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Dysfunction of HPV16-specific CD8+ T cells derived from oropharyngeal tumors is related to the expression of Tim-3 but not PD-1

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ABSTRACT

Background: Human papillomavirus (HPV) type 16 infection is one of the most important etiological agents of oropharyngeal squamous cell carcinoma. Patients with HPV-associated carcinomas of the head and neck were reported to have a better clinical outcome than patients with HPV-negative tumors. Because HPV16 E6 and E7 oncoproteins are highly immunogenic and constitutively expressed, HPV-specific T cell immunity may play the key role in improving the prognosis of these patients.
Methods: Tumor-derived T cells were expanded in high levels of IL-2 and stimulated with HPV16 E6/E7 peptides in the presence or absence of anti-PD-1 monoclonal antibody nivolumab and soluble Tim-3.
Results: HPV16-specific tumor-infiltrating T cells were present in 73.1% of HPV-associated oropharyngeal tumors. HPV16 specific CD8+ TILs were able to produce IFNγ upon specific stimulation and predominantly expressed PD-1 but not Tim-3. Specific IFNγ production was further enhanced after a blockade of both PD-1 and Tim-3 pathways but not after a PD-1 blockade alone. Additionally, the specific stimulation of anti-HPV16 CD8+ T cells suppressed Tim-3 upregulation after the PD-1 blockade.
Conclusion: Our data provide the rationale for combination cancer immunotherapy approaches, including the dual blockade of PD-1 and Tim-3 and, potentially, the use of HPV16-directed therapeutic vaccines.

Introduction

Over the past two decades, an increase in incidence of oropharyngeal squamous cell carcinoma (OPC) has been reported in patients with no history of alcohol and tobacco use. Human papillomavirus type 16 has been identified as the most important etiological agent of this malignancy [1]. Following the standard treatment regimes, patients with HPV-associated tumors have a better clinical outcome than patients with tobacco-related carcinomas; however, standard chemo- and radiotherapy is still associated with considerable morbidity and toxicity in these patients. Therefore, more effective and less toxic therapeutic strategies are needed. However, no new targeted therapies have been approved since cetuximab in 2006, which, as a monotherapy, shows a therapeutic benefit in only 10–15% of head and neck squamous cell carcinoma (HNSCC) patients [2]. Similarly, clinical trials with PD-1:PD-L1 targeting agents, which are promising in other tumor histologies, only report modest response rates (13–18%) in HNSCC, including HPV-associated OPC [3–5]. Recently, preclinical studies have shown that targeting PD-1 pathway simultaneously with an alternative checkpoint molecule, T cell immunoglobulin and mucin domain 3 (Tim-3), emerges as a promising approach for improvement of current immunotherapy [6,7].

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In the development of efficient immunotherapeutic strategies, the identification of tumor-specific antigens remains essential. In HPV-associated cancers, the HPV E6 and E7 oncoproteins represent optimal specific antigens. They are constitutively expressed and presented by cancer cells and they are highly immunogenic [8]. Indeed, preclinical studies have reported that anti-HPV E7 vaccines elicited E7-specific CD8+ T cells in tumor-bearing mouse models. The presence of HPV-specific T cells was associated with partial regression of E7-expressing TC-1 tumors in immunized mice [9–11]. Moreover, therapeutic vaccines targeted against HPV16 E6 and E7 oncoproteins have been reported to induce a complete response in 47% of patients with HPV-associated vulvar intraepithelial neoplasia in a phase II clinical trial [12]. With HPV16 being a leading etiological agent in oropharyngeal cancer (OPC), the induction of a robust HPV-specific immune response may represent a promising therapeutic strategy. However, there are only a few reports concerning the existence of HPV16-specific T cell immunity in HNSCC patients, and most are focused on the detection of virus-specific T cells in the peripheral blood [13–16].

The aim of this study was to analyze the frequency, phenotype and function of HPV16 E6/E7-specific tumor-infiltrating T cells (TILs) in oropharyngeal tumors and to test the effect of anti-PD1 mAb (nivolumab), soluble Tim-3 (sTim-3) and homeostatic *in vitro* expansion on these characteristics.

Materials and methods

Patients and samples

Blood samples and primary oropharyngeal squamous cell carcinoma specimens were obtained from 51 patients immediately after radical surgery at the Department of Otorhinolaryngology and Head and Neck Surgery, Motol University Hospital in Prague between April 2015 and August 2017. Patients enrolled in this study had not received any neoadjuvant chemo- or radiotherapy. All of the patients signed an informed consent approved by the Institutional Review Board of the University Motol. The clinical-pathological characteristics of the patients are summarized in Table 1.

The tumor tissues were processed as described previously [17]. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by centrifugation on a Ficoll-Paque density gradient (GE Healthcare, Waukesha, WI).

Table 1
Clinical-pathological characteristics of the patients.

Variable	No.	%
Total no. of patients	51	
Age		
Mean	59	
Range	36–75	
Sex		
Male	36	70.6
Female	15	29.4
Nodal status		
N0	9	17.6
N1-N3	42	82.4
Stage		
I	1	1.9
II	8	15.7
III	13	25.5
IV	29	56.9
Tumor site		
Base of tongue	10	19.6
Tonsil	32	62.7
Oropharynx	9	17.7
HPV status		
HPV-	10	19.6
HPV+	41	80.4

TIL expansion

All T cell cultures were performed in RPMI 1640 supplemented with 10% human AB serum, penicillin-streptomycin, L-glutamine (all from Invitrogen, Carlsbad, USA) and 450 U IL-2 (Proleukin, Prometheus Laboratories Inc., San Diego, USA). Freshly isolated TILs at a concentration of 3×10^5 cells/ml were expanded for two weeks with the addition of fresh medium and IL-2 every 3 days. After 2 weeks of homeostatic expansion, the cells were harvested, and their phenotype was analyzed using flow cytometry and qPCR. Proportional frequency of TILs within fresh tumor single cell suspension and expanded TILs is shown in Fig. S1.

For quantification of cytokines, free fatty acids, adenosine and sPD-1 production, expanded tumor-derived TILs and PBMCs (1×10^6 cells/ml) were cultured in culture medium in the presence or absence of HPV16 E6/E7 peptide-loaded autologous monocytes. After 24 h of incubation, the culture supernatants were harvested and stored at −80 °C until use.

Flow cytometry

Single cell suspensions derived from tumor tissues were labeled using monoclonal antibodies (mAbs) against CD3 (Exbio, Vestec, Czech Republic), CD4 (eBioscience, San Diego, USA), CD8 (Exbio), PD-1 and Tim-3 (both from BioLegend, San Diego, USA). For the intracellular detection of cytokines, the cells were fixed and permeabilized with a FoxP3 Staining Buffer Set (eBioscience) and intracellularly labeled with mAbs against IFNγ (BD Biosciences) and TNFα (BioLegend). The cells were then analyzed on a BD FACSCanto II (BD Biosciences) and evaluated with FlowJo software (TreeStar, Ashland, OR).

Detection of HPV16-specific T cells

Monocytes from autologous PBMCs were isolated using a Human CD14 Positive Selection Kit (Stemcell Technologies, Vancouver, Canada). The obtained monocytes were loaded with HPV16 E6 and E7 peptide pools (5 μg/ml) (JPT, Berlin, Germany) and added to expanded TILs at a ratio of 1:10. The cells were incubated for 6 h in the presence of Brefeldin A (BioLegend). After the 6-h incubation, the cells were stained with antibodies for the intracellular detection of cytokines as described above. Cut off was calculated as the mean proportion of IFNγ-/TNFα-positive CD8+ T cells detected upon specific stimulation in HPV-negative patients + 3SD.

For *in vitro* blocking studies, anti-PD-1 mAb (10 μg/ml) (nivolumab, Bristol-Myers-Squibb, New York, USA) and soluble Tim-3 (5 μg/ml) (Recombinant Human Tim-3 protein, Abcam, Cambridge, UK) were added to the TIL cultures 42 h prior to specific stimulation with HPV16 E6 and E7 peptide-loaded autologous monocytes.

Detection of cytokines, soluble PD-1, free fatty acids and free adenosine in culture supernatants

To detect the concentrations of cytokines released into culture supernatant, the MILLIPLEX™ Human Cytokine Kit (Millipore, Billerica, MA) was used. Levels of soluble PD-1, free fatty acids and adenosine were analyzed using a PD-1 Human ELISA Kit (Thermo Fisher Scientific), Free Fatty Acid Quantification Kit (Abcam) and Adenosine Assay Kit (BioVision, Milpitas, USA), respectively. All assays were performed according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from 1×10^6 tumor-tissue derived cells