >2 SDs above baseline expression for 13 patients with RRP and 8 controls are shown for the T_H1-like cytokines (IFN-γ, IL-12, and IL-18) (A) and for the T_H2-like cytokines (IL-4 and IL-10) (B). Comparisons between patients and controls were made by using a two-tailed, unpaired *t* test with a Welch correction.



RESULTS

Concentration of E6 and E7 proteins required to induce cytokine-specific mRNA responses by PBMC. PBMC from patients with RRP and from controls were analyzed for cytokine mRNA responses to various concentrations of purified E6 or E7 proteins. To determine the minimal protein concentration of each protein required to induce a change in cytokine mRNA expression (T_H1-like [IFN-γ, IL-2, IL-12, and IL-18] and T_H2-like [IL-4 and IL-10]), serial dilutions of E6 or E7 protein (range, 50 to 0.2 ng/ml) were used to stimulate 10^6 PBMC. Figure 1 shows a representative dose-response curve for IFN-γ mRNA expression in response to E6 protein. After similar dose-response curves were generated for each patient and control for each cytokine and protein stimulator, these data were collapsed into threshold values, as described above. Figure 2A shows the lowest concentration (threshold) of E6 that was sufficient to induce an increase in T_H1-like cytokine expression. PBMC from patients required significantly more E6 protein to induce an increase in IFN-γ ($P = 0.02$) and IL-12 ($P = 0.04$), although there was marked variation among patients. A similar trend was observed in the requirement to induce IL-18 ($P = 0.08$). Of note, all of the patients with mild-to-moderate disease were included in the cluster of patients who were comparable to controls. The pattern for T_H2-like responses made by PBMC in response to E6 challenge is shown in Fig. 2B. As with the T_H1-like cytokines, some patients required more E6 to induce IL-10 mRNA ($P = 0.054$). Patients with mild-to-moderate disease were found to have a threshold for IL-10 mRNA expression similar to that of controls. Both patients and controls responded equally when IL-4 mRNA was analyzed, and the threshold for IL-4 induction was higher than that for all of the other cytokines analyzed. In contrast to the results with E6, only minimal cytokine responses were made even at the highest concentrations of E7, and there was no difference between patients and controls (data not shown). The lack of a cytokine mRNA response to E7 serves as a control to confirm that the responses seen to E6 are not the result of an immune response to the His_6 residues added to both the E6 and E7 proteins, or trace contaminants that may have copurified with these antigens.

Comparison of threshold cytokine mRNA responses to E6 and disease severity. Because there was marked variability in

TABLE 2. Number of nonadherent cells expressing IFN-γ or IL-10 protein in patients with RRP and in controls[a]

Group	Cytokine	Mean no. of cells ± SD[a]		
		Untreated	E6 treated	tat-E6 treated
Patients	IFN-γ	12 ± 5	44 ± 7*	152 ± 20**
	IL-10	90 ± 23	>500†	>500‡
Controls	IFN-γ	9 ± 5	47 ± 25*	64 ± 27**
	IL-10	58 ± 29	11 ± 6†	37 ± 9‡

[a] Results are the mean numbers of cytokine-producing cells per 10^5 nonadherent cells ± the standard deviations as analyzed by ELISPOT assay for four different patients and four controls. *, Comparison of IFN-γ expression between patients and controls responding to E6 ($P = 0.83$); **, comparison of IFN-γ expression between patients and controls responding to tat-E6 ($P = 0.0034$); †, comparison of IL-10 expression between patients and controls responding to E6 ($P = 0.0023$); ‡, comparison of IL-10 expression between patients and controls responding to tat-E6 ($P = 0.0027$).

the responsiveness of patients to E6 protein, and patients with mild-to-moderate disease responded similarly to controls, we sought to determine whether the expression of IFN-γ, IL-12, IL-18, and IL-10 correlated with disease severity. We compared the ability of patients and controls to respond to low concentrations (≤3.12 ng/ml) of E6 (Fig. 3). This concentration was selected because only one of the controls had failed to make IFN-γ mRNA at this concentration. Only a subset of the patients responded to ≤3.12 ng of E6/ml by expressing any of the cytokines. Significant differences from the controls were observed with IL-12 mRNA ($P = 0.025$) and IL-18 mRNA ($P = 0.05$) when all patients with RRP were compared to controls. In addition, the severe patients were deficient in expressing IL-12 ($P = 0.002$) and IL-18 ($P = 0.045$), whereas a trend for decreased IFN-γ ($P = 0.075$) expression by these patients was noted compared to controls. However, severe patients' expression of IL-10 ($P = 0.15$) was comparable to controls. Thus, almost all patients with severe disease required higher concentrations of E6 to induce T_H1-like cytokine mRNA responses, whereas only some required higher concentrations of E6 to express IL-10 compared to controls. This suggests that patients with RRP may not generate an HPV-specific, cytokine environment that could support effective clearance of HPV-infected keratinocytes.

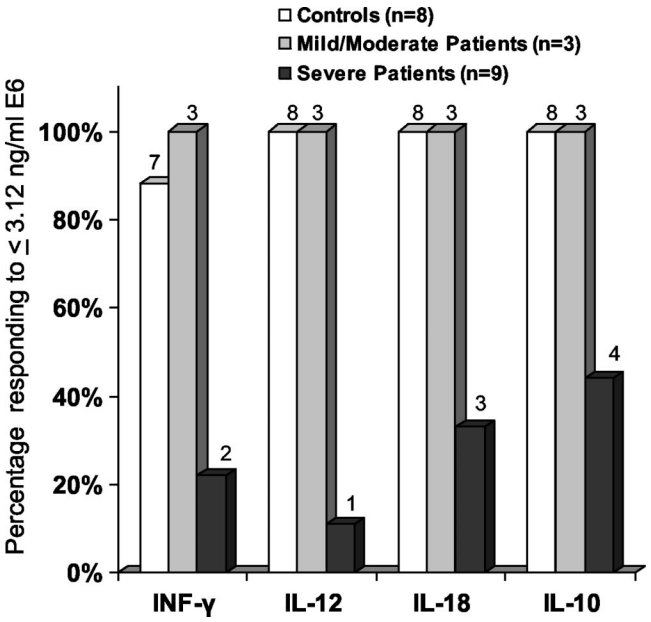


FIG. 3. Ability of PBMC to respond to low concentrations of E6 protein. Open bars show the percentage of controls ($n = 8$) responding to ≤3.12 ng of recombinant E6/ml for IFN-γ, IL-12, IL-18, and IL-10. Gray bars show the percentage of mild-to-moderate patients with RRP ($n = 3$) responding, and black bars show the percentage of patients with severe disease ($n = 9$) responding. Comparisons between controls, and each group of patients were performed by using a two-sided Fisher exact test with Bonferroni correction.

Transduction of PBMC by tat-E6/tat-E7. PBMC exposed exogenously to proteins antigens present peptides of these antigens primarily on MHC class II molecules to T cells, whereas viral proteins expressed in the cytoplasm of antigen-presenting cells are presented as peptides primarily on MHC class I molecules, although not exclusively (25). We constructed a pair of vectors that produced HPV-11 E6 and E7 proteins fused in frame to the 11-amino-acid transduction domain of the HIV-1 tat protein at their N termini (34, 43) and refer to these transducing, fusion proteins as tat-E6 and tat-E7 hereafter. Using these reagents, we then transduced tat-E6 or tat-E7 into the cytoplasm of PBMC. We confirmed the ability of the recombinant proteins to transduce PBMC by labeling both the native and the tat fusion proteins with FITC and then measuring by flow cytometry the uptake of fluorochrome after transduction. Figure 4 shows the increase in fluorescence intensity associated with the presence of the transduction domain in the tat-E7 fusion protein (peak D) compared to FITC-labeled nontransducing E7 (peak C), FITC-labeled BSA (peak B), or untreated cells (peak A). This was consistent with previous reports that amino acids 46 to 56, which comprise the transducing domain of the HIV-1 protein tat, allow for the rapid transduction of all proteins which contain this sequence into the cytoplasm of a wide variety of cells (34).

Minimal protein concentration (threshold) of tat-E6 or tat-E7 required to elicit a change in cytokine mRNA expression. PBMC from patients and controls were transduced with tat-E6 or tat-E7 fusion proteins over the same concentration range used for E6 and E7. Figure 5 shows the comparison of the lowest concentration of tat-E6 that was necessary to induce an increase in IFN-γ or IL-10 expression for each patient or control. PBMC from patients required a significantly higher concentration of tat-E6 protein to induce an increase in IFN-γ ($P = 0.01$) compared to control PBMC. In addition, all of the patients that required more tat-E6 to induce IFN-γ mRNA had severe disease. However, both patients and controls showed indistinguishable IL-10 mRNA expression, even at the lowest tat-E6 concentration tested. Of note, no differences in the concentration of protein required to induce an IFN-γ or IL-10 mRNA response were observed when PBMC from either patients or controls were exposed to tat-E7, and responses were minimal (data not shown). Again, the response with tat-E7 served as a control for possible differences between patients and controls to the tat sequences fused to the viral proteins.

There was an unanticipated IL-2 and IL-18 cytokine mRNA response to tat-E6. In contrast to the cytokine expression patterns observed for all other cytokines, where higher levels of

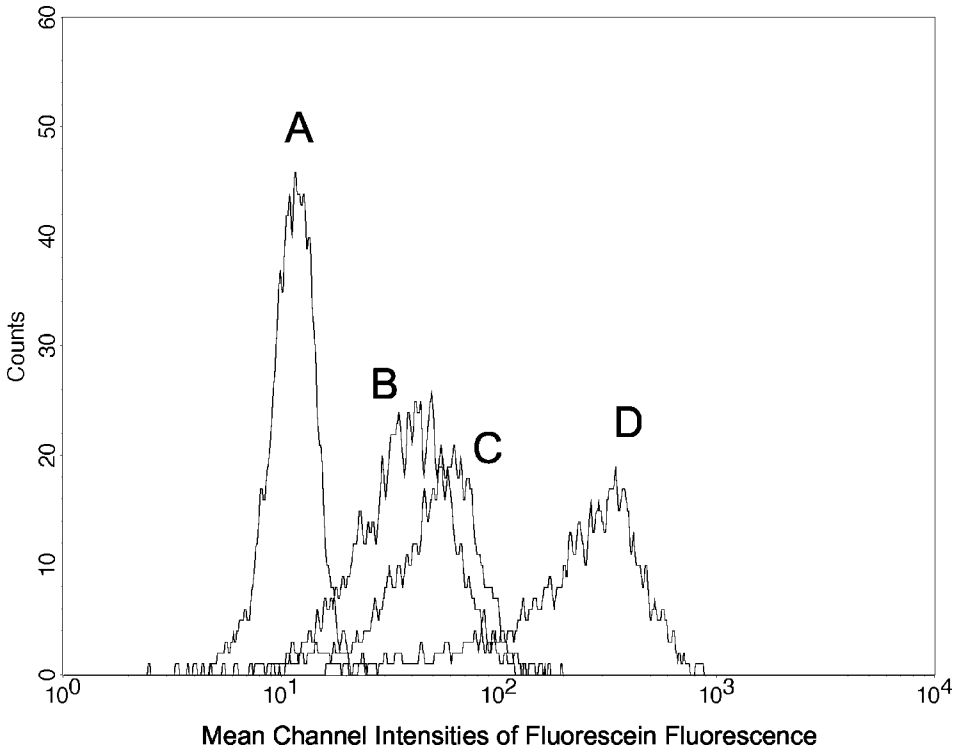


FIG. 4. Cytofluorometric analysis of PBMC transduced with recombinant E7 proteins conjugated with FITC. Peak A represents PBMC that were untreated, peak B represents PBMC containing FITC-labeled bovine serum albumin, peak C represents PBMC containing FITC-labeled E7 protein, and peak D represents PBMC containing FITC-labeled tat-E7.

E6 protein always induced higher levels of cytokine-specific mRNA amounts, high levels of tat-E6 failed to induce or actually, in some instances, decreased the mRNA expression of both IL-2 and IL-18. Of note, at low concentrations of tat-E6, PBMC from four of the original eight controls were able to express a measurable IL-2 or IL-18 response, whereas only one of the eight patients' PBMC was able to do so at any of the tat-E6 concentrations tested (data not shown). At the highest concentrations of E6, a decrease in IL-2 mRNA was seen in two of eight patients and two of eight controls. Figure 6 shows the IL-2, IL-18, and IL-10 responses made by one of these patients. In addition, transduction of PBMC with tat-E7 did

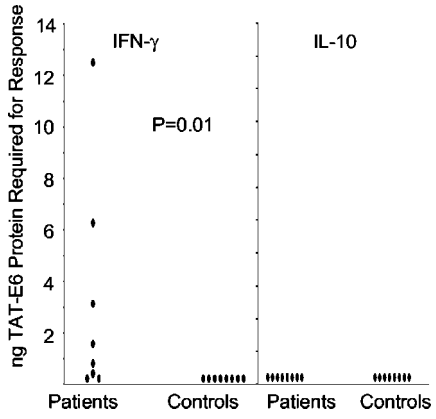


FIG. 5. IFN-γ and IL-10 cytokine mRNA expression by PBMC to purified tat-E6 protein. The minimal concentrations, or threshold values, of recombinant tat-E6 required to induce an increase of >2 SDs above baseline expression for eight patients with RRP and eight controls are shown. Comparisons between patients with RRP and controls were made by using a Mann-Whitney test.

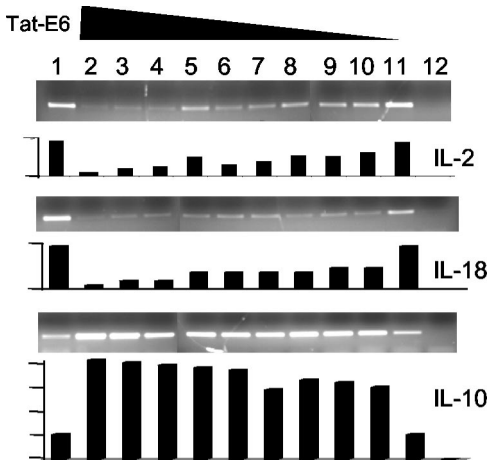


FIG. 6. Inhibitory effect of high tat-E6 concentrations on IL-2 and IL-18 mRNA expression. Each figure set (IL-2, IL-18, and IL-10) shows the actual PCR product, and the corresponding densitometry as the fold changes, for a patient with RRP. In each figure set, lanes 1 and 11 are baseline untreated PBMC expression. Lanes 2 to 10 show the IL-2, IL-18, and IL-10 mRNA levels induced by tat-E6 concentrations ranging from 50 to 0.2 ng/ml. Lane 12 is mRNA not reverse transcribed into cDNA as a control. Two of eight patients and two of eight controls showed this same pattern.

not induce the negative effect on IL-2 or IL-18 mRNA expression that was observed with PBMC exposure to tat-E6 (data not shown). These results imply that E6, but not E7, has immunoregulatory effects on the pivotal cytokine IL-2 and the IFN-γ-inducing cytokine, IL-18.

Cytokine-specific ELISPOT assay. To determine the frequency of cells in PBMC that express IFN-γ and IL-10, we performed cytokine-specific ELISPOT analysis after a 72-h exposure of total PBMC, or the nonadherent fraction of PBMC from the same patients and controls, to E6, tat-E6, E7, or tat-E7 proteins. Table 2 shows the average number of IL-10- and IFN-γ-secreting nonadherent fractions of PBMC from four patients with RRP and four controls (Table 2) exposed to E6 or E7 proteins. To standardize this assay, PBMC were either treated with phorbol 12-myristate 13-acetate–ionomycin (positive control), which routinely yielded >500 spots/well, or BSA (negative control), which yielded similar spot production as shown in Table 2 for unstimulated, nonadherent cells in this assay. In agreement with the results obtained by measuring mRNA induction (Fig. 2A and B and 5), tat-E6 was a more potent inducer of cytokine-producing cells than E6, and both were more active than tat-E7 and E7 in patients with RRP. We observed a marked reduction in the number of IL-10-producing cells, ca. 50%, when total PBMC from patients were compared to the nonadherent cell fraction (data not shown). However, only a minimal reduction (<5%) was observed in the number of IFN-γ-producing cells when total PBMC were again compared to the nonadherent cell fraction of PBMC from patients. The ELISPOT results confirm that the mRNA responses shown in Fig. 2, 3, and 5 predict that changes in mRNA expression that ultimately result in secretion of cytokine proteins. Of note, the number of IL-10-producing, nonadherent cells was significantly greater than the corresponding number of IFN-γ-producing cells for each protein tested in patients with RRP (Table 2). However, in the control nonadherent PBMC population, there was a marked reduction of IL-10-secreting cells responding to E6 and tat-E6 proteins (Table 2). This lack of induction of IL-10 producing cells was confirmed by IL-10-specific, RT-PCR which showed IL-10 mRNA expression at levels comparable to the expression found in untreated PBMC (data not shown). These data suggest that the strong IL-10 mRNA responses made by controls after a short incubation with E6 at 4 h, or tat-E6 at 16 h, is not maintained over a longer period of time. However, in patients with RRP, there is sustained expression of IL-10 by nonadherent cells. In contrast, 72 h of incubation with E6 or tat-E6 resulted in comparable numbers of IFN-γ-expressing nonadherent cells in PBMC from controls and patients with RRP.

Determination of IFN-γ and IL-10 expression by T cells and non-T cells. To determine whether T cells in PBMC exposed to E6 or tat-E6 express IFN-γ and IL-10, we positively and negatively selected T cells from PBMC by using microbead, magnetic separation. Figure 7A shows the purity of the negative selection of T cells from non-T cells in PBMC after E6 or tat-E6 exposure with G3PDH, CD3δ, and IL-2-specific primers in RT-PCR. Equal amounts of mRNA were studied from both cell fractions exposed to E6 or tat-E6 compared to untreated PBMC (Fig. 7A), as shown by the G3PDH products. Equal amounts of T-cell mRNA (CD3δ) were present in each of the T-cell-enriched cell lanes (Fig. 7A); however, no detectable

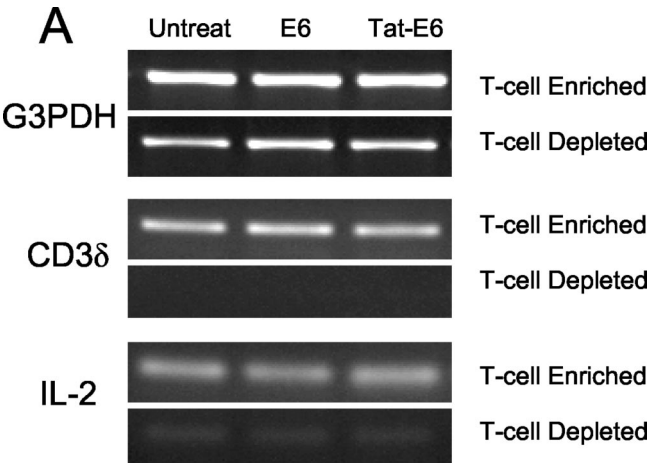


FIG. 7. Cytokine responses of T-cell-enriched and T-cell-depleted fractions of PBMC. (A) Negative selection for T cells was performed with anti-CD14, -CD16, -CD19, and -CD56 antibodies to obtain the T-cell-enriched and the T-cell-depleted fractions. Equal amounts of cDNA from untreated (left column), E6-treated (middle column), or tat-E6-treated (right column) T-cell enriched fractions (upper row), or T-cell depleted fractions (lower row) were amplified with specific primers for G3PDH (upper), CD3δ (middle), or IL-2 (lower). (B) Equal amounts of cDNA from a control (left) or patient with RRP (right) were amplified with IFN-γ (upper) or IL-10 (lower).

CD3δ was present in each of the T-cell-depleted lanes, confirming the efficiency of the microbead magnetic separation. IL-2 mRNA was also only present in the T-cell-enriched fraction, a finding consistent with the fact that only T cells make IL-2 (Fig. 7A).

Figure 7B shows the expression of IFN-γ and IL-10 mRNA after negative selection of T cells obtained from E6- or tat-E6-pulsed PBMC by using microbead magnetic separation. Figure 7B shows the cytokine mRNA expression of the T-cell-enriched and T-cell-depleted fractions of PBMC, pulsed with E6 or tat-E6, from a patient with RRP and a control. Of note, the T-cell-depleted fractions from the patient expressed little IFN-γ mRNA but substantial amounts of IL-10 mRNA. Furthermore, the T-cell fraction of the patient expressed comparable amounts of IFN-γ and IL-10 mRNA when challenged with E6. In contrast, both the T-cell-enriched and T-cell-depleted fractions from the control expressed both IFN-γ and IL-10 in response to E6. Although these results are semiquantitative, T cells from the control expressed more IFN-γ than IL-10, whereas the T cells from the patient produced comparable amounts of IFN-γ and IL-10. In addition, the T-cell-depleted fraction from the control expressed comparable amounts of IFN-γ and IL-10, whereas the T-cell-depleted fraction from the patient expressed more IL-10 than IFN-γ. The results in the patient and control are consistent with our findings shown in Fig. 2, 3, and 5 above. Positive selection of T cells from non-T cells yielded similar results (data not shown).

DISCUSSION

Our results suggest that HPV-11 E6 skews IL-10 and IFN-γ expression by patients with RRP toward greater expression of IL-10 than IFN-γ. In addition, there is a general cytokine

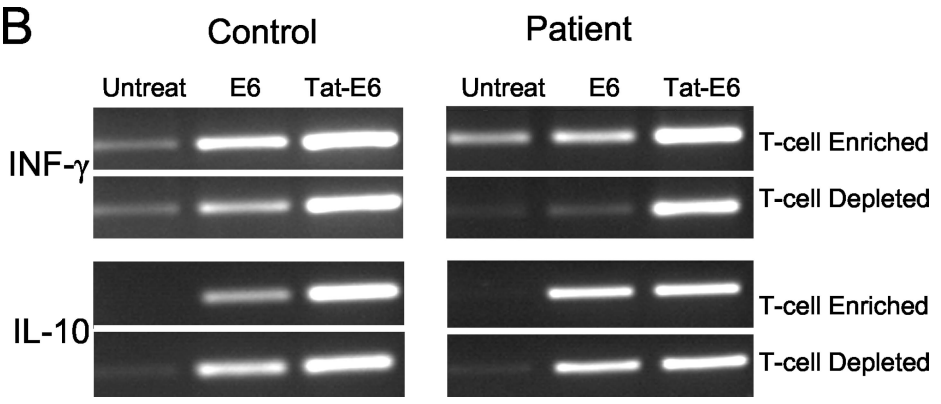


FIG. 7—*Continued*.

hyporesponsiveness to E6 that is more prominent for T_H1-like cytokines expressed by patients with severe disease. We propose that this poor response contributes to the failure of the immune system to control recurrent disease. There are several possible explanations for these findings, which also address clinical observations in RRP.

We also report the paucity of IL-2 and IL-18 mRNA expression by some patients, when other cytokine mRNAs, notably IL-10, are relative robustly expressed at the same concentration of E6. Our findings of decreased IL-18 mRNA expression are consistent with a recent report describing the direct inhibition of IL-18 by E6 in humans and mice (13). To our knowledge, we provide the first evidence that IL-2 mRNA expression may be similarly inhibited by E6. Furthermore, decreased IL-2 expression with relatively robust expression of IL-10 would constitute a cytokine microenvironment unfavorable for $CD8^+$ T-cell maturation, in contrast to a balanced IL-2 and IL-10 response to E6 that would support T_H1-like expression of IFN-γ (42).

One intriguing possibility supported by the relative paucity of T_H1-like cytokine mRNA expression with a concomitant and robust expression of IL-10 by non-T cells, which also correlates with disease severity, is that there may be an E6-induced, non-T-cell defect in RRP. Specifically, myeloid subtype 1 dendritic cell (DC) (31, 47) responses to E6 may be dysfunctional in RRP. IL-12 and IL-18 are essential cytokines constitutively expressed by human DCs that support T_H1-like cell maturation and IFN-γ expression (49, 57). Although HPV does not infect antigen-presenting cells (44), HPV could enter the endogenous antigen processing pathway by DC cross-presentation (3, 16, 45). Thus, HPV peptides could be presented to $CD4^+$ and $CD8^+$ T cells on class I and II MHC molecules by DCs (3, 16, 45). A failure of cross-presentation by DCs would inhibit HPV-specific, cytotoxic-T-lymphocyte (CTL) development and thereby explain the absence of HPV-specific CTL, which is a hallmark of RRP and other HPV-induced diseases (44).

HPV E6 could also activate DCs and support T_H2-like hyporesponsiveness through IL-10 expression and thereby suppress T_H1-like responses (6, 11, 17, 18, 30, 50). We observed a relatively robust IL-10 mRNA response in patients, compared to controls, in both our ELISPOT and T-cell enrichment experiments, suggesting that IL-10-expressing memory T cells are likely active in patients with RRP. It is intriguing to speculate that the cytokine imbalance seen in patients with RRP may be due to either the presence of suppressor T cells or the lack of IFN-γ-producing DCs that may predispose RRP patients to recurrent disease. Taken together, our results suggest that the inability to clear the virus in RRP may reflect E6-induced DC cell dysfunction that ultimately shifts HPV-specific, immune responses by T cells toward IL-10 expression.

It is also possible that decreased IFN-γ expression by PBMC may reflect immunoregulatory T-cell (T_H3-like or Tr-1 cells) activity that suppresses T_H1-like, T-cell function (40). An imbalance in IL-10 and IFN-γ expression induced by E6 may also reflect T_C2-like, $CD8^+$ T-cell activity in RRP (22, 46, 56). HPV-infected, cervical cancer tissues also polarize T cells into T_H2- and T_C2-like T cells by their expression of IL-10 and/or transforming growth factor β (TGF-β) (46) and T-cell-depleted, respiratory papillomas can also express TGF-β (V. R. Bonagura, unpublished data). This suggests that mucosal HPV-induced lesions have a common ability to polarize T cells away from HPV-specific, CTL responses capable of eradicating this virus. We previously reported increased numbers of $CD8^+$ $CD28^-$ T cells in tumor-infiltrating lymphocytes and in PBMC exposed to autologous papilloma tissue, a finding that correlated with disease severity (8). They may be T_C2-like T cells, which are heterogeneous in function (5, 26–28), are able to express IL-10, and behave poorly as CTLs in other viral diseases and in tumors (5, 12, 26–28, 32). Alternatively, they may be immature T_C1-like CTLs that fail to mature in a cytokine microenvironment containing little IL-2, IFN-γ, IL-12, and IL-18 but relatively more IL-10.

Other mechanisms may also predispose HPV-exposed individuals to develop RRP and explain in part the clinical variability in this disease. Select class II MHC alleles are commonly found in patients with RRP but not in HPV-exposed and asymptomatic controls (9, 19, 51). Some of these alleles have been associated with aggressive forms of chronic bacterial and viral infections (7, 35, 36, 41). In addition, HPV proteins themselves may account for deficient, HPV-specific CTL responses in RRP. We have reported elsewhere that HPV-11 E7 interacts with the transporter associated with antigen-processing 1 (TAP1) (52), possibly preventing peptide presentation to HPV-specific, $CD8^+$ T cells on class I MHC molecules.

In summary, E6 and E7 proteins from HPV-11 have unique

immunoregulatory functions. Clearly, E6 is the dominant inducer of cytokine expression by PBMC, whereas E7 appears to have a minimal role in this capacity. Moreover, neither E6 nor E7 alone are responsible for the robust expression of IL-4 that we previously observed when PBMC from patients were exposed to autologous papilloma tissue (8). Therefore, another HPV protein or a combination of proteins is likely responsible for inducing IL-4 expression that would downregulate IFN-γ expression (39). Ultimately, immunologic mapping E6, and possibly E7, to identify specific peptides within these proteins that can induce potent expression of IFN-γ, IL-12, IL-18, and IL-2 may yield a therapeutic vaccine to prevent the relentless recurrence of respiratory papillomas.

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