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Original Article

HPV-induced tongue cancer patients develop different types of HPV-specific CD4$^+$ T cell responses, which is associated with disease prognosis

Xiangfeng Pan, Minchao Zhao, Lijun Cheng, Dawei Wang, Zhiqiang Feng, Jianfeng Dong

Department of Oral and Maxillofacial Surgery, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, Hebei, China

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Abstract: Infection by high-risk HPV 16 is usually self-limiting, asymptomatic and subclinical. However, in a minor subset of patients, HPV 16 could induce malignant transformation of infected cells, and is currently accounting for an increasing number of oropharyngeal cancer cases. To examine the host factors that could result in failed HPV clearance and oral cancer development, we examined the CD4$^+$ T cell responses to HPV stimulation in gender- and age-matched tongue cancer patients and asymptomatic controls. We observed that in HPV 16-infected asymptomatic controls, the proliferating CD4$^+$ T cells in response to HPV stimulation contained significantly higher frequencies of Th1 and lower Th17 and Treg cells, than in tongue cancer patients. The CD4$^+$ T cell cytokine secretion profile after HPV-stimulation in asymptomatic controls was also more biased toward IFN-g and IL-17 than IL-4 and IL-10, compared to tongue cancer patients. These observations were not due to tumor-mediated immunosuppression because the overall strength of HPV-specific CD4$^+$ T cell activation was higher in HPV-associated tongue cancer patients than in asymptomatic controls. Together, these results indicate that the types of HPV-specific CD4$^+$ T cell responses induced in HPV-infected individuals were associated with different infection outcomes. Furthermore, within the tongue cancer patients, those with low HPV-specific Th1 and high HPV-specific Treg were more likely to die, demonstrating that the types of CD4$^+$ T cell response toward HPV stimulation is associated with tumor prognosis.

Keywords: HPV, CD4, T cell, tongue cancer

Introduction

The head and neck cancers represent the sixth most common cancers with a growing incidence worldwide [1]. Oncogenic human papillomavirus (HPV) is currently detected in 25.9% of head and neck cancers, a statistic predicted to increase to more than 50% in the future in oropharyngeal cancers [2]. HPV is a double-stranded virus with over 40 sexually transmitted subtypes and represents the most common sexually transmitted pathogen [3]. In most cases, the infection is undetected, asymptomatic and subclinical [4, 5]. But persistent infection of high-risk HPV subtypes, most notably HPV 16, significantly elevates the risk of cancer development both in the oral cavity and the cervix [6-8]. Despite this, the majority of HPV 16-infected individuals spontaneously resolves the infection within two years, or maintains a disease-free low viral load with no symptoms [9-11]. Very little is understood in the underlying mechanisms of the different clinical manifestations in different individuals.

Cancer development and the adaptive immune system are fundamentally interrelated, as tumors can be immunogenic. This is especially true in HPV-related cancers, since blood and tumor-infiltrating HPV-specific adaptive T cells can be detected in HPV-associated cancer patients [12]. Whether the types and strengths of HPV-specific T cell responses in different individuals could directly lead to different infection outcomes need to be examined. In particular, the CD4$^+$ adaptive T cells are made up of a heterogeneous group of cells with very different immune functions. The interferon-gamma (IFN-g)-producing T helper 1 (Th1) cells could provide help to cytotoxic T cells, which in turn

Table 1. Demographic and clinicopathological characteristics of study participants. Student's *t* test or Fisher's exact test were applied where appropriate

	Control	Patient	P
N	16	16	
Gender (M/F)	8/8	8/8	> 0.05
Age, y (median, range)	46 (38-57)	48 (37-55)	> 0.05
Smoking (N/Y)	16/0	16/0	> 0.05
Alcohol (N/occasional/frequent)	3/7/6	2/7/7	> 0.05
Tumor status (T1+T2/T3+T4)	-/-	9/7	-
Node status (N0+N1/N2+N3)	-/-	13/3	-
Clinical staging (I+II/III+IV)	-/-	8/8	-

induce direct lysis of tumor cells. IFN-g could also exert direct anti-proliferative and pro-apoptotic effects to tumor cells [13]. Another major $CD4^+$ T cell subset, the Th17 cells, was also shown to boost cytotoxic immunity via IL-17 production and reduced the susceptibility of developing lung melanoma [14]. The Th2 cells, on the other hand, could stimulate the tumor-associated macrophages through IL-4 and IL-13 to transition toward the regulatory M2 type and thereby support tumor metastasis [15]. The regulatory T (Treg) cells could also exert tumor-promoting effects by suppressing antitumor immunity [16]. These distinctive anti-tumor or tumor-promoting roles of $CD4^+$ T cell subsets suggest that the frequency and activation status of each subset in cancer patients are associated with prognosis. For example, in breast cancer, high Th2/Th1 ratios in tumor-infiltrating cells are indicative of poor prognosis [17]; in colorectal cancer, poor prognosis is associated with high Th17 and low Th1 gene expression [18]; elevated frequencies of Treg cells were also associated with poor prognosis in hepatocellular carcinoma [19]. Interestingly, the same T cell subset could exert completely opposite functions in different cancers, for reasons still unclear. The role of $CD4^+$ T cells in inducing the outcome of HPV infections and HPV-associated oral cancers is still unknown.

To answer this question, the $CD4^+$ T cell subsets in HPV-infected tongue cancer patients, representing the most frequent type of HPV-associated oropharyngeal cancers, were examined and compared to those in HPV-infected asymptomatic patients. Our results indicate that the types of HPV-specific $CD4^+$ T cell responses induced in HPV-infected individuals were associated with different infection outcomes, and that patients with lower HPV-specific Th1 and higher HPV-specific Treg were more likely to die within five years.

Patients and methods

Study participants

Tongue cancer patients with primary tongue squamous cell carcinoma without any prior history of chemotherapy or radiotherapy were recruited at the Third Hospital of Hebei Medical University, and were screened by the Hybrid Capture II HPV test (Qiagen, Hilden, Germany) [20]. HPV-infected asymptomatic controls were recruited by screening a large set of healthy volunteers, who presented no sign or symptom of HPV infection nor had any other inflammatory diseases such as hepatitis B, rheumatoid arthritis and diabetes, for the presence of HPV 16 genomic sequences at the cervical specimens. To control for potential differences caused by different HPV genotypes, only participants who were positive for HPV 16 but not any other HPV genotypes were included. The asymptomatic controls and tongue cancer patients were gender- and age-matched. The demographic and clinicopathological characteristics of participants are presented in **Table 1**. All participants gave written informed consent in accordance with our institutional guidelines. The study protocols were reviewed and approved by the Research Ethic Committee of the Third Hospital of Hebei Medical University.

HPV 16 antigen preparation

Pool of 20-mer peptides spanning the entire E6 and E7 proteins of the HPV 16 strain, designed with a 4-mer overlap between each peptide, were purchased from PolyPeptide Laboratories (Strasbourg, France) and were dissolved in DMSO and diluted with PBS to a stock concentration of 4 mg/mL and stored at -20°C. Recombinant HPV 16 full-length protein expressed in *E. coli* was purchased from Abcam (Cambridge, UK) and stored at -80°C. To stimulate the cells, both proteins and peptide pools were added to the cell culture at a final concentration of 2 μg/mL each.

Flow cytometry

PBMCs were either stained directly *ex vivo* after thawing with RPMI complete medium supple-

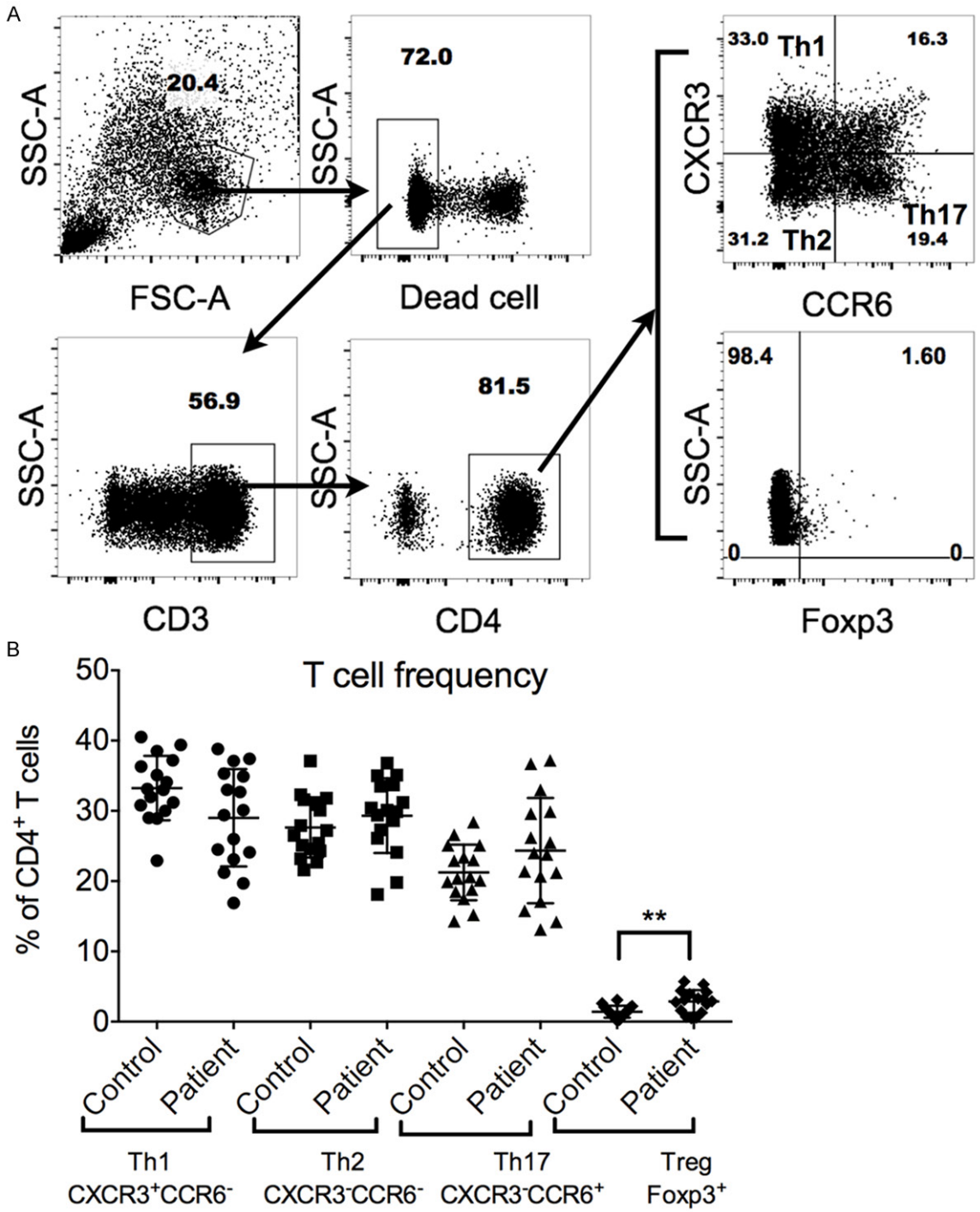

Figure 1. Identification of $CD4^+$ T cell subsets in asymptomatic controls and tongue cancer patients. PBMCs were surface-stained with anti-CD3, CD4, CXCR3, and CCR6, and then intracellular-stained with Foxp3. A. Identification of Th1 ($CXCR3^+CCR6^-$), Th2 ($CXCR3^-CCR6^-$), Th17 ($CXCR3^-CCR6^+$) and Treg ($Foxp3^+$) $CD4^+$ T cells in one representative individual. Greater than 10^5 events were collected at the lymphocyte gate. B. The frequencies of Th1, Th2, Th17, and Treg cells as a percentage of total $CD4^+$ T cells, in all asymptomatic controls and tongue cancer patients. Mean ± SD. Unpaired *t* test with Welch's correction. **: $P < 0.01$.

mented with 10% FCS and 1 μg/mL DNase (Sigma-Aldrich, St. Louis, MO), or after 6-day incubation in RPMI complete medium at 37°C and 5% CO_2 with or without HPV 16 antigen

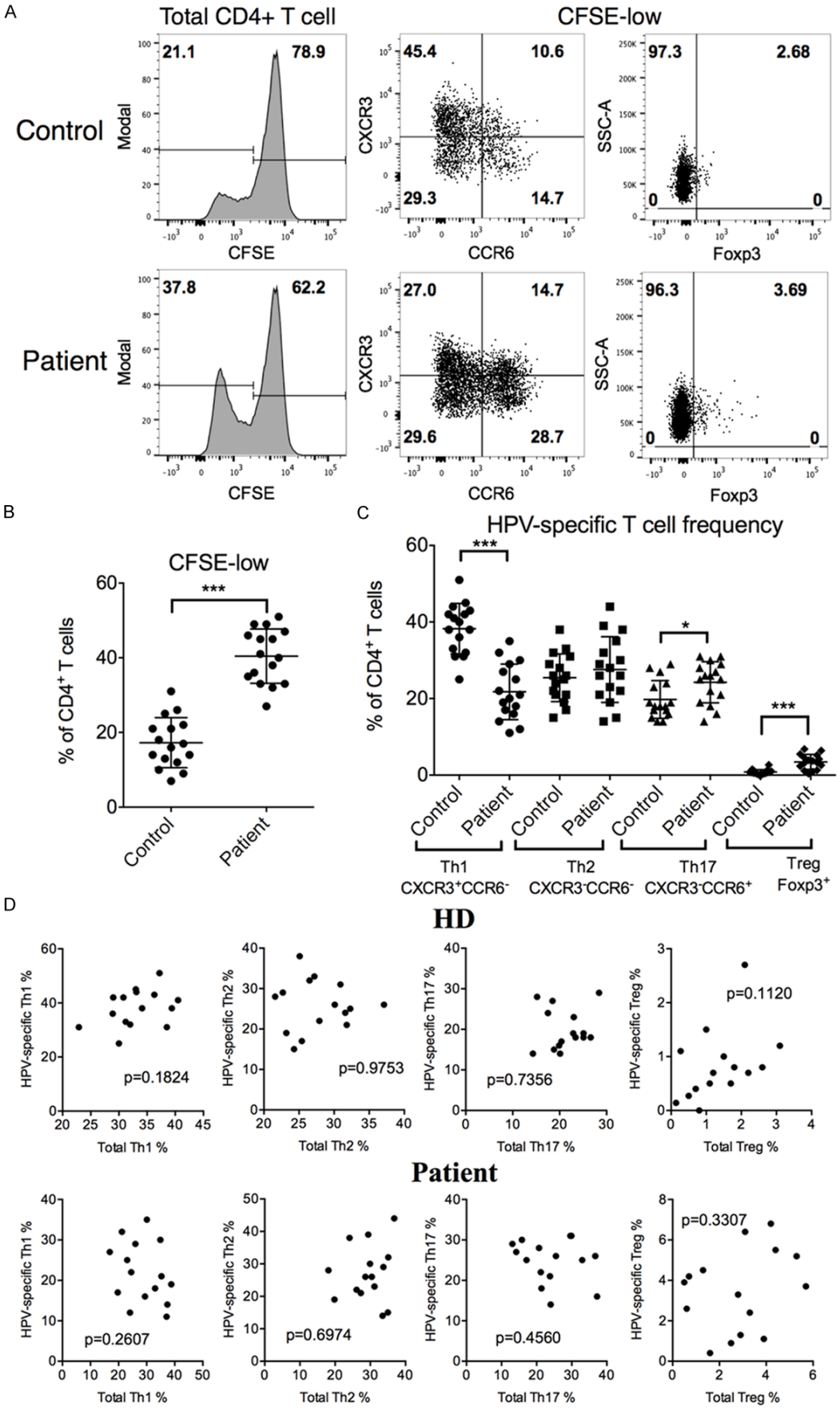
A
Total CD4+ T cell
CFSE-low
Control
Patient
Modal
CFSE
CXCR3
CCR6
SSC-A
Foxp3
21.1
78.9
45.4
10.6
97.3
2.68
29.3
14.7
0
0
37.8
62.2
27.0
14.7
96.3
3.69
29.6
28.7
0
0
B
CFSE-low
% of CD4+ T cells
***
Control
Patient
C
HPV-specific T cell frequency
% of CD4+ T cells
***
*
***
Control
Patient
Th1
CXCR3+CCR6-
Th2
CXCR3-CCR6-
Th17
CXCR3-CCR6+
Treg
Foxp3+
D
HD
Patient
HPV-specific Th1 %
Total Th1 %
p=0.1824
HPV-specific Th2 %
Total Th2 %
p=0.9753
HPV-specific Th17 %
Total Th17 %
p=0.7356
HPV-specific Treg %
Total Treg %
p=0.1120
p=0.2607
p=0.6974
p=0.4560
p=0.3307

Figure 2. Identification of HPV-specific CD4+ T cells in asymptomatic controls and tongue cancer patients. PBMCs were labeled with CFSE and incubated in the presence or HPV 16 antigens for 6 d. The PBMCs were then stained with anti-CD3, CD4, CXCR3, CCR6 and Foxp3. A. Identification of CFSE-low CD4+ T cells and CD4+ T cell subsets in one representative individual. B. The percentage of CFSE-low cells as the fraction of total CD4+ T cells, in asymptomatic controls and tongue cancer patients. C. The frequencies of HPV-specific (identified as CFSE-low) Th1, Th2, Th17 and Treg cells as a percentage of total CD4+ T cells in asymptomatic controls and tongue cancer patients. Mean ± SD. Unpaired *t* test with Welch's correction. *: $P < 0.05$. ***: $P < 0.001$. D. The correlation between the frequencies of total Th1, Th2, Th17 or Treg cells and their HPV-specific counterparts, in asymptomatic controls and tongue cancer patients. p represents the Pearson correlation coefficient. $P < 0.05$ is considered significant.

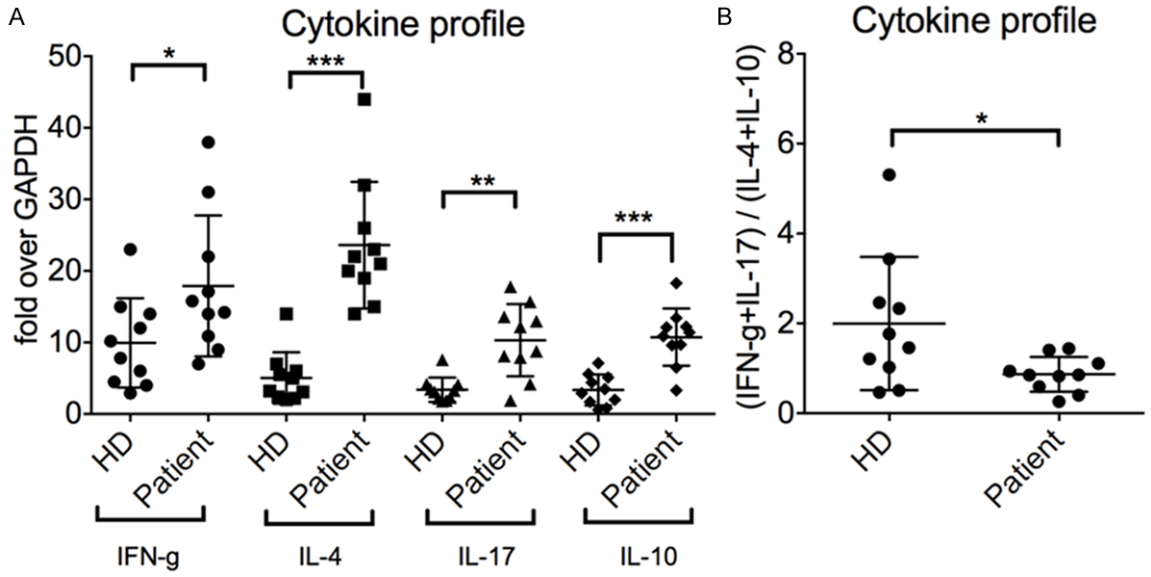

Figure 3. Cytokine expression by CD4+ T cells after stimulation with HPV 16 antigen. PBMCs were incubated in the presence or absence (background) of HPV 16 antigens for 72 h. CD4+ T cells were negatively purified and washed. Reverse transcription and quantitative PCR were then performed to measure the transcript levels in purified CD4+ T cells. Cytokine mRNA level after subtraction of background was expressed as fold over GAPDH. A. IFN-g, IL-4, IL-17 and IL-10 expression after HPV stimulation in asymptomatic controls and tongue cancer patients. B. The ratio of IFN-g + IL-17 expression to IL-4 + IL-10 expression in asymptomatic controls and tongue cancer patients. Mean ± SD. Unpaired *t* test with Welch's correction. *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$.

stimulation. Labeling of CFSE was done using CFSE Cell Label Kit (Abcam) following the kit's instructions before the start of incubation. After incubation, cells were stained with 2 μg/mL anti-human CD3, CD4, CXCR3, and CCR6 antibodies (Biolegend, San Diego, CA), and Aqua Dead Cell Stain (Invitrogen, Waltham, MA), for 30 min at 4°C. Cells were then washed twice and treated with Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA) for staining with anti-Foxp3 antibody (eBioscience). At the end, cells were fixed in 2% formalin to be acquired by BD FACSCanto and analyzed in FlowJo software.

RT-PCR

PBMCs were incubated in the presence or absence of HPV 16 antigens for 72 h, after which the CD4+ T cells was negatively selected using EasySep Human CD4+ T Cell Enrichment Kit (Stemcell, Vancouver, Canada) and lysed with Buffer RLT (Qiagen) containing 1% 2-ME. mRNA was isolated with the RNAeasy Kit (Qiagen) and treated for 20 min at 37°C with DNase. The DNase was heat-inactivated for 10 min at 65°C. Reverse transcription was performed by 0.5 U AMV Reverse Transcriptase (Promega, Madison, WI) for 1 h at 42°C. The cDNA levels of IFN-g, IL-4, IL-17 and IL-10 were determined by PCR with the ABI Prism 7900 system (Applied Biosystems, Foster, CA). The primer sequences are (5'-3', forward and reverse): IFN-g: TGCAGGTCATTCAGATGTAG and AGCCATCACTTGGATGAGTT; IL-4: TGCCTCCAAGAACACAACTG and AACGTACTCTGGTTGGCTTC; IL-17: GTGGTTGACCCGGAGTTACTG and CCTTCGGGAAATGGAATAAAAA; IL-10: TACCTGGGTTG-

Table 2. HPV-specific CD4$^+$ T cell frequency and 5-year survival rates of all tongue cancer patients. Fisher's exact test

HPV-specific cell type (median, %)	Alive, N (%)	Deceased, N (%)	P
Th1 (20.5)			0.0406
High	7	1	
Low	2	6	
Th2 (27)			1.0000
High	4	4	
Low	5	3	
Th17 (25.5)			0.3147
High	6	2	
Low	3	5	
Treg (3.5)			0.0406
High	2	6	
Low	7	1	

CCAAGCCTT and GTTCACAGAGAAGCTCAGT. All RT-PCR experiments were performed in duplicates. GADPH was used as an internal control.

Statistics

All statistical analyses were performed in Prism 6 software. Differences between HPV-infected asymptomatic controls and tongue cancer patients were examined by unpaired *t* test with Welch's correction. Correlation between total and HPV-specific CD4$^+$ T cells was examined by Pearson correlation. Differences in 5-year survival rate were calculated by Fisher's exact test. $P < 0.05$ is considered significant.

Results

HPV-specific CD4$^+$ T cells in tongue cancer patients and healthy donors have different compositions

Adaptive CD4$^+$ T cell immunity is a critical component in establishing local inflammatory environment and assisting CD8$^+$ T cell trafficking to the inflamed site to enable virus clearance. Th1, Th2, Th17 and Treg cells are among the best studied CD4$^+$ T cell subsets, with distinctive proinflammatory or antiinflammatory activities. Dysregulation of CD4$^+$ T cell subsets is thought to contribute to the induction of inflammatory diseases and limit pathogen clearance [21-26]. We took advantage that human Th1, Th2 and Th17 can be identified by surface CXCR3$^+$CCR6$^-$, CXCR3$^-$CCR6$^-$, and CXCR3$^-$CCR6$^+$ expression, and that Foxp3 is a transcription factor for Treg cells (**Figure 1A**) [27-31]. We found that the frequencies of Th1, Th2 and Th17 cells were not significantly altered in tongue cancer patients compared to HPV-infected healthy donors (**Figure 1B**). The frequencies of Tregs, on the other hand, were significantly upregulated compared to that in healthy donors, but with high variability from patient to patient (**Figure 1B**).

The overall frequencies of T cell subsets may not be good predictors of the inflammatory outcomes, since a particular antigen might not activate all T cells. We therefore examined the HPV-specific T cells. Recognition of specific antigen at the CD3 complex could lead to T cell proliferation. We therefore treated CFSE-labeled PBMCs with HPV 16 antigen from these two strains for 6 d, and identified CFSE-low cells as HPV-specific CD4$^+$ T cells. The Th1 (CXCR3$^+$CCR6$^-$), Th2 (CXCR3$^-$CCR6$^-$), Th17 (CXCR3$^-$CCR6$^+$) and Treg (Foxp3$^+$) CD4$^+$ T cells were then gated in the CFSE-low population (**Figure 2A**). The frequencies of total CFSE-low CD4$^+$ T cells were significantly higher in tongue cancer patients than in HPV-infected healthy donors (**Figure 2B**), reflecting ongoing infection. However, when we examined the T cell subset composition within the CFSE-low portion, we found that healthy donors had significantly higher HPV-specific Th1, while tongue cancer patients had significantly higher HPV-specific Th17 and Treg (**Figure 2C**). The frequencies of HPV-specific CD4$^+$ T cell subsets were not correlated with the frequencies of total CD4$^+$ T cell subsets (**Figure 2D**). These data suggested that different CD4$^+$ T cell subsets were upregulated by HPV infection in tongue cancer patients and healthy donors.

Tongue cancer patients and healthy donors have different cytokine signature after HPV stimulation

Although chemokine receptors are critical in the development of Th1, Th2 and Th17 cells and could be used as markers of CD4$^+$ T cell subsets, cytokine secretion could provide direct information in CD4$^+$ T cell function. We therefore stimulated PBMCs with HPV antigen and examined the transcription levels of characteristic CD4$^+$ T cell subset cytokines in purified CD4$^+$ T cells, including IFN-g (Th1), IL-4 (Th2), and IL-17 (Th17), as well as IL-10, in tongue cancer patients and healthy donors. We found that the mRNA transcript levels of all four

cytokines were significantly higher in tongue cancer patients than in healthy donors (**Figure 3A**), consistent with the higher frequencies of HPV-specific $CD4^+$ T cells in tongue cancer patients (**Figure 2B**). IFN-g and IL-17 are generally considered proinflammatory cytokines, while IL-4 and IL-10 are generally considered antiinflammatory cytokines [32]. We found that the ratio of proinflammatory-to-regulatory cytokine transcripts in tongue cancer patients were significantly lower than that in healthy donors (**Figure 3B**).

Five-year survival of tongue cancer patients were associated with the types of HPV-specific $CD4^+$ T cells

These previous data suggested that the difference in the types of anti-HPV $CD4^+$ T cell responses being induced in the individuals might contribute to the final outcome of HPV infection, leading to resolution or tongue cancer. Whether it could affect tongue cancer prognosis is still unclear. In our tongue cancer patient cohort, using median cell frequency as a cutoff, we found that patients with high Th1 and low Treg HPV-specific $CD4^+$ T cells had significantly better 5-year survival rate (**Table 2**), suggesting a role of HPV-specific Th1 and Treg cells in cancer prognosis. No association between the total frequencies of $CD4^+$ T cells and the 5-year survival rate in tongue cancer patients was found (data not shown).

Discussion

Our results indicate that the types of HPV-specific $CD4^+$ T cell responses induced in HPV-infected individuals were associated with different infection outcomes, and that patients with lower HPV-specific Th1 and higher HPV-specific Treg were more likely to die within five years. We demonstrated these findings with the following experiments. First, we showed that in HPV 16-infected asymptomatic controls, the proliferating $CD4^+$ T cells in response to HPV stimulation contained significantly higher frequencies of Th1 and lower Th17 and Treg cells, than in tongue cancer patients. The $CD4^+$ T cell cytokine secretion profile after HPV-stimulation was also more biased toward the proinflammatory IFN-g and IL-17 than the anti-inflammatory IL-4 and IL-10 subtypes in asymptomatic controls, compared to tongue cancer patients. Together, these data demonstrated that HPV-induced different $CD4^+$ T cell responses in asymptomatic controls and tongue cancer patients. Furthermore, within the tongue cancer patients in our cohort, those with low HPV-specific Th1 and high HPV-specific Treg were more likely to die, demonstrating that the types of $CD4^+$ T cell response toward HPV stimulation is associated with tumor prognosis.

Multiple inhibitory mechanisms exist in the tumor microenvironment to specifically suppress tumor antigen-specific T cells and tumor-infiltrating T cells, including antigen-presentation by $IL\text{-}10^{hi}IL\text{-}12^{lo}$ M2-type tumor-associated macrophages and defective dendritic cells [33-35], hypoxia-induced immune suppression and resistance to cytotoxicity [36, 37], constitutive expression of pro-apoptotic molecule PD-L1 and CTLA-4 on tumor cells [38, 39], and infiltration of regulatory T cells and B cells [40, 41]. However, the altered HPV-specific $CD4^+$ T cell composition in our cohort of tongue cancer patients is unlikely due to these potential tumor-mediated suppression of proinflammatory T cells, since the overall strength of HPV-specific $CD4^+$ T cell activation was higher in HPV-associated tongue cancer patients than in asymptomatic controls, evident by the higher frequencies of CFSE-low proliferating $CD4^+$ T cells and the higher mRNA levels of all cytokines examined. This suggests that the initial types of $CD4^+$ T cell response, rather than the overall strength, is important for the more favorable outcome of HPV infection.

Currently, it is unknown why different individuals induced different types of immune responses to the same HPV genotype, but the viral load and the presence of concurrent infections, as well as the route of infection, are likely playing a role. More research is necessary to examine the host immune status in relation to HPV infection and resolution. In addition, the results presented here suggest that HPV-specific $CD4^+$ T cell composition might act as a prognostic indicator of HPV infection outcome with the potential to assist early diagnosis and treatment, which would need validation in a larger cohort of HPV-infected subjects in future studies.

Disclosure of conflict of interest

None.

Address correspondence to: Minchao Zhao, Department of Oral and Maxillofacial Surgery, The Third

Hospital of Hebei Medical University, 139 Ziqiang Road, Shijiazhuang 050051, Hebei, China. E-mail: minchaozhao_sjz@sina.com

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