Impaired HPV-Specific T-Cell Response Characterized with Skewed Cytokine Profile and T-Cell Dysfunction in Juvenile-Onset Recurrent Respiratory Papillomatosis Patients

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Research Article

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Abstract

Background

Immunologic dysfunction is one of the most important mechanisms underlying the initiation and development of Juvenile-onset Recurrent Respiratory Papillomatosis (JORRP). The study aimed to explore whether HPV-specific T-cell response was impaired in JORRP patients.

Methods

A total of 46 JORRP patients and 93 age- and sex- matched healthy controls were enrolled. The plasma concentrations of various cytokines were measured using the Luminex. The cytokine mRNA profiles of PBMCs were quantified with the real-time PCR. The T-cell subsets, HPV-specific T-cell activation, proliferation, apoptosis and expression of replicative senescent T-cell markers CD57 were measured with the flow cytometry. The cytokine secretion of HPV-specific T cells was assessed by the ELISpot.

Results

JORRP patients had a Th2-biased cytokine profile correlated with disease severity in the JORRP peripheral system. JORRP patients had an increased percentage of memory T cells and a reduced percentage of naive T cells in circulation. Upon HPV6/11 antigens stimulation, T cells from JORRP patients exhibited a greater activation profile as judged by a higher CD25 and CD69 expression. Of note, JORRP patients presented with a greater number of IL-10- and IL-4-secreting HPV6/11 antigen responding cells than that of IFN-γ and TNF-α secreting responders in the ELISpot experiment. Furthermore, in response to HPV6/11 antigen stimulation, JORRP patients showed a reduced level of cell proliferation, an increased level of apoptosis and higher percentage of the differentiated T cells expressing the replicative senescent cell marker CD57.

Conclusions

Impaired HPV-specific T-cell responses could be partly responsible for JORRP development.

Introduction

Recurrent respiratory papillomatosis (RRP) is a benign disease of the upper aero-digestive tract caused by human papillomavirus (HPV) infection, in particular low-risk virus type HPV6 and HPV11[1, 2]. RRP typically presents in a bimodal age pattern occurring either during adult life or early childhood [3]. Adult-onset RRP (AORRP) typically emerges in early adulthood, the most vulnerable age window when HPV is readily acquired by sexual transmission. By contrast, juvenile-onset RRP (JORRP) appears in early childhood and usually is transmitted vertically during pregnancy or is acquired at birth from an HPV-
infected mother [3]. Of note, JORRP has a more aggressive and recurring clinical course, perhaps in part because infants have smaller airways or because of immune compromise [4]. Surgical excision is the current standard of care for RRP, which is directed at preventing upper airway compromise and improving vocal function while preserving laryngeal tissues [5]. However, there is no cure for RRP so far. Typical JORRP patients require nearly 20 surgical procedures throughout their lifetimes, many of which occur early in the children’s lives. During the initial years of the disease, a child is estimated to require slightly more than 4 surgeries per year [6]. Thus, this disease not only affects individual patients significantly but also places a large economic burden on their families and society in general [7]. To date, it is unclear why some individuals do not mount a sufficient anti-HPV response to the initial HPV6- and HPV11 infection [8].

Researchers found evidence that T cell immune dysfunction may contribute to the development of RRP. One group observed that IL-4 and IL-10 were dominantly expressed, compared with IFN-γ, in T cells that infiltrate papilloma of RRP patients and this imbalance correlated with disease severity [9]. Another study proposed that RRP patients have more circulating CD4+ T cells that constitutively express the Th2-like cytokines IL-4 and IL-10 [10, 11]. Furthermore, HPV-11 E6 protein has been shown to cause the Th1/Th2 cytokine imbalance in papilloma and in peripheral blood mononuclear cells (PBMCs), manifested as up-regulate the expression of IL-10 and IL-4 and reduce the expression of IFN-γ, IL-12 and IL-18 [12]. These altered T cell immune response, in combination with the skewed cytokine expression, may reveal the polarization of the adaptive immune system toward a Th2-like in RRP patients, which subsequently suppress Th1 cell-mediated clearance of the infection [10]. However, the functional profile of HPV-specific T-cell responses remain unclear, and these studies mainly focused on AORRP patients [13]. Indeed, JORRP usually runs a more aggressive clinical course, requiring a more thorough study for its antiviral immunity. In our previous studies, it has been revealed that children with JORRP exhibited a Th2-biased cytokine profile [14]. Furthermore, we found that impaired T cell-dependent humoral immune response supported persistent HPV infection in JORRP [15]. These studies exposed the abnormal immune response of T cells to HPV in JORRP.

In this study, we performed a further assessment of circulating cytokines expression in JORRP, and explored the state of the antiviral immune response mediated by T cells. More important, Upon HPV6/11 antigens stimulation, functional changes in the circulating HPV-specific T-cell responses were scrutinized, including T cell activation, proliferation, senescence, and apoptosis. We found that the HPV-specific T cell immune response in JORRP children was impaired, which may be the major contributing factor to the recurrence of JORRP.

**Materials And Methods**

**Subjects and sample collection**

Demographic and clinical characteristics of JORRP patients and healthy controls are described in Table 1. Overall 46 JORRP patients from Beijing Children’s hospital admitted at the period from January 2018 to
December 2019 were enrolled. Ninety three age- and sex- matched healthy controls subjected to the routine physical examination between January 2018 and December 2019 at Beijing Children’s Hospital were recruited. The study was approved by the Medical Ethics Committee of Beijing Children’s Hospital, Capital Medical University (Grant No. 2019-k-43), which acted in compliance with ethical standards defined by the Declaration of Helsinki. Written consents were signed by all the participants or their legal guardians. Peripheral blood samples from healthy controls and JORRP patients were collected in BD Vacutainer™ plastic blood collection tubes with EDTA K2 as anticoagulant. Plasma samples were collected by a centrifugation at 600 g for 5 min at room temperature and the supernatant were aliquoted and stored at -80 °C.

| Table 1 |
| Baseline characteristics of the study participants |
| JORRP group | Healthy controls |
| --- | --- |
| Total number | 46 | 93 |
| Mean age, month | 48.7 ± 28.9 | 45.8 ± 27.8 |
| Gender | 26 females, 20 males | 48 females, 45 males |
| Aggressiveness of course | Yes = 24 (52.2%), no = 22 (47.8%) | - |
| Total number of surgeries | Median = 5, range= (1, 16) | - |
| Max number of surgeries in one year | Median = 2, range= (1, 9) | - |
| Recurrence interval (month) | Median = 6.1, range= (1.3, 18) | - |
| Extra laryngeal Spread | 2 | - |
| Required Tracheostomy | 2 | - |
| Other Comorbidities | 1 with stenosis of larynx, 1 with patient foramen ovale | - |

JORRP, Juvenile-onset recurrent respiratory papillomatosis; -, none.

Assessment of JORRP severity

To provide an overall assessment of disease severity, patients were categorized as having ‘aggressive’ or ‘non-aggressive’ disease according to Doyle et al. criteria as previously reported [15]. This binomial classification has been applied clinically by other researchers [16]. The characteristics of aggressive disease include 10 or more total procedures with three or more procedures within a 1-year period and/or spread of disease distal to the subglottis. By contrast, the characteristics of non-aggressive disease
include less than 10 total procedures, less than three procedures within a 1-year period, and the absence of distal spread.

**Plasma cytokine concentrations determined by a Luminex 200 system**

Plasma concentrations of TNF-α, IFN-γ, IL-10 and IL-4 were determined using a human cytokine panel (MILLIPLEX MAP KIT) and read by a Luminex 200 system (Merck Millipore, Darmstadt, Germany). Luminex was performed as previously described [18]. All samples were measured in duplicate.

**PBMCs isolation**

Freshly isolated EDTA anticoagulated blood was diluted with PBS solution and layered carefully on Ficoll-Hypaque density gradients. After being centrifuged at 1000 g for 20 min interphase cell layer was carefully transferred into a 15 ml tube. Then the 15 ml tube was filled with 10 ml PBS, and cell pellet was collected after centrifugation at 500 g for 5 min.

**Real-time PCR**

To confirm the differential expression level of cytokines between patients and healthy controls, total RNA was extracted from PBMCs using the Direct-zol RNA Miniprep (ZYMO research, USA), and the first strand cDNA was synthesized by a RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, USA). SYBR Green real-time PCR was performed with corresponding primers in a QuantStudio 6 flex real-time PCR system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal standard for final normalization. The primer sequence is listed in Table 2.

**Table 2**

| Gene     | Sense primer (5’→3’) | Antisense primer (5’→3’) |
|----------|----------------------|--------------------------|
| GAPDH    | CAACGAATTGGCTACAGCA  | AGGGGTCTACATGGCAACTG     |
| TNF-α    | CAGCCTCTTCCTCCTTGAT  | GCCAGAGGGCTGATTAGAGA     |
| IFN-γ    | TTTTGAAGAATTGGAAAGAGGA | CACTTGGATGAGTTGAGTATTTG  |
| IL-10    | AGGGAGCCCCTTTTGATGAT | GGTTGGGAATGAGGTAGG       |
| IL-4     | TACAGCCACCATGAGAAGGAC | TGATCGTTTTAGCCTTTCCA     |

**Synthetic HPV6 and 11 antigens**

The HPV6 and HPV11 recombinant antigens for in vitro cell culture were synthesized commercially (ProSpec, USA) as described in previous report [19]. Recombinant HPV6 antigen and HPV11 antigen are sequences of immunodominant antigens that are expressed in E. coli. Recombinant HPV6 antigen
(Catalogue No. HPV 003) is a 55.6 kDa protein covering the full-length of HPV6 major capsid while recombinant HPV11 antigen (Catalogue No. HPV 004) is a 58.1 kDa protein covering the full-length of HPV11 major capsid (Table S1). Lyophilized antigens were dissolved in phosphate-buffered saline (PBS) at a concentration of 100 mg/ml (stock) and the aliquots were stored at -80°C for use. The titrated dose of 2.5 µg/ml of both antigens were used in culture.

In vitro stimulation of PBMCs and cell culture

For each sample, the PBMCs were isolated, suspended in 10% FBS RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) (5×10⁵ cells/well) in 96-well plates (round-bottomed) at 37°C humidified cell incubator with 5% CO₂. Recombinant HPV6 and HPV11 antigens (HPV6/11 antigens) were added to the culture for a 48-hr stimulation. All samples were further processed for cell surface staining with titrated fluorochrome-labelled antibody.

Elispot assay

The HPV-specific response was displayed with a human Elispot assay kits determining the number of responding cells releasing IFN-γ, IL-10, IL-4 or TNF-α after a 24-hr incubation with HPV6/11 recombinant antigens in 96-well round bottom plates (5×10⁵ cells/well). The experiment was implemented according to the manufacturer’s instruction [12]. In each assay, bovine serum albumin (BSA) was added instead of HPV antigen as a negative responder control, and cell mitogen phytohemagglutinin (PHA) was used as a positive responder control (Supplementary 1).

Carboxyfluorescein succinimidyl ester (CFSE) proliferation assay

PBMCs were resuspended in 2 ml RPMI 1640 medium and incubated with 2 µM CFSE (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 10 min. The reaction was terminated by cold medium with 10 % FBS and placed on ice for 5 min. The stained PBMCs were washed and resuspended with RPMI 1640 medium plus 10 % FBS. Then the washed PBMCs (1×10⁶/ml) were stimulated by HPV6/11 antigens, seeded in 96-well round bottom plates and cultured for 3 days (Supplementary 2). Samples were analyzed by FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Flow cytometry

Freshly isolated PBMCs were resuspended in cell staining buffer (PBS + 3% FBS). For surface marker labeling, 1×10⁶ cells were incubated with different combinations of human antibodies. Human FITC-conjugated anti-CD45RA, PE-conjugated anti-CD4, anti-CD57 and anti-CD8, APC-conjugated anti-CD3, anti-CD25, anti-CD69 and Annexin V (AV) were purchased from Biolegend (San Diego, CA, USA). PerCP-Cy5.5-conjugated anti-CD45RO was purchased from BD Pharmingen (San Diego, CA, USA). Isotype antibodies conjugated with same fluorochrome were used to exclude the non-specific staining. After 20 min incubation at 4°C in dark, samples were washed twice in PBS before final resuspension in 300 µl PBS subjected to flow cytometry analysis. Cell events were acquired with a BD FACS Calibur flow cytometer.
Statistical analyses

The data were represented as the mean ± SD. Significant differences between JORRP patients and healthy controls were determined by the Student t-test or rank sum test according to the normal distribution test. Statistical analysis was performed with Prism (version 5.04) software. p < 0.05 was considered to be significantly different.

Results

Th1/Th2 cytokine imbalance correlated with disease severity in JORRP

Cytokines expression profile is one of the main components shaping the anti-infection immunity and mediating the pathogenesis of tissue damage. We were interested in knowing if pathogen and host interaction during HPV infection led to a skewed cytokine expression profile in the JORRP peripheral system. With this aim, we first measured the plasma concentration of the multiple cytokines from 46 JORRP patients and 93 healthy controls using a Luminex 200 system. The result indicated that the plasma concentration of TNF-α was significantly lower in JORRP patients than in healthy controls, while the IFN-γ, IL-10 and IL-4 were not evidently different (Fig. 1A). More importantly, we noted a significant negative correlation between the plasma concentration of TNF-α and the number of surgeries, the index for disease recurrence frequency. In contrast, IL-10 and IL-4 presented a positive correlation with the number of surgeries (Fig. 1B).

Secondly, in order to observe the transcription levels of these cytokines in circulating immune cells, we compared the mRNA expression in PBMCs from JORRP patients with those from healthy controls by real-time PCR. The results showed that JORRP patients had a down-regulated mRNA expressions of IFN-γ and TNF-α and an up-regulated mRNA expressions of IL-10 and IL-4 (Fig. 1C). Association analysis further revealed that IL-10 mRNA expression was significantly positively correlated with the number of surgeries, while IFN-γ, TNF-α and IL-4 presented no significant correlation (Fig. 1D).

JORRP patients had a positive memory T cell immune response to HPV

The view that the altered T cell immune response plays an important role in the progression of JORRP disease is now widely accepted [10]. We speculated that the changes of various cytokines in plasma and PBMC of JORRP children would closely relate to the behavior of T cells in peripheral blood. To test this, we measured the proportion of total T cells (CD3+) and T cell subsets, including CD4+ and CD8+ T cells in peripheral blood. The results showed that neither the percentage of total T cells in PBMC nor the ratio of any of T cell subpopulations changed in JORRP group compared with the healthy controls. (Fig. 2A-C). Unexpectedly, we noted a significant reduction in the proportion of CD45RA expressing naïve T in JORRP
patients compared to healthy controls. In contrast, the percentages of CD45RO expressing memory T cells were increased in JORRP patients (Fig. 2D).

To further assess the responsiveness of T cells to HPV, we stimulated PBMCs isolated from JORRP patients or healthy controls with HPV6/11 antigens for 48h, and then analyzed the activation phenotype of T cells in PBMC by flow cytometry. Our result indicated that the active markers CD25 (Fig. 2G) and CD69 (Fig. 2H) were significantly increased in T cells from JORRP patients.

**HPV induced the polarization of T cells to a Th2-like phenotype**

The phenotype of the T cells was further analyzed for the expression of Th1/Th2 cytokines. Here we performed a cytokine-specific elispot assay and then found that HPV6/11 antigens restimulation induced a higher number of IFN-γ, IL-10 and IL-4 secreting cells in PBMC from JORRP patients compared with that from the healthy controls, while the number of TNF-α secreting cells decreased (Fig. 3A-D). Of note, the number of IL-10 (892 ± 77) and IL-4 producing cells (832 ± 91) was significantly greater than the number of IFN-γ producing cells (240 ± 91) and TNF-α producing cells (723 ± 74) in JORRP patients (Fig. 3E). In other words, the PBMCs, primarily T cells, that respond to HPV antigen presented an Th2-like phenotypic polarization in JORRP children.

**The proliferation, senescence and apoptosis of HPV mediated T-cell in JORRP**

Now that we have known that there is a skewed phenotype of HPV antigen stimulated T cells in children with JORRP, we were eager to know if HPV could induce functional changes in T cells. First, we examined the effect of HPV on T cell proliferation. The isolated PBMC were labeled by CFSE and stimulated by HPV6/11 antigens in vitro for 3 days. The flow analysis results revealed that T cells from healthy controls generated more progeny through cell divisions than T cells from JORRP patients (Fig. 4A-B).

Next, flow cytometry was used to analyze the expression of cell surface marker CD57, a general marker of highly differentiated or senescent T cells [20]. We observed that the frequencies of CD57 in total T cells and memory T cells were increased in JORRP patients compared with healthy controls after HPV 6/11 antigens stimulation (Fig. 4C-D). As to apoptosis, based on our AV + assay by flow cytometry, the expression of AV in total T, naive T and memory T cells showed a remarkable increase in JORRP patients (Fig. 4E-G), which was consistent with CD57-expressing T cells. Taken together, these analyses maybe suggest that HPV induce T cells to turn into a senescent phenotype and underwent apoptosis in a shorter time among JORRP patients.

**Discussion**

Activation and inhibition of the cellular immune responses are linked to cytokines expression in humans. The dysregulated expression of cytokines in cellular immunity against HPV infection may facilitate the development of RRP. However, the current literature presented different cytokine expression profiles in RRP disease and lacked a systematic study for JORRP patients. In this study we perform a
comprehensive evaluation of the expression of 4 representative T cell associated cytokines in 34 JORRP children (Fig. 1). Compared with that in healthy controls, the plasma level of TNF-α was found significantly reduced in JORRP patients and were negatively correlated with the number of surgeries. This agreed with findings in AORRP which suggested a suppression of pro-inflammatory Th1 cytokines [12]. Interestingly, while IL-10 and IL-4 presented with comparable levels as those in control group, they exhibited a positive relationship with the number of surgeries. Surprisingly, different from other investigations [21], in our hand, we never tracked a significant decrease in plasma IFN-γ. This could be a reflection of undisturbed protein synthesis and secretion of IFN-γ in JORRP immune cells as our previous study. We have reported that upon a phorbol 12-myristate 13-acetate stimulation, the expression of intracellular protein level of IFN-γ in CD4 T cells was not altered in a noticeable way [15]. Nevertheless, in our study, the mRNA level of IFN-γ was shown dramatically decreased in PBMCs of patients. Indeed, there was a general reduction in the expression of pro-inflammatory cytokines at mRNA level in PBMCs from JORRP patients. Besides IFN-γ, the mRNA expression of TNF-α decreased in JORRP patients accompanied with an increase in the mRNA expression of anti-inflammatory cytokine IL-10 and IL-4, in which IL-10 presented a positive relationship with the number of surgeries. These results agreed with previous study in which mRNA of these cytokines was measured in PBMCs or papilloma of RRP patients [22, 12]. In conclusion, the skewed cytokine expression in plasma and PBMC could be the synergic molecular modulations for the shift of the T cell polarity in response to HPV infection in JORRP patients.

The view that RRP patients establish “low level” tolerance [8] to the initial HPV-6 or -11 infection, and develop an exaggerated tolerogenic response to these viruses, rather than anti-HPV responses that effectively clear or contain them is now widely accepted. Cumulative evidence has suggested that T cells, as the most critical effector cells against HPV, are involved in the tolerance regulation of HPV [10]. Thus, understanding the mechanism(s) by which HPV-6 and -11 polarizes the T cell immune response towards tolerance in RRP, as opposed to the development of cell-mediated immune clearance of these viruses, is critical in developing novel therapies that would prevent disease recurrence and/or reduce disease severity. In the present study, we did not observe the difference in the proportion of circulating total T cells (CD3+), CD4 + T and CD8 + T between JORRP children and healthy controls, which was in line with some of other studies (Fig. 2A-C) [23]. Interestingly, we found that JORRP patients had an increased proportion of memory T cells and a decreased proportion of naïve T cells. This finding may attribute to the inhibitory cycle of immunocytes in RRP (Fig. 2D-F). The inhibition cycle model indicated that memory Th2-like T cells expressing IL-4, IL-10 and TGF-β alternatively activate macrophages to express the Th2-like chemokines CCL17 and CCL18, which polarize naïve CD4 + T cells to become memory Th2-like T cells and Tregs [10]. In light of that, we further analyzed the phenotypes of T cells stimulated by HPV6/11 antigens and found that compared with healthy controls, T cells in JORRP patients showed a higher HPV antigenic reactivity, manifested by up-regulated expression of activation markers CD25 and CD69 (Fig. 2G-H). In previous studies, CD4 + CD25bright CD127- Foxp3 + Treg cells expressing PD-1 and CD69 have been shown to be enriched in papillomas [24]. The functional Tregs would likely circulate to the periphery and suppress HPV-specific T cell immune response [10]. Thus, upon HPV antigen restimulation,
JORRP subject may present increased circulating CD25 bright and CD69+ Treg cells, which would trigger immune tolerance to HPV.

In addition, the phenotype of the T cells was further analyzed for the expression of Th1/Th2 cytokines. The cytokine-specific elispot assay showed that the PBMCs, primarily T cells, that responding to HPV antigen presented an Th2-like phenotypic polarization in JORRP patients, with a dominant expression of IL-10 and IL-4 compared to IFN-γ and TNF-α, which would block effector Th1-like responses to HPV-6 and −11 in patients (Fig. 3). James E et al. previously observed that HPV E2/E6-specific CD4+ T-cell clones from the RRP subjects produced lower levels of IFN-γ and higher levels of IL-13 protein, also tended to exhibit reduced TNF-α secretion compared with healthy control subjects [25]. Taken together, all these evidences suggested that HPV prevents an effective anti-viral T-cell response in JORRP.

Although the phenotypic changes in T cell induced by HPV have been exposed, few studies have focused on the ultimate fate of T cells in chronic HPV infection. In our study, we found the expression of CD57+ and AV+ T cells in JORRP patients responding to HPV6 /11 antigen was increased, while the proliferation of T cells was decreased (Fig. 4). Expression of CD57 usually represented the phenotype associated with replicating senescent T cells and is thought to be responsible for the inability of these T cells to proliferate [26, 27, 28]. This proliferative defect was found in all CD57-expressing CD4+ and CD8+ T cells and NK cells and could not be overcome by the addition of exogenous IL-2 or IL-15 [29]. These T cells commonly were found in individuals with chronic immune activation [30, 31], and they increased in frequency with age [32]. In Bonagura et al. ’s study, the CD57+ CD4+ T-cell subsets were significantly elevated in papillomas of RRP susceptible population who lacked both KIR3DS1 and KIR2DS1 genes compared with patients who expressed one or both of these KIR genes [33]. It is precisely because of the evidence that restricted T-cell clones in papillomas circulate in the blood of RRP patients [10] that it is not difficult to understand that the increased CD57+ T cells were found in peripheral circulation of JORRP patients responding to HPV6 /11 antigen in our study (Fig. 4C and 4D). These results may imply that chronic HPV infection induced T cells to turn into a senescent phenotype and underwent apoptosis.

Interestingly, we also found that memory T cells from JORRP have increased apoptosis and tended to exhibit increased expression of CD57 in response to HPV antigen restimulation (Fig. 4G). The data from James E et al. suggested that HPV-specific CD4+ T cells from RRP have reduced STAT-5, a crucial regulator of antigen restimulation-induced T cell death (RICD) in memory T cells in mice and humans [25, 34]. we hypothesized that the deficient STAT5 signaling may drive RICD in effector memory T (TEM) cells from JORRP, and induced TEM cell exhaustion.

These evidences suggested that JORRP patients have established T cell immune tolerance, manifested as the activation of functional Treg and skewed Th2-like immunophenotype, and the TEM exhaustion mediated by the deficient STAT5 signaling, may together contribute to HPV-specific T cell function impairment.

In sum, we have demonstrated that the HPV infection led to the polarization of circulating T cells toward memory Th2-like phenotypes, and ultimately to senescence and apoptosis, which could be an important
cause of impaired T cell immune response in JORRP children. We believe that the results of our current study provide the basis for understanding and accurate assessment of the immune cell regulatory mechanisms in JORRP.

**Declarations**

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**Conflict of Interest** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Availability of Data And Material** All data generated or analysed during this study are included in this published article.

**Code Availability** Not applicable.

**Author's Contributions** XN and JG designed the research, and supervised the work; YX and WW performed the research, analyzed data, and drafted the manuscript; HW, XW, JZ, JZ and GW screened patients, performed sample collection, collected clinical data, and helped to revise the manuscript. All authors have read and approved the final manuscript.

**Ethics Approval** This study was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University (Grant No. 2019-k-43).

**Consent to participate** Written informed consent was obtained from individual or guardian participants.

**Consent for publication** Not applicable.

**References**

1. Derkay CS, Wiatrak B. Recurrent respiratory papillomatosis: a review. Laryngoscope. 2008;118(7):1236–47.

2. Ivancic R, Iqbal H, Desilva B, Pan Q, Mattrka L. Immunological tolerance of low-risk hpv in recurrent respiratory papillomatosis. Clinical Experimental Immunology. 2020;199(2):131–42.

3. Venkatesan NN, Pine HS, Underbrink MP. Recurrent respiratory papillomatosis. Otolaryngol Clin North Am. 2012;45(3):671–94.

4. Reeves WC, Ruparelia SS, Swanson KI, Derkay CS, Marcus A, Unger ER. National registry for juvenile-onset recurrent respiratory papillomatosis. Archives of otolaryngology–head neck surgery. 2003;129(9):976–82.

5. Ruiz R, Achlatis S, Verma A, Born H, Kapadia F, Fang Y, et al. Risk factors for adult-onset recurrent respiratory papillomatosis. Laryngoscope. 2015;124(10):2338–44.
6. Armstrong LR, Derkay CS, Reeves WC. Initial results from the national registry for juvenile-onset recurrent respiratory papillomatosis. Archives of otolaryngology–head neck surgery. 1999;125(7):743–8.

7. Venkatesan NN, Pine HS, Underbrink MP. Recurrent respiratory papillomatosis. Otolaryngol Clin North Am. 2012;45(3):671–94.

8. Faria AMC, Weiner HL. Oral tolerance. Immunol Rev. 2005;206(1):232–59.

9. Bonagura VR, Hatam L, Devoti J, Zeng F, Steinberg BM. Recurrent respiratory papillomatosis: altered CD8(+) T-cell subsets and TH1/TH2 cytokine imbalance. Clinical Immunology. 1999;93(3):302–11.

10. Bonagura VR, Hatam LJ, Rosenthalal DW, Voti JAD, Lam F, Steinberg BM, et al. Recurrent respiratory papillomatosis: a complex defect in immune responsiveness to human papillomavirus-6 and – 11. Apmis. 2010;118(6–7):455–70.

11. Romagnani S. TH1 and TH2 in human diseases. Clin Immunol Immunopathol. 1996;80(3):225–35.

12. Devoti JA, Steinberg BM, Rosenthal DW, Hatam L, Vambutas A, Abramson AL, et al. Failure of gamma interferon but not interleukin-10 expression in response to human papillomavirus type 11 e6 protein in respiratory papillomatosis. Clinical Diagnostic Laboratory Immunology. 2004;11(3):538–47.

13. Rosenthal DW, Devoti JA, Steinberg BM, Abramson AL, Bonagura VR. TH2-like chemokine patterns correlate with disease severity in patients with recurrent respiratory papillomatosis. Mol Med. 2012;18(9):1338–45.

14. Xiao Y, Wu X, Ma L, Gui J, Bai L, Ni X, et al. Enhanced th2-like peripheral adaptive immune responses in Juvenile-onset Recurrent Respiratory Papillomatosis (JORRP). Immunol Lett. 2017;191:31–4.

15. Wu X, Wang G, Chen X, Zhang J, Zhao J, Wang J, et al. Impaired t cell-dependent humoral immune response associated with juvenile-onset recurrent respiratory papillomatosis progression. Sci Rep. 2016;6:36378.

16. Doyle DJ, Gianoli GJ, Espinola T, et al. Recurrent respiratory papillomatosis: juvenile versus adult forms. Laryngoscope. 1994;104(5 Pt 1):523–7.

17. Omland T, Akre H, Lie KA, et al. Risk factors for aggressive recurrent respiratory papillomatosis in adults and juveniles. PLoS One. 2014;9(11):e113584.

18. Wang X, Mou W, Han W, Xi Y, Chen X, Zhang H, et al. (2019). Diminished cytolytic activity of γδ T cells with reduced DNAM-1 expression in neuroblastoma patients. Clinical immunology. 2019, 203, 63–71.

19. Singh M, Thakral D, Rishi N, Kar HK, Mitra DK. Functional characterization of CD4 and CD8 T cell responses among human papillomavirus infected patients with ano-genital warts. Indian J Virol. 2017;28(2):133–40.

20. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8 + T cells. Blood. 2003;101(7):2711–20.
21. Nan B, Chen B, Zhang C, Jiang M, Zhang Y, Xiang H, Yu X. (2013). Levels of serum IL-4, IFN-γ, IL-32 in children with juvenile-onset recurrent respiratory papillomatosis. Lin chang er bi yan hou tou jing wai ke za zhi = Journal of clinical otorhinolaryngology, head, and neck surgery, 27(12), 651–653.

22. Holm A, Nagaeva O, Nagaev I, Loizou C, Laurell G, Mincheva-Nilsson L, et al. Lymphocyte profile and cytokine mRNA expression in peripheral blood mononuclear cells of patients with recurrent respiratory papillomatosis suggest dysregulated cytokine mRNA response and impaired cytotoxic capacity. Immunity Inflammation Disease. 2017;5:541–50.

23. Bonagura VR, Siegel FP, Abramson AL, Santiagoschwarz F, O'Reilly ME, Shah K, et al. Enriched hla-dq3 phenotype and decreased class I major histocompatibility complex antigen expression in recurrent respiratory papillomatosis. Clin Diagn Lab Immunol. 1994;1(3):357–60.

24. Hatam LJ, Devoti JA, Rosenthal DW, Lam F, Abramson AL, Steinberg BM, et al. Immune suppression in premalignant respiratory papillomas: enriched functional CD4 + Foxp3 + regulatory T cells and PD-1/PD-L1/L2 expression. Clin Cancer Res. 2012;18(7):1925–35.

25. James EA, Devoti JA, Rosenthal DW, Hatam LJ, Steinberg BM, Abramson AL, et al. Papillomavirus-specific CD4 + T cells exhibit reduced STAT-5 signaling and altered cytokine profiles in patients with recurrent respiratory papillomatosis. J Immunol. 2011;186:6633–40.

26. Sze MY, Giesajtis G, Brown RD, Raitakari M, Joshua DE. Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+)CD57(+)CD28(-) compartment. Blood. 2001;98(9):2817–27.

27. Champagne P, Ogg G, King A, Knabenhans C, Ellefsen K, Nobile M, et al. Skewed maturation of memory hiv-specific CD8 T lymphocytes. Nature. 2001;410:106–11.

28. Bandrés E, Merino J, Vázquez B, Inogés S, Moreno C, Subirá ML, et al. The increase of IFN-γ production through aging correlates with the expanded CD8(+ high) CD 28(-)CD 57(+) subpopulation. Clinical Immunology. 2000;96(3):230–5.

29. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8 + T cells. Blood. 2003;101(7):2711–20.

30. Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. CD4+, CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. Arthritis Rheumatology. 2010;44(1):13–20.

31. Rowbottom AW, Garland RJ, Lepper MW, Kaneria SS, Goulden NJ, Oakhill A, et al. Functional analysis of the CD8 + CD57 + cell population in normal healthy individuals and matched unrelated T-cell-depleted bone marrow transplant recipients. Br J Haematol. 2000;110(2):315–21.

32. Tarazona R, Delarosa O, Alonso C, Ostos B, Solana R. Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells. Mechanisms of Ageing Development. 2001;121(1–3):77–88.

33. Bonagura VR, Du Z, Ashouri E, Luo L, Hatam LJ, DeVoti JA, et al. Activating killer cell immunoglobulin-like receptors 3DS1 and 2DS1 protect against developing the severe form of recurrent respiratory papillomatosis. Hum Immunol. 2010;71(2):212–9.
34. Majri SS, Fritz JM, Villarino AV, Zheng L, Kanellopoulou C, Chaing-Delalande B, et al. STAT5B: A Differential Regulator of the Life and Death of CD4 + Effector Memory T Cells. J Immunol. 2018;200(1):110–8.

**Supplementary Tables**

Table S1 is not available with this version.

**Figures**

**Figure 1**
Identification of the T cell associated cytokine levels in plasma and PBMCs of JORRP patients. (A) Circulating cytokine levels in plasma from healthy controls and patients with JORRP were analyzed by Luminex 200 platform. (B) The correlation analysis between the number of surgeries and plasma cytokine concentration in patients with JORRP. (C) The mRNA expression of cytokines in PBMCs from healthy controls and patients with JORRP were analyzed by real-time PCR. (D) The correlation analysis between the number of surgeries and cytokine transcription levels in patients with JORRP. mRNA expression results of cytokines were represented by relative quantification (RQ). Spearman correlation coefficients (r and p-value) were shown. * p < 0.05, ** p < 0.01, and *** p < 0.001 compared with the healthy controls.
Figure 2

The phenotype and activation of circulating T cells in patients with JORRP. The proportion of the different T cell subsets in PBMCs were analyzed by flow cytometry. Statistics of the proportion of (A) the CD3+ T cells (in lymphocytes), (B) CD4+T (in CD3+T) and (C) CD8+T (in CD3+T) in healthy controls and patients. (D-F) The proportion of (E) CD45RA+CD45RO- naive T cells and (F) CD45RO+CD45RA- memory T cells in CD3+ T cells was shown in healthy controls and patients. (G-H) The PBMCs were isolated from JORRP...
patients or healthy controls and stimulated with HPV6/11 antigens for 48h. The expression frequencies of the active markers CD25 (G) and CD69 (H) on CD3+T cells were shown. Statistically significant changes are designated as follows * p < 0.05 and ** p < 0.01.

Figure 3

HPV6/11 antigens restimulation induced a skewed Th2-like cytokine expression in PBMC from JORRP patients. The cytokine-specific elispot assay was performed and analyzed for the cytokine secretion in PBMC from healthy controls and JORRP patients. Number of cells expressing (A) IFN-γ, (B) TNF-α, (C) IL-10 and (D) IL-4 protein response to HPV6 and HPV 11 antigens was shown. (E) Comparison of the number of these cytokines producing cells in JORRP patients. Statistically significant changes are designated as follows * p < 0.05, ** p < 0.01, and *** p < 0.001.
Figure 4

The proliferation, senescence and apoptosis of HPV mediated T-cell in JORRP. To detect T cell proliferation, isolated PBMC were labeled with CFSE and stimulated with HPV6/11 antigen in vitro for 3 days from healthy controls and JORRP patients. (A) Analytical strategies for the detection of T cell proliferation by flow cytometry. (B) Statistics of the proportion of CFSE on T cells. To detect T cell senescence and apoptosis, Frequencies of peripheral CD57+ cells in (C) CD3+ T cells and (D) in CD3+CD45RO+CD45RA- memory T cells response to HPV6/11 antigens were determined in both healthy controls and JORRP patients. Frequencies of peripheral Annexin V+ cells (E) in CD3+ T cells, (F) in CD3+CD45RA+ CD45RO- naive T cells and (G) in CD3+CD45RO+CD45RA- memory T cells response to HPV6/11 antigens were determined in both healthy controls and JORRP patients. Statistically significant changes are designated as follows * p < 0.05 and ** p < 0.01.