

Distinct patterns of intratumoral immune cell infiltrates in patients with HPV-associated compared to non-virally induced head and neck squamous cell carcinoma

Simona Partlová^{1,2}, Jan Bouček^{3,4}, Kamila Kloudová^{1,2}, Eva Lukešová^{5,6}, Michal Zábrodský³, Marek Grega⁷, Jitka Fučíková^{1,2}, Iva Truxová¹, Ruth Tachezy^{5,6}, Radek Špišek^{1,2}, and Anna Fialová^{1,2,*}

¹Sotio, Prague, Czech Republic; ²Department of Immunology; 2nd Faculty of Medicine; Charles University; Motol University Hospital; Prague, Czech Republic; ³Department of Otorhinolaryngology and Head and Neck Surgery; 1st Faculty of Medicine; Charles University and Motol University Hospital; Prague, Czech Republic; ⁴Institute of Microbiology ASCR; Prague, Czech Republic; ⁵Department of Experimental Virology; Institute of Hematology and Blood Transfusion; Prague, Czech Republic; ⁶Department of Genetics and Microbiology; Faculty of Science; Charles University; Prague, Czech Republic; ⁷Department of Pathology and Molecular Medicine; 2nd Faculty of Medicine; Charles University and Motol University Hospital; Prague, Czech Republic

Keywords: CD8⁺ T lymphocytes, HNSCC, HPV, PD-1, Tim-3

Abbreviations: Cox-2, cyclooxygenase 2; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; mDC, myeloid dendritic cell; PD-1, programmed cell death 1; pDC, plasmacytoid dendritic cell; PD-L1, programmed cell death-ligand 1; Tim-3, T cell immunoglobulin and mucin protein 3; Treg, regulatory T cell

Human papillomavirus (HPV) infection is one of the most important etiologic causes of oropharyngeal head and neck squamous cell carcinoma (HNSCC). Patients with HPV-positive HNSCC were reported to have a better clinical outcome than patients with HPV-negative cancers. However, little is known about the possible causes of different clinical outcomes. In this study, we analyzed a detailed immune profile of tumor samples from HNSCC patients with respect to their HPV status. We analyzed the characteristics of immune cell infiltrates, including the frequency and distribution of antigen-presenting cells and naïve, regulatory and effector T cells and the cytokine and chemokine levels in tumor tissue. There was a profound difference in the extent and characteristics of intratumoral immune cell infiltrates in HNSCC patients based on their HPV status. In contrast to HPV-negative tumor tissues, HPV-positive tumor samples showed significantly higher numbers of infiltrating IFN γ ⁺ CD8⁺ T lymphocytes, IL-17⁺ CD8⁺ T lymphocytes, myeloid dendritic cells and proinflammatory chemokines. Furthermore, HPV-positive tumors had significantly lower expression of Cox-2 mRNA and higher expression of PD1 mRNA compared to HPV-negative tumors. The presence of a high level of intratumoral immune cell infiltrates might play a crucial role in the significantly better response of HPV-positive patients to standard therapy and their favorable clinical outcome. Furthermore, characterization of the HNSCC immune profile might be a valuable prognostic tool in addition to HPV status and might help identify novel targets for therapeutic strategies, including cancer immunotherapy.

Introduction

HNSCC is a heterogeneous group of tumors located in the oral cavity, oropharynx, hypopharynx and larynx. Originally, tobacco and/or alcohol exposure were the main risk factors for HNSCC, but in an expanding subset of patients with oropharyngeal carcinoma, HPV infection has been described in the last two decades as a crucial etiologic agent.^{1,2} Although patients with HPV-associated tumors are more often diagnosed at advanced

stages of the disease with large metastatic lymph nodes, their prognosis is reported to be significantly better than that of patients with non-HPV induced cancers.^{3,4}

Despite the improved response of HPV-positive HNSCC to conventional treatment involving a combination of surgery, radiation therapy and chemotherapy, HPV-positive cell lines were shown to be more resistant to radiation and cisplatin *in vitro* when compared to HPV-negative cells. However, *in vivo*, HPV-positive tumors were more sensitive to

© Simona Partlová, Jan Bouček, Kamila Kloudová, Eva Lukešová, Michal Zábrodský, Marek Grega, Jitka Fučíková, Iva Truxová, Ruth Tachezy, Radek Špišek, and Anna Fialová

*Correspondence to: Anna Fialová; Email: Askalova@centrum.cz

Submitted: 07/04/2014; Revised: 09/05/2014; Accepted: 09/10/2014

<http://dx.doi.org/10.4161/21624011.2014.965570>

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

radio- and chemotherapy in immunocompetent mice. Importantly, neither radiotherapy nor cisplatin therapy cured immunocompromised mice, indicating an important role for the immune system in HPV-positive HNSCC outcome.⁵ Although contradictory results recently published by Kimple et al.⁶ showed enhanced radiation sensitivity in HPV-positive cancer cell lines, this finding does not explain why HPV-positive patients treated with surgery alone also have a better prognosis.³ Moreover, in addition to the high proportion of relapses, especially in HPV-negative patients, conventional therapy remains associated with significant toxicity. Therefore, there is great interest in developing less toxic and more targeted therapies, including immunotherapy. Consequently, a better understanding of the interplay between the tumor microenvironment, HPV and the infiltrating immune cells is essential.

Indeed, characterization of the adaptive immune response has been shown to be an important prognostic tool in a wide range of carcinomas, potentially even more relevant than the current cancer staging system.⁷⁻¹¹ In HNSCC, recently published studies indicate that the assessment of the level of circulating CD8⁺ T lymphocytes,¹² the extent of tumor infiltration by CD8⁺ T lymphocytes and the ratio of infiltrating CD8⁺/FoxP3⁺ T lymphocytes^{13,14} might have a prognostic significance. However, a complex profile of the particular tumor-infiltrating immune cell subsets, including antigen-presenting cells, has not been evaluated to date.

In this study, we analyzed the distribution and phenotype of CD8⁺ and CD4⁺ T cell subsets, dendritic cell subsets (DCs) and monocytes/macrophages as well as the chemokine and cytokine profile in fresh HNSCC samples with regard to HPV status. Our findings confirm that HPV-positive tumor samples show a distinct immunologic profile compared to HPV-negative samples, with high levels of infiltrating IFN γ ⁺ CD8⁺ T lymphocytes, IL-17⁺ CD8⁺ T lymphocytes (Tc17 lymphocytes), myeloid DCs and spontaneously produced proinflammatory chemokines and cytokines. Additionally, HPV-positive samples expressed significantly lower levels of *Cox-2* mRNA and higher levels of *PD-1* mRNA than HPV-negative samples.

Results

HPV-positive tumors are mostly localized in the oropharynx

As expected, only the HPV 16 type was detected in all of the samples that were positive for HPV DNA. The expression of

HPV 16 E6 mRNA was detected in 45.5% ($n = 20$) of patients; 55.0% ($n = 11$) of the HPV-positive tumors were localized in the tonsils, 40.0% ($n = 8$) at the base of the tongue and 5.0% ($n = 1$) at the base of the oral cavity (Table 1). Lymph node metastases were histologically confirmed (pN⁺) in 90.5% of HPV-positive and 66.7% of HPV-negative patients, but this difference did not reach a statistical significance. The tumor grade and the stage were equivalent in patients with HPV-positive and HPV-negative HNSCC.

Patients with HPV-positive HNSCC have significantly higher levels of tumor-infiltrating CD8⁺ T cells with the capacity to produce IFN γ and IL-17 after *in vitro* stimulation.

The presence of tumor-infiltrating leukocytes, particularly CD8⁺ T cells, was shown to be a strong prognostic marker in various types of cancer; therefore, we analyzed the numbers and proportions of tumor-infiltrating immune cells in HPV-positive and HPV-negative HNSCC patients. The levels of tumor-infiltrating CD45⁺ leucocytes were significantly higher in HPV-positive tumor samples (Fig. 1A). As expected, there were significantly higher numbers of CD8⁺ T cells in HPV-positive tumor tissue samples compared to HPV-negative samples (Fig. 1B). Additionally, significantly higher proportions of CD8⁺ cells from HPV-positive samples produced IFN γ (Fig. 1C and D) or IL-17 (Fig. 1E and F) upon PMA and ionomycin stimulation *in vitro*.

Patients with HPV-positive HNSCC have higher numbers of CD4⁺ T cells in tumor tissue

We observed a trend to an increase in the numbers of total CD4⁺ T cells as well as IFN γ -producing CD4⁺ cells (Th1 cells) in the HPV-positive tumor samples ($p < 0.1$) (Fig. 2A-C). The proportion of Th17 cells did not show any differences between HPV-positive and -negative tumors (Fig. 2D and E); however, single cell suspensions isolated from HPV-positive tumors produced significantly higher levels of IL-17 upon PMA and ionomycin stimulation *in vitro* ($p = 0.030$) (Fig. 4C). Additionally, we observed a slightly lower proportion of Tregs in HPV-positive tumors (Fig. 2G). None of the subsets of CD4⁺ T cells listed above showed any statistically significant differences between patients with HPV-positive and -negative tumors, but significantly higher numbers of naïve T cells were detected in HPV-positive tumor tissues compared to HPV-negative tumor samples ($p = 0.018$) (Fig. 2H and I).

HPV-positive HNSCC samples have increased numbers of tumor-infiltrating antigen presenting cells

Additionally, we also analyzed subsets of antigen presenting cells, namely mDCs, pDCs and monocytes/macrophages in the tumor tissue. We observed an increased frequency of all of these cell populations in patients with HPV-positive tumors. The total numbers of mDCs in HPV-positive tumor samples showed a statistically significant increase compared to HPV-negative samples; however, the frequency of pDCs and monocytes/macrophages were not statistically

Table 1. Frequency of HPV-positive and HPV-negative tumors

	HPV negative -		HPV positive +	
	N	%	N	%
Total	24	54.5	20	45.5
Tongue	9	37.5	8	40.0
Tonsil	2	8.3	11	55.0
Larynx	8	33.3	0	0
Others	5	20.8	1	5.0

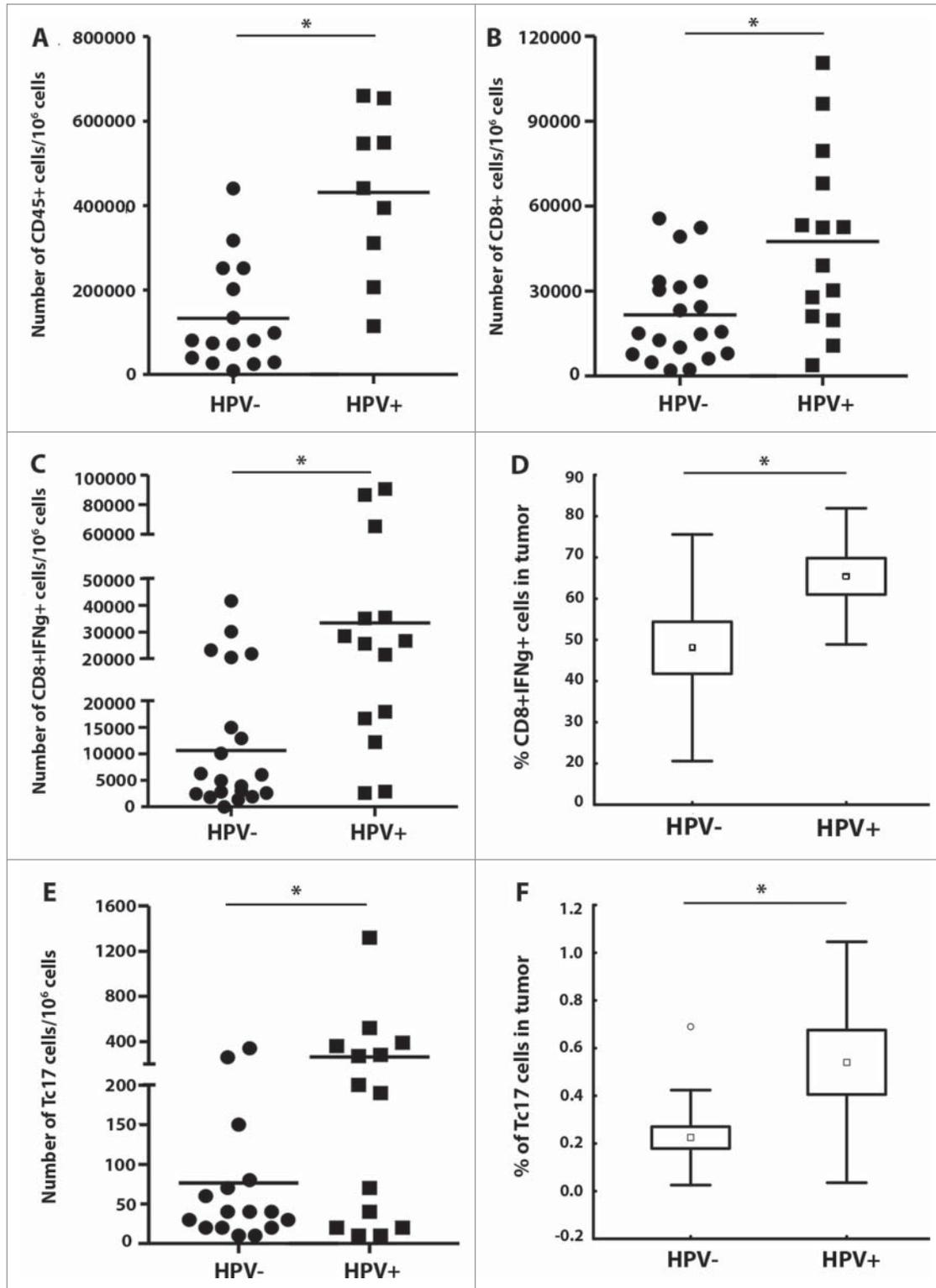


Figure 1. Proportions of leukocytes within tumor tissues of head and neck squamous cell carcinoma (HNSCC) patients ($n = 44$) according to human papillomavirus (HPV)-status. To evaluate the pattern of the immune cell infiltrates, tumor-derived single cell suspensions were stimulated with PMA and ionomycin in the presence of Brefeldin A and analyzed by flow cytometry. **(A–C, and E)** The data are expressed as the numbers of CD45⁺ cells, CD8⁺ cells, CD8⁺IFN γ ⁺ cells and Tc17 in 1×10^6 isolated tumor-derived cells; the line represents the mean value. **(D and F)** Box plots represent the proportions of CD8⁺IFN γ ⁺ cells and CD8⁺IL-17⁺ (Tc17 cells) among the tumor-infiltrating CD8⁺ T cells. The boundaries of the box indicate the standard error of the mean (SEM), and the lines in the box represent the median. Whiskers indicate the standard deviation (SD). * $p < 0.05$ (General Linear Models; age was added as a covariate).

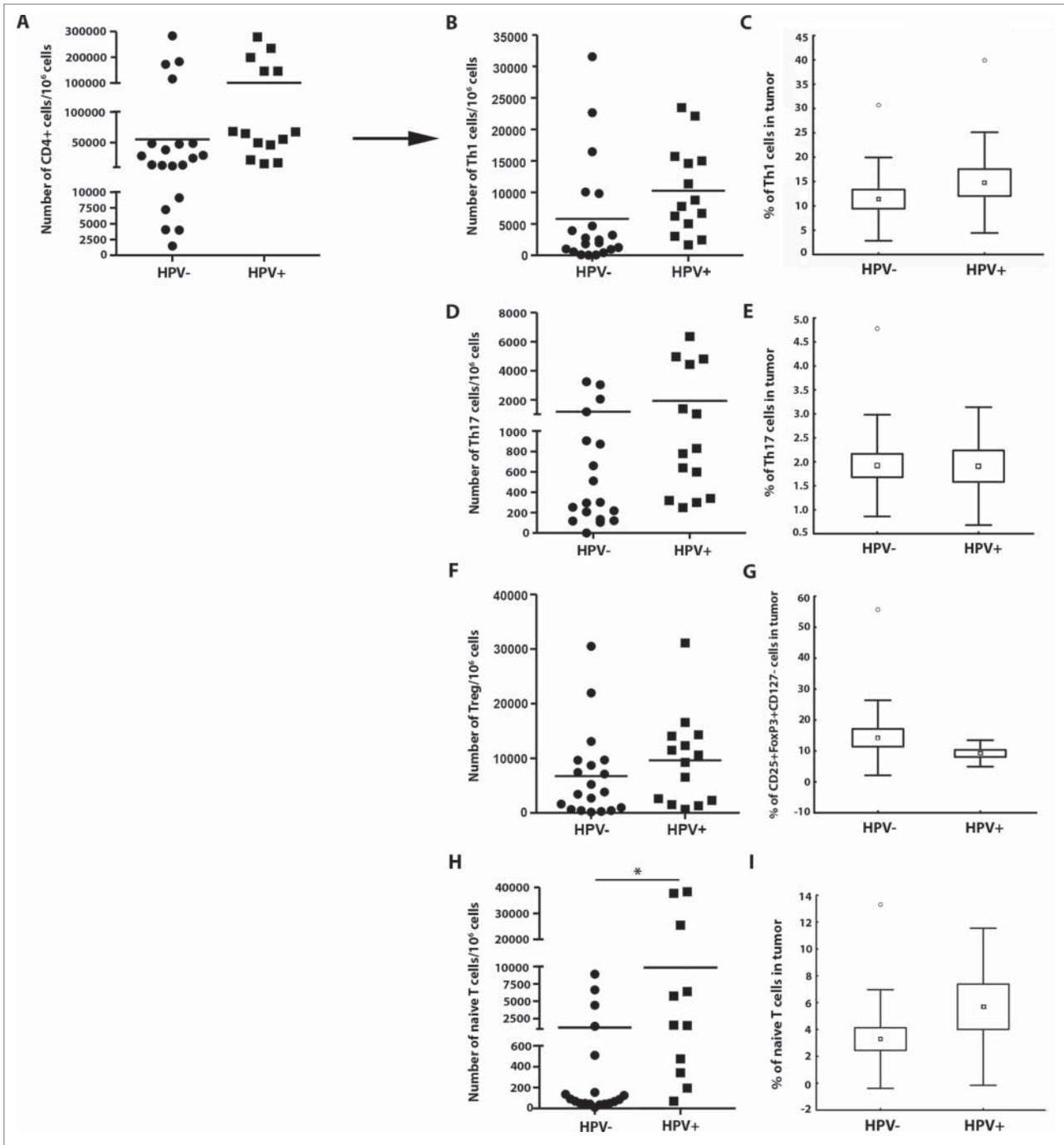


Figure 2. The frequency of CD4⁺ T cell subsets in the tumor tissues ($n = 44$) with regard to human papillomavirus (HPV)-status. To evaluate the subtypes of tumor-infiltrating CD4⁺ T cells, tumor-derived single cell suspensions were stimulated with PMA and ionomycin in the presence of Brefeldin A and analyzed by flow cytometry. **(A, B, D, F, and H)** The plots represent the numbers of Th1 cells, Th17 cells, Tregs or naïve T cells within 10^6 isolated tumor-derived cells; the lines in the box represent the median. **(C, E, G, and I)** The data are expressed as the proportion of Th1 cells, Th17 cells, Tregs and naïve T cells, respectively, among the tumor-infiltrating CD4⁺ cells. The boundaries of the box indicate the SEM, and the lines in the box represent the mean. Whiskers indicate the SD * $p < 0.05$ (General Linear Models; age was added as a covariate).

significantly different (Fig. 3). As expected, we detected high proportions of CD16⁺ HNSCC-infiltrating mDCs, but these proportions did not differ between HPV-negative and HPV-positive tumor tissues ($70.9 \pm 4.5\%$ and $65.5 \pm 2.3\%$, respectively).

HPV-positive tumor tissue-derived single cell suspensions produced higher levels of chemokines, but the cytokine profile was not significantly different

To evaluate whether the increased frequency of leukocytes in HPV-positive tumor samples could be caused by the recruitment

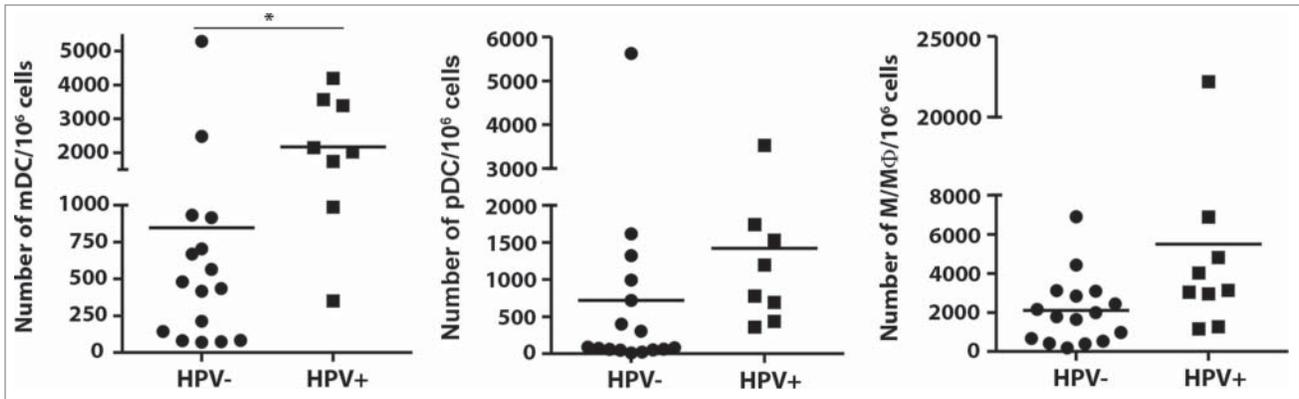


Figure 3. Numbers of dendritic cells (DCs) and monocytes/macrophages within the tumor tissue of head and neck squamous cell carcinoma (HNSCC) patients ($n = 26$) in relation to human papillomavirus (HPV)-status. To assess the pattern of tumor-infiltrating antigen presenting cells, fresh tumor-derived single cell suspensions were analyzed by flow cytometry. (A) The data are expressed as the numbers of myeloid dendritic cells (mDCs, characterized as CD45+LIN-HLA-DR+CD14-CD11c+), plasmacytoid dendritic cells (pDCs, characterized as CD45+LIN-HLA-DR+CD14-CD123+) and monocytes/macrophages (Mo/M Φ , characterized as CD45+LIN-HLA-DR+CD14+) in 10^6 isolated tumor-derived cells. The lines represent the mean value. * $p < 0.05$ (General Linear Models; age was added as a covariate).

of leukocytes to tumor sites rather than their polarization *in situ*, as shown in our previous study,¹⁵ we analyzed the chemokine and cytokine profiles of the tumor-derived cell culture supernatants.

In the supernatants from unstimulated cell cultures, high levels of CXCL9, CXCL10, CXCL12, CCL20, CCL21 and CCL22 were detected. Of the other chemokines measured, only CCL5, CCL17 and CCL19 were at low levels (Fig. 4A). Markedly higher production of CXCL9, CXCL12, CCL17 and CCL21 was detected in the supernatants of HPV-positive patient samples compared to HPV-negative patient samples; however, only the levels of CCL17 and CCL21 showed statistically significant differences ($p = 0.023$ and $p = 0.040$, respectively). As expected, the levels of CXCL12 positively correlated with the lymph node status (average values: N0 = 0 pg/mL and N1-3 = 8331.3 ± 2357.1 pg/mL).

Surprisingly, even in unstimulated cell cultures, we were able to detect most of the cytokines analyzed with the exception of IL-4 and IL-12 (Fig. 4B). HPV-positive cell cultures tended to produce higher levels of IL-2, IL-17, IL-23 and IFN γ and lower levels of IL-1 β , but these differences were not statistically significant. In supernatants of HPV-positive tumors upon PMA and ionomycin stimulation we detected higher levels of IL-10, IL-17, IL-21, TNF α and IFN γ compared to supernatants of HPV-negative tumor samples; however, only the levels of IL-17 showed statistically significant differences ($p = 0.030$) (Fig. 4C).

HPV-positive tumor samples expressed lower levels of *Cox-2* and *Tim-3* mRNA and higher levels of PD-1 mRNA

In addition to the flow cytometry data described above, we analyzed the mRNA expression levels of four regulatory genes, *Cox-2*, *PD-1*, *PD-L1* and *Tim-3*, in the tumor tissue samples and

in metastatic and control lymph nodes of patients with HPV-positive and HPV-negative tumors.

We observed markedly higher expression of all of the analyzed genes except *PD-1* in tumor tissues and metastatic lymph nodes compared to control lymph nodes, regardless of HPV status (Fig. 5A). In comparison to the HPV-positive tumor tissues, we detected a significant increase in the expression of the negative prognostic marker *Cox-2* in the HPV-negative samples ($p = 0.016$). On the contrary, we observed a significantly higher expression of PD-1 in HPV-positive tumor tissues compared to HPV-negative samples ($p = 0.018$). The mRNA expression level of *Tim-3* was similar in both groups of patients (Fig. 5E). However, because we observed markedly higher numbers of CD45⁺ cells in the HPV-positive tumor tissues (Fig. 1A), we decided to normalize the expression levels of PD-1 and *Tim-3* mRNA to the expression level of CD45⁺ mRNA. When the results were normalized to CD45⁺ mRNA expression, we observed higher levels of PD-1 and lower levels of *Tim-3* in HPV-positive tissue samples compared to HPV-negative tumors, but these differences were not statistically significant (Fig. 5F).

In addition to mRNA expression, we analyzed the expression of *PD-1* and *Tim-3* on freshly isolated tumor-derived cells using flow cytometry. Both *PD-1* and *Tim-3* were mainly expressed on CD3⁺ T lymphocytes (Fig. 5C). Whereas *PD-1* was frequently expressed on CD8⁺ and CD4⁺ T cells in all of the tissues analyzed, particularly in the peripheral blood, control lymph nodes, metastatic lymph nodes and tumor tissue, *Tim-3* was mainly expressed on tumor-infiltrating T-lymphocytes (Fig. 5D). As expected, we observed substantially decreased production of IFN γ by *Tim-3*+PD-1+ CD8⁺ T cells compared to both *Tim-3*-PD-1-CD8⁺ T cells and *Tim-3*-PD-1+ CD8⁺ T cells (Fig. 5B), whereas there was no difference in IFN γ production in CD4⁺ T cells with regard to the expression of PD-1 and *Tim-3*.

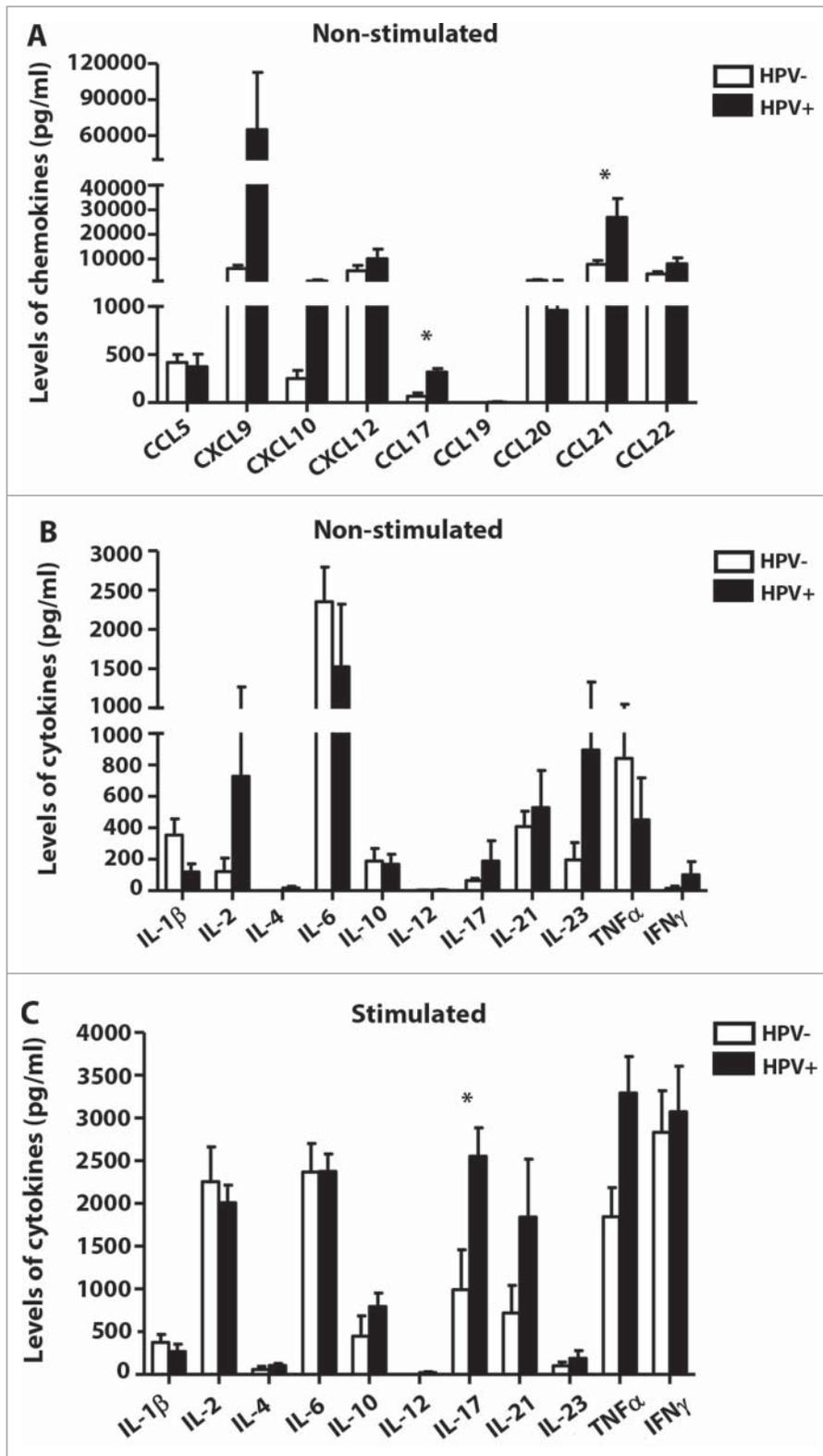


Figure 4. Chemokine and cytokine profiles of tumor-derived single cell suspensions (HPV-negative samples: $n = 7$; HPV-positive samples: $n = 7$). Supernatants of tumor-derived single cell suspension cultures were analyzed using a Quantibody Array Kit (Raybiotech, Norcross, GA). (A) The white columns represent the mean spontaneous chemokine production after 24 h for culture supernatants from HPV-negative patients; the black columns represent the mean production from human papillomavirus (HPV)-positive patients. (B) The white columns represent the mean spontaneous cytokine production after 24 h for culture supernatants from HPV-negative patients; the black columns represent the mean production from HPV-positive patients. (C) The white columns represent the mean cytokine production upon PMA and ionomycin stimulation after 24 h for culture supernatants from HPV-negative patients; the black columns represent the mean production from HPV-positive patients. All of the error bars indicate the SEM * $p < 0.05$ (General Linear Models; age was added as a covariate).

infection. HPV-associated tumors are known to show a different molecular profile than tobacco/alcohol-induced cancers, similar to HPV-positive cervical tumors. Although HPV-positive HNSCC patients are often diagnosed at a late stage of the disease with developed nodal metastases, their prognosis is significantly better compared to HPV-negative cancers.² It has been suggested that the improved response of HPV-positive HNSCC patients to the conventional treatment was related to the immune system⁵; however, a detailed analysis of the pattern of tumor-infiltrating immune cells in HPV-positive compared to HPV-negative HNSCC tissues has not been performed to date.

In this prospective study, we analyzed the immune profile of 54 fresh HNSCC samples with regard to their HPV status. We report that HPV-positive tumors have a markedly different immunologic profile compared to HPV-negative tumors, with high levels of infiltrating CD8⁺ IFN γ ⁺ T lymphocytes, Tc17 lymphocytes, naïve CD4⁺ T lymphocytes and myeloid DCs. HPV-positive tumor tissue-derived cell cultures produced markedly higher levels of chemokines, namely CXCL9, CXCL10,

CXCL12, CCL17 and CCL21, and slightly higher levels of the cytokines IL-2, IL-17, IL-23 and IFN γ . Additionally, HPV-positive samples expressed significantly lower levels of *Cox-2* mRNA and higher levels of PD-1 mRNA.

Discussion

There are two major causative agents of HNSCC, namely tobacco and/or alcohol consumption and a high-risk HPV

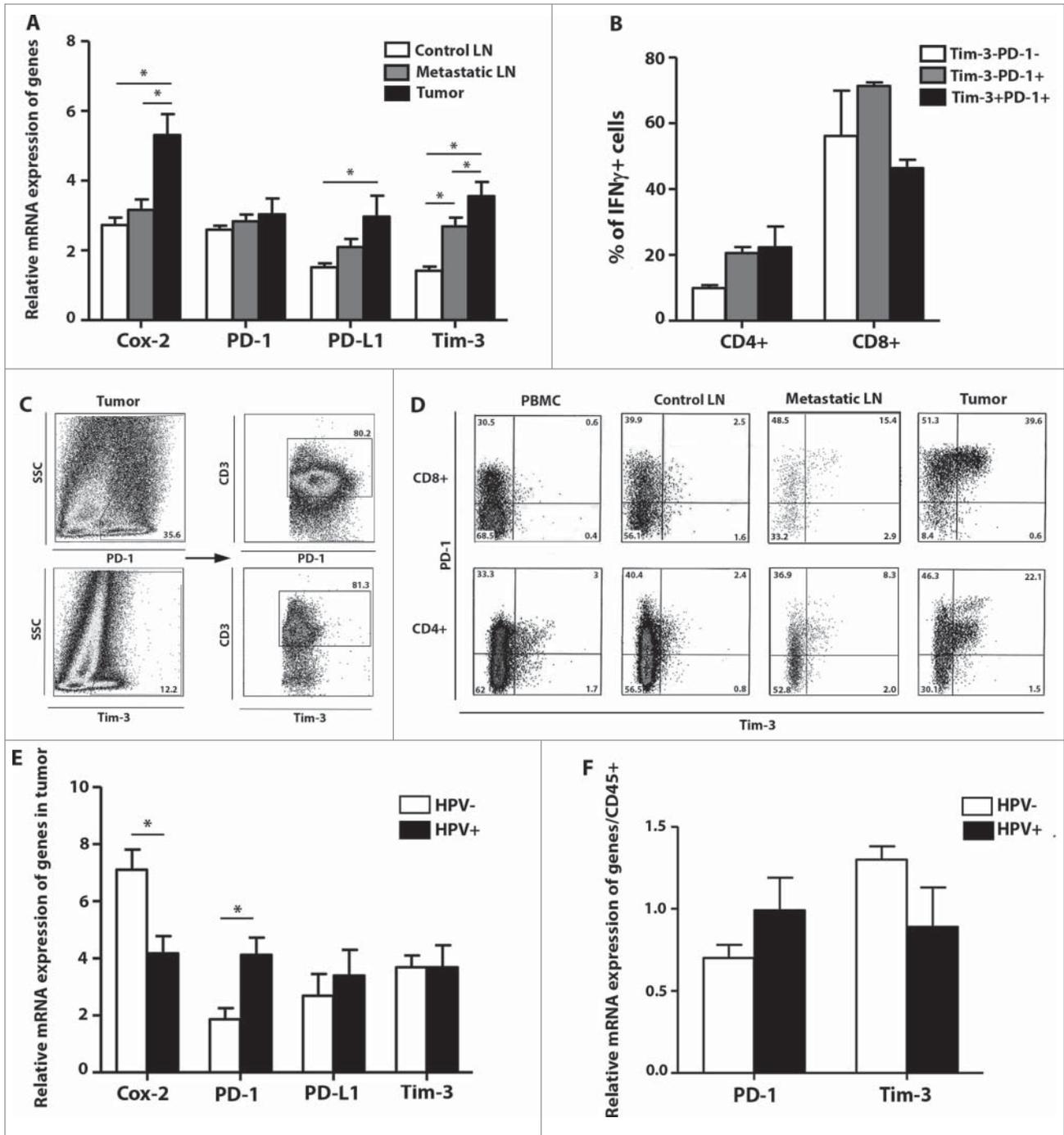


Figure 5. The levels of cyclooxygenase 2 (*Cox-2*), programmed cell death 1 (*PD-1*), programmed cell death-ligand 1 (*PD-L1*) and T cell immunoglobulin and mucin domain containing protein-3 (*Tim-3*) in control and metastatic lymph nodes and tumor tissue from head and neck squamous cell carcinoma (HNSCC) patients. **(A)** The white columns represent the relative mRNA expression of *Cox-2*, *PD-1*, *PD-L1* and *Tim-3* in control lymph nodes (LN; $n = 14$); the gray columns represent the relative mRNA expression of genes in metastatic lymph nodes ($n = 14$); the black columns represent the relative mRNA expression of these genes in tumor tissues ($n = 14$). To assess the expression levels of mRNA, cDNA was synthesized from total tumor and lymph node RNA and amplified by quantitative real time PCR. As an internal reference, β -actin housekeeping gene was used. **(B)** The columns represent the mean proportion of $\text{IFN}\gamma^+$ cells among *Tim-3*-*PD-1*⁻ (white column), *Tim-3*-*PD-1*⁺ (gray column) and *Tim-3*+*PD-1*⁺ (black column) cells in the tumor tissue ($n = 6$). To analyze the $\text{IFN}\gamma$ production, tumor-derived single cell suspensions were stimulated with PMA and ionomycin in the presence of Brefeldin A and analyzed by flow cytometry. **(C)** Dot plots show the expression of CD3^+ on *PD-1*⁺ or *Tim-3*⁺ tumor-infiltrating cells from a representative patient. **(D)** Dot plots are gated on $\text{CD3}^+\text{CD8}^+$ cells (upper line) and $\text{CD3}^+\text{CD4}^+$ cells (lower line) and show the expression of *Tim-3* and *PD-1* in the peripheral blood, control LN, metastatic LN and tumor tissue from a representative patient. The columns represent the mean relative mRNA expression of *Cox-2*, *PD-1*, *PD-L1* and *Tim-3* or *PD-1* and *Tim-3*, respectively, in HPV⁻ (white columns) and HPV⁺ (black columns) tumor samples ($n = 14$) with **(F)** or without **(E)** normalization to the expression level of CD45^+ mRNA. All of the error bars indicate the SEM * $p < 0.05$ (**A**, paired t-test; **B**, Friedman ANOVA; **E and F**, General Linear Models; age was added as a covariate).

It has been convincingly shown that a high density of tumor-infiltrating CD8⁺ T lymphocytes is predictive of a favorable clinical outcome in different types of cancers, including HNSCC.^{7,9,10,14,16} Because most of the studies focusing on the quantification of tumor-infiltrating immune cells in HNSCC were retrospective and based on immunohistochemical data, the detailed phenotype of CD8⁺ T cells had not yet been evaluated. Importantly, recent HNSCC studies^{17,18} suggest that other characteristics of the intratumoral CD8⁺ T lymphocytes, such as their location within the tumor, PD-1 expression, as well as the expression of HLA-class I, SCINDERIN and EPHRIN-A1 in the tumor cells might have a strong impact on the prognostic value of the CD8⁺ T cells. Therefore, not only a quantitative, but also a qualitative analysis of the immune cell infiltrates seems to be crucial for the identification of clinically relevant prognostic markers. Our data show that tumor-infiltrating CD8⁺ T cells in HPV-positive tumor samples not only were more frequent but also had a higher capacity to produce IFN γ and IL-17 upon PMA and ionomycin stimulation compared to HPV-negative tumors, indicating a stronger immune response.

The role of CD4⁺ T cells in anticancer immunity is more controversial. Most of the studies examining tumor-infiltrating CD4⁺ T cells as possible prognostic markers focused on regulatory T cells. The role of Tregs seems to differ according to the type and etiology of the cancer. On one hand, Tregs are known to be the key mediators of tumor immune suppression, and high numbers of tumor-infiltrating Tregs have been related to poor outcome in many types of cancer.¹⁹⁻²² On the other hand, Tregs have been described as a positive prognostic factor in colorectal cancer and lymphomas.^{23,24} In HNSCC, Badoual et al.²⁵ showed that high numbers of tumor-infiltrating Tregs positively correlated with locoregional control. Similarly, other groups studying these cells confirmed the positive correlation between the numbers of intratumoral Tregs and the overall survival.^{26,27} On the contrary, Nasman et al.¹³ demonstrated that a high CD8⁺/FoxP3⁺ T cell ratio correlated with longer disease-free survival. With regard to the significance of peripheral blood Tregs, Boucek et al.²⁸ showed that high levels of Tregs in the peripheral blood of HNSCC patients at the time of primary diagnosis might predict an early recurrence of the disease. In our study, we found higher numbers of CD4⁺ T cells in HPV-positive tumor samples, with slightly higher proportions of Th1 cells and a significantly higher number and frequency of naïve T cells. No statistically significant differences were observed in the numbers and proportions of Tregs and Th17 cells. For understanding the possible relationship between the increased immune infiltration of HPV-positive tumors and HPV-specific immune response, it will be important to analyze the specificity of infiltrating T cells. It is conceivable that, at least some, infiltrating T cells in HPV-positive head and neck tumors will be HPV specific as already suggested by recent studies.²⁹ These results are comparable to what is found in anogenital HPV16 induced lesions.^{30,31} However, the detailed analysis of tumor specificity and viral specificity of T cells present in the head and neck tumors needs to be performed in future studies.

In addition to the increased lymphocytic infiltration, we observed higher numbers of mDCs and slightly higher numbers of pDCs and monocytes/macrophages in HPV-positive tumors. High numbers of CD68⁺ macrophages infiltrating HNSCC were shown to correlate with lymph node metastasis, extracapsular spread and an advanced stage of disease.³² In accordance with these results, we found that the numbers of monocytes/macrophages significantly positively correlated with the lymph node status ($p = 0.048$, data not shown). The prognostic impact of DC subtypes on the tumor microenvironment is less clear. Most likely, the phenotype, not only the number, of DCs might be crucial.¹⁰ It was shown that in HNSCC, monocytes and DCs express the low-affinity Fc γ RIII (CD16).³³ As HNSCC patients are known to have high levels of antigen-antibody immune complexes³⁴ that activate monocytes and DCs via CD16,³⁵ CD16 crosslinking might promote pro-tumor and angiogenic activities.³⁶ Indeed, in our study, we also found a markedly higher number of tumor-infiltrating CD16⁺ mDCs ($70.9 \pm 4.5\%$ for HPV-negative tumors and $65.5 \pm 2.3\%$ for HPV-positive tumors) than CD16-mDCs. However, the prognostic value of these mDC subtypes remains to be elucidated.

In addition to differences in immune cell infiltrates, we also observed a markedly different chemokine profile in HPV-positive and HPV-negative tumor tissue-derived cell cultures. HPV-positive tumor tissue-derived cell cultures produced much higher levels of chemokines, namely CXCL9, CXCL10, CXCL12, CCL17 and CCL21. The chemokine CXCL12 (SDF-1) and its receptor CXCR4 were shown to play a key role in regulating the trafficking of cancer cells to sites of metastases.^{37,38} Indeed, in our study, only cell cultures derived from patients with lymph node metastases produced CXCL12 ($N0 = 0$ pg/mL compared to $N1-3 = 8331.3 \pm 2357.1$ pg/mL). As nodal metastases were detected in 90.5% of the patients with HPV-positive and 66.7% of the patients with HPV-negative tumor samples, the differences in CXCL12 levels most likely reflect this fact. The levels of CCL17 (TARC), a ligand for CCR4, positively correlated with the numbers of tumor-infiltrating Th17, Th1 and CD8⁺ T lymphocytes. In mice, CCL17 was shown to be mainly produced by mature DCs of myeloid origin.³⁹ We did not observe any correlation between CCL17 production and the numbers of mDCs in the tumor tissue; however, we observed a significant positive correlation between the CCL17 levels and the numbers of tumor-infiltrating pDCs ($r = 0.91$; $p < 0.001$). Surprisingly, the levels of CCL21, a ligand for CCR7, positively correlated only with the frequency of Th17 cells but not with the number or frequency of DCs and naïve T cells. Although tumor-derived cells of HPV-positive tumors also expressed higher levels of proinflammatory cytokines, namely IL-2, IL-17, IL-23 and IFN γ , these differences did not reach statistical significance due to high variability in the samples.

Analysis of the mRNA expression levels of four regulatory genes, *Cox-2*, *PD-1*, *PD-L1* and *Tim-3*, also showed differences associated with HPV status. The expression of *Cox-2*, which specifically catalyzes the production of prostaglandins, is undetectable in most healthy tissues but is usually overexpressed in inflammation, premalignant lesions and tumors. Functionally,

Cox-2-derived prostaglandins were shown to promote angiogenesis and induce tumor invasion.^{40,41} Consequently, high *Cox-2* expression was associated with an unfavorable outcome in breast cancer patients.⁴² We detected basal expression of *Cox-2* in control lymph nodes and significant overexpression of *Cox-2* in tumor tissues from our cohort of HNSCC patients (Fig. 5A). The levels of *Cox-2* mRNA negatively correlated with the numbers of tumor-infiltrating Th1 and Th17 lymphocytes ($p = 0.002$ and $p = 0.003$, respectively, data not shown) and positively correlated with the mRNA expression of *Tim-3* ($p = 0.008$, data not shown). Interestingly, the HPV-positive tumor samples expressed significantly lower levels of *Cox-2* mRNA compared to the HPV-negative samples, which is in accordance with a previous report stating an improved outcome in HPV-positive HNSCC patients.²

The surface receptors *PD-1* and *Tim-3* belong to a group of immune checkpoint proteins that decrease T-cell reactivity and were identified, together with CTLA-4 and Lag-3, as benchmarks for exhausted T cells.⁴³ Dysfunctional, exhausted T cells develop after long-term exposure to a high antigen load⁴⁴ and are incapable of exhibiting robust effector responses to further antigen re-challenge.^{45,46} Interestingly, in cancer, dysfunctional T cells expressing *Tim-3* and *PD-1* were only found in tumor tissue but not in the peripheral blood.⁴⁷ Importantly, tumor-infiltrating CD8⁺ Tim-3+ PD-1+ cells exhibited a surface phenotype that is consistent with effector/memory T cells, indicating that exhausted T cells emerge from effector T cells.⁴⁸ Indeed, it was recently shown that exhausted T cells are successfully re-functionalized by blocking checkpoint receptors. Consequently, cancer immunotherapy using T-cell checkpoint inhibitors has become one of the most promising new therapeutic approaches.

In agreement with published data, we only found high proportions of Tim-3+ PD-1+ T cells in tumor tissue but not in the peripheral blood and control lymph nodes of HNSCC patients. On the contrary, Tim-3-PD-1+ T cells were observed in all of the tissues analyzed. Consistent with the flow cytometry data, we detected significantly higher levels of Tim-3 but not *PD-1* mRNA in tumor tissue compared to control lymph nodes. To examine the capacity of Tim-3-PD-1-, Tim-3-PD-1+ and Tim-3+PD-1+ tumor-infiltrating T cells to produce IFN γ , we analyzed the phenotype of these cells after *in vitro* stimulation with PMA and ionomycin using flow cytometry. Although we detected IFN γ -producing cells in all of the subtypes of T cells tested, the proportions of IFN γ ⁺ T cells were markedly lower in CD8⁺PD-1+Tim-3+ T cells compared to CD8⁺PD-1+Tim-3- and CD8⁺PD-1-Tim-3- T cells, as expected. These data indicate that Tim-3 together with PD-1 might be considered a better exhaustion marker in HNSCC-infiltrating CD8⁺ T cells than PD-1 alone.

Badoual et al.¹⁸ showed that high levels of tumor-infiltrating PD-1+ T cells correlated with better survival in HNSCC patients. In agreement with these results, we observed higher *PD-1* expression in HPV-positive tumor samples compared to HPV-negative tumors. In a preclinical model, Badoual et al.¹⁸ showed that partial grafting of the HPV E7-expressing TC-1 epithelial

cell line, which constitutively expresses PD-L1, is dependent on the presence of HPV-specific PD-1+ CD8⁺ T cells. An anti-PD-L1 monoclonal antibody vaccine further enhanced this immune response. Here, we confirmed that *PD-L1* was markedly more expressed in tumor tissue than in the control lymph nodes. As *Tim-3* expression was also tumor tissue specific, these two molecules, instead of *PD-1* alone, might be a very promising target for immunotherapy in HNSCC.

Taken together, our data show that HPV-positive tumor tissues have a distinct immune profile compared to HPV-negative tumors. Substantial infiltrates of immune cells are usually associated with a good prognosis and indicate a strong past antitumor immune response in HPV-positive tumors, which might be reactivated/reprogrammed by not only a targeted immunotherapy approach, but even during the standard therapy. Better understanding of targets of the immune response in HPV-positive vs. HPV-negative tumors and of mechanisms directing the recruitment of immune cells to the tumor will hopefully lead to the design of successful immunotherapeutic strategies.

Materials and Methods

Patients and samples

Blood samples, primary HNSCC specimens metastatic and control lymph nodes were obtained from 54 patients immediately after radical surgery at the Department of Otorhinolaryngology and Head and Neck Surgery, 1st Faculty of Medicine, Charles University and Motol University Hospital in Prague between April 2011 and November 2013. The 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 2.

The tumor tissues, metastatic and control lymph nodes were minced into small pieces with scissors and digested in RPMI 1640 containing 1 mg/mL of Collagenase D and 0.05 mg/mL of DNase I (both from Roche, 11088866001, 11284932001) for 30 min at 37°C under permanent gentle rocking motion. Afterwards, the specimens were passed through a 100- μ m nylon cell strainer (BD Biosciences, 352360) and washed with PBS. The PBMCs were isolated from the peripheral blood by centrifugation on a Ficoll-Paque density gradient (GE Healthcare, 17-1440-03).

Flow cytometry

Single cell suspensions from peripheral blood, tumor tissue and control lymph nodes were used for cell surface labeling using monoclonal antibodies (mAbs) against CD3, CD8⁺, CD11c, CD14, CD16, CD19, CD20, CD45, CD45RA, CD45RO, CD56, CD62L (Exbio), CD4⁺, CD123 (eBioscience, 12-1239-41), HLA-DR (BD Biosciences, 335830) and CCR7 (BioLegend, 353220) for detection of myeloid DCs (mDCs characterized as CD45+LIN-HLA-DR+CD14-CD11c+), plasmacytoid DCs (pDCs; CD45+LIN-HLA-

Table 2. Clinical-pathological characteristics of the patients

Variable	No.	%
Total no. of patients	54	
Age		
Mean	62	
Range	38–78	
Sex		
Male	44	81.5
Female	10	18.5
Tumor grade		
1	8	14.8
2	30	55.6
3	16	29.6
4	0	0
Nodal status		
N0	13	24.1
N1	11	20.4
N2	28	51.9
N3	2	3.7
Stage		
I	3	5.6
II	5	9.3
III	9	16.7
IV	37	68.5
Tumor site		
Tongue	20	37
Tonsil	17	31.5
Larynx	10	18.5
Verbal base	3	5.6
Hypopharynx	2	3.7
Gl. submandibularis	1	1.9
Floor of mouth	1	1.9
HPV status		
HPV–	24	44.4
HPV+	20	37
Non-defined	10	18.5

DR+CD14-CD123+), monocytes/macrophages (M/M ϕ ; CD45+LIN-HLA-DR+CD14+) and naïve T lymphocytes (defined as CD3+CD4+CD45RA+CD45RO-CCR7+CD62L+). The following anti-human mAbs were used for staining regulatory T cells (Tregs): anti-CD3 (Exbio, A7-202-T100), anti-CD4⁺ (eBioscience, 25-0049-42), anti-CD8⁺ (Exbio, 1 T-207-T100), anti-CD25 and anti-CD127 (BioLegend, 302626, 351318) for surface labeling, which was followed by fixation and permeabilization using the FoxP3 staining buffer set (eBioscience) and intracellular staining with anti-FoxP3 (eBioscience, 53-4776-42) and anti-Helios (BioLegend, 137216) antibodies. For analysis of Th17 and Th1 lymphocytes, cell suspensions were stimulated for 4 h with 50 ng/mL of PMA and 1 μ g/mL of ionomycin (Sigma-Aldrich, P8139-1MG, I0634-1MG) in the presence of Brefeldin A (BioLegend, 420601) before intracellular staining. Next, the cells were stained with anti-CD3 (Exbio), anti-CD4⁺ (eBioscience), anti-CD8⁺ (Exbio), anti-PD-1 (BioLegend, 329908) and anti-Tim-3 (BioLegend, 345006) antibodies, fixed, permeabilized and labeled with mAbs against IL-17 (BioLegend, 512310) and IFN γ (BD Biosciences, 554551). The cells were

analyzed on a BD FACSCanto II (BD Biosciences) and evaluated with FlowJo software (TreeStar).

Chemokine and cytokine analysis

For the analysis of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IL-21, IL-23, TNF α , IFN γ , CCL5, CCL17, CCL19, CCL20, CCL21, CCL22, CXCL9, CXCL10, and CXCL12 in culture supernatants harvested from tumor tissue-derived cell suspensions, a Quantibody Array Kit (Raybiotech) was used. Cell suspensions at the concentration of 1 \times 10⁶/mL in the presence or absence of PMA and ionomycin were cultured for 24 h in RPMI 1640 supplemented with 10% FCS, Glutamax and penicillin-streptomycin (Invitrogen, A12860-01, 15140-130). The supernatants were then collected and stored at -80°C until use.

RNA extraction and quantitative real time PCR

Total RNA was extracted from 2 \times 10⁶ tumor-tissue derived cells using an RNA Easy Mini Kit (Qiagen). RNA concentrations were determined with a NanoDrop[®] 2000 c UV-Vis spectrophotometer (Thermo Scientific), and the RNA integrity was assessed using an Experion automated system (BioRad). cDNA was synthesized from total RNA using the M-MLV reverse transcriptase (Invitrogen) and amplified by quantitative real time PCR in the presence of primers and TaqMan[®] probes specific for *Cox-2*, *PD-1*, *PD-L1* and *Tim-3*, as well as the β -actin housekeeping gene, which was used as an internal reference. All primers and probes were commercially synthesized (TIB MOLBIOL). The identity of the qPCR products in each assay was verified by sequencing. The relative expression of the target genes was normalized to the expression of β -actin.

HPV Detection

Tumor samples

The pathologist obtained two side-by-side sections of the tumor from the primary site. One of the paired sections from each anatomical location was labeled, snaps frozen in liquid nitrogen, and stored for future analysis. The other section from each pair was fixed in 10% neutral formalin and paraffin embedded. From each paraffin block, the first and last sections were histologically analyzed to confirm that the sections in between that were designated for the detection of viral nucleic acids and immuno-histochemical (IHC) analysis contained at least 10% of the tumor cells from the entire volume of the sample. Both DNA and RNA, were extracted from the tumor tissue using the Ambion Recover All TM Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Bioscience) as previously reported.⁴⁹ Care was taken to avoid sample cross-contamination.

PCR

All procedures have been described in detail previously.^{49,50} HPV DNA detection was performed by PCR with primers specific for the L1 region (GP5⁺/GP6⁺) as described previously.⁵¹ As an internal control, a 110-bp fragment of the human β -globin gene was amplified.⁵² HPV typing was performed by reverse line

blot hybridization (RLB) with probes specific for 37 types as specified in detail by van den Brule et al.⁵³ From the total RNA, cDNA was prepared by reverse transcription. The absence of contaminating DNA was confirmed by amplification of the internal GAPDH internal control gene.⁵⁴ As a control for the integrity of the mRNA, the β -globin gene was amplified. Amplification of the HPV 16E6*I mRNA oncoprotein was performed with primers that amplify the 86-bp fragment.⁵⁵

Immunohistochemical analysis

IHC examination was performed as described previously.⁴⁹ Briefly, the antibody p16INK4 a (purified mouse anti-human p16, Clone G175-405, BD PharMingen TM, dilution 1:100) was used. The intensity of staining (graded + to +++) and the proportion of cells stained (scored in percentages) were evaluated. For p16 immunostaining, the location of the signal (cytoplasmic and/or nuclear) was also specified. A semi-quantitative evaluation was performed. The samples considered positive for p16 expression exhibited more than 50% positive cells and nuclear and/or cytoplasmic staining.

Statistical analysis

Statistical analyses were performed using Statistica® 10.0 software (StatSoft).

The parametric assumptions of the data were verified using the Kolmogorov–Smirnov test for normality. The homogeneity of the variances was tested by the Levene test. The

differences between HPV-positive and HPV-negative tumor samples were analyzed using a general linear model (GLM), and age was added as a covariate. The differences between control lymph nodes and tumor tissue were analyzed using a paired t-test. The results were considered statistically significant when $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr. Ludek Sojka for the analysis and interpretation of mRNA data.

Funding

This project was supported by research grant NT 11542 from the Grant Agency of the Ministry of Health of the Czech Republic, GAUK 5734/2012 from Charles University, grant 00064203 from University Hospital Motol, Prague, Czech Republic, grants SVV 266513; UNCE 204013 and PRVOUK 27-1, Biocev, the joint project of Academy of Science and Charles University (CZ.1.05/1.1.00/02.0109) and from the European Regional Development Fund.

References

- Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, Ahrlund-Richter S, Marklund L, Romanitan M, Lindquist D et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer* 2009; 125:362-6; PMID:19330833; <http://dx.doi.org/10.1002/ijc.24339>
- Lajer CB, von Buchwald C. The role of human papillomavirus in head and neck cancer. *APMIS* 2010; 118:510-9; PMID:20553531; <http://dx.doi.org/10.1111/j.1600-0463.2010.02624.x>
- Licitra L, Perrone F, Bossi P, Suardi S, Mariani L, Artusi R, Oggionni M, Rossini C, Cantu G, Squadrelli M et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2006; 24:5630-6; PMID:17179101; <http://dx.doi.org/10.1200/JCO.2005.04.6136>
- Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4 A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. *J Clin Oncol* 2009; 27:1992-8; PMID:19289615; <http://dx.doi.org/10.1200/JCO.2008.20.2853>
- Spanos WC, Nowicki P, Lee DW, Hoover A, Hostager B, Gupta A, Anderson ME, Jeh JH. Immune response during therapy with cisplatin or radiation for human papillomavirus-related head and neck cancer. *Arch Otolaryngol Head Neck Surg* 2009; 135:1137-46; PMID:19917928; <http://dx.doi.org/10.1001/archoto.2009.159>
- Kimple RJ, Harari PM, Torres AD, Yang RZ, Soriano BJ, Yu M, Armstrong EA, Blitzer GC, Smith MA, Lorenz LD et al. Development and characterization of HPV-positive and HPV-negative head and neck squamous cell carcinoma tumorgrafts. *Clin Cancer Res* 2013; 19:855-64; PMID:23251001; <http://dx.doi.org/10.1158/1078-0432.CCR-12-2746>
- Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; 313:1960-4; PMID:17008531; <http://dx.doi.org/10.1126/science.1129139>
- Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 1996; 77:1303-10; PMID:8608507; [http://dx.doi.org/10.1002/\(SICI\)1097-0142\(19960401\)77:7%3c1303::AID-CNCR12%3e3.0.CO;2-5](http://dx.doi.org/10.1002/(SICI)1097-0142(19960401)77:7%3c1303::AID-CNCR12%3e3.0.CO;2-5)
- Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnajatic S, Ambrosone C et al. Intraepithelial CD8⁺ tumor-infiltrating lymphocytes and a high CD8⁺ regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 2005; 102:18538-43; PMID:16344461; <http://dx.doi.org/10.1073/pnas.0509182102>
- Kocian P, Sedivcova M, Drgac J, Cerna K, Hoch J, Kodet R, Bartunkova J, Spisek R, Fialova A. Tumor-infiltrating lymphocytes and dendritic cells in human colorectal cancer: their relationship to KRAS mutational status and disease recurrence. *Hum Immunol* 2011; 72:1022-8; PMID:21884745; <http://dx.doi.org/10.1016/j.humimm.2011.07.312>
- Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishiwaki Y, Gemma A et al. Predominant infiltration of macrophages and CD8(+) T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. *Cancer* 2008; 113:1387-95; PMID:18671239; <http://dx.doi.org/10.1002/cncr.23712>
- Wansom D, Light E, Worden F, Prince M, Urba S, Chepeha DB, Cordell K, Eisbruch A, Taylor J, D'Silva N et al. Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. *Arch Otolaryngol Head Neck Surg* 2010; 136:1267-73; PMID:21173378; <http://dx.doi.org/10.1001/archoto.2010.211>
- Nasman A, Romanitan M, Nordfors C, Grun N, Johansson H, Hammarstedt L, Marklund L, Munck-Wikland E, Dalianis T, Ramqvist T. Tumor infiltrating CD8⁺ and Foxp3⁺ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. *PLoS One* 2012; 7:e38711; PMID:22701698; <http://dx.doi.org/10.1371/journal.pone.0038711>
- Nordfors C, Grun N, Tertipis N, Ahrlund-Richter A, Haegglblom L, Sivals L, Du J, Nyberg T, Marklund L, Munck-Wikland E et al. CD8(+) and CD4(+) tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. *Eur J Cancer* 2013; 49:2522-30; PMID:23571147; <http://dx.doi.org/10.1016/j.ejca.2013.03.019>
- Fialova A, Partlova S, Sojka L, Hromadkova H, Brtnicky T, Fucikova J, Kocian P, Rob L, Bartunkova J, Spisek R. Dynamics of T-cell infiltration during the course of ovarian cancer: the gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T-cells. *Int J Cancer* 2013; 132:1070-9; PMID:22865582; <http://dx.doi.org/10.1002/ijc.27759>
- Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. CD8⁺ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998; 58:3491-4; PMID:9721846
- Hasmim M, Badoual C, Vielh P, Drusch F, Marty V, Laplanche A, de Oliveira Diniz M, Roussel H, De Guillebon E, Oudard S et al. Expression of EPHRIN-A1, SCINDERIN and MHC class I molecules in head and neck cancers and relationship with the prognostic value of intratumoral CD8⁺ T cells. *BMC Cancer*

- 2013; 13:592; PMID:24330498; <http://dx.doi.org/10.1186/1471-2407-13-592>
18. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, Levionnois E, Nizard M, Si-Mohamed A, Besnier N et al. PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. *Cancer Res* 2013; 73:128-38; PMID:23135914; <http://dx.doi.org/10.1158/0008-5472.CAN-12-2606>
 19. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; 10:942-9; PMID:15322536; <http://dx.doi.org/10.1038/nm1093>
 20. Miracco C, Mourmouras V, Biagioli M, Rubegni P, Mannucci S, Monciatti I, Cosci E, Tosi P, Luzi P. Utility of tumour-infiltrating CD25+FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. *Oncol Rep* 2007; 18:1115-22; PMID:17914561; <http://dx.doi.org/10.3892/or.18.5.1115>
 21. Bohling SD, Allison KH. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. *Mod Pathol* 2008; 21:1527-32; PMID:18820666; <http://dx.doi.org/10.1038/modpathol.2008.160>
 22. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006; 12:5423-34; PMID:17000676; <http://dx.doi.org/10.1158/1078-0432.CCR-06-0369>
 23. Salama P, Phillips M, Grieff F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009; 27:186-92; PMID:19064967; <http://dx.doi.org/10.1200/JCO.2008.18.7229>
 24. Alvaro T, Lejeune M, Salvado MT, Bosch R, Garcia JF, Jaen J, Banham AH, Roncador G, Montalban C, Piris MA. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. *Clin Cancer Res* 2005; 11:1467-73; PMID:15746048; <http://dx.doi.org/10.1158/1078-0432.CCR-04-1869>
 25. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, Mosseri V, Laccourreye O, Bruneval P, Fridman WH et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 2006; 12:465-72; PMID:16428488; <http://dx.doi.org/10.1158/1078-0432.CCR-05-1886>
 26. Bron L, Jandus C, Andrejevic-Blant S, Speiser DE, Monnier P, Romero P, Rivals JP. Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma. *Int J Cancer* 2013; 132:E85-93; PMID:22815199; <http://dx.doi.org/10.1002/ijc.27728>
 27. Loose D, Signore A, Bonanno E, Vermeersch H, Dierckx R, Deron P, Van de Wiele C. Prognostic value of CD25 expression on lymphocytes and tumor cells in squamous-cell carcinoma of the head and neck. *Cancer Biother Radiopharm* 2008; 23:25-33; PMID:18298326; <http://dx.doi.org/10.1089/cbr.2007.0373>
 28. Boucek J, Mrkván T, Chovanec M, Kuchar M, Betka J, Boucek V, Hladikova M, Betka J, Eckschlager T, Rihova B. Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck. *J Cell Mol Med* 2010; 14:426-33; PMID:19183242; <http://dx.doi.org/10.1111/j.1582-4934.2008.00650.x>
 29. Heusinkveld M, Goedemans R, Briet RJ, Gelderblom H, Nortier JW, Gorter A, Smit VT, Langeveld AP, Jansen JC, van der Burg SH. Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer. *Int J Cancer* 2012; 131:E74-85; PMID:22020783; <http://dx.doi.org/10.1002/ijc.26497>
 30. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ, Melief CJ, Hellebrekers BW, Fleuren GJ, Kenter GG et al. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. *Int J Cancer* 2008; 122:486-94; PMID:17955486; <http://dx.doi.org/10.1002/ijc.23162>
 31. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, Drijfhout JW, Valentijn AR, Wafelman AR, Oostendorp J et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin Cancer Res* 2008; 14:178-87; PMID:18172269; <http://dx.doi.org/10.1158/1078-0432.CCR-07-1880>
 32. Marcus B, Arenberg D, Lee J, Kleer C, Chepeha DB, Schmalbach CE, Islam M, Paul S, Pan Q, Hanash S et al. Prognostic factors in oral cavity and oropharyngeal squamous cell carcinoma. *Cancer* 2004; 101:2779-87; PMID:15546137; <http://dx.doi.org/10.1002/cncr.20701>
 33. Lam-ubol A, Hopkin D, Letuchy EM, Kurago ZB. Squamous carcinoma cells influence monocyte phenotype and suppress lipopolysaccharide-induced TNF-alpha in monocytes. *Inflammation* 2010; 33:207-23; PMID:20084448; <http://dx.doi.org/10.1007/s10753-009-9175-6>
 34. Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. *Curr Opin Immunol* 2007; 19:209-16; PMID:17276050; <http://dx.doi.org/10.1016/j.coi.2007.01.001>
 35. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 2007; 81:584-92; PMID:17135573; <http://dx.doi.org/10.1189/jlb.0806510>
 36. Ramanathapuram LV, Hopkin D, Kurago ZB. Dendritic Cells (DC) facilitate detachment of squamous carcinoma cells (SCC), while SCC promote an immature CD16(+) DC phenotype and control DC migration. *Cancer Microenviron* 2013; 6:41-55; PMID:21809059; <http://dx.doi.org/10.1007/s12307-011-0077-4>
 37. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; 410:50-6; PMID:11242036; <http://dx.doi.org/10.1038/35065016>
 38. Cojoc M, Peitzsch C, Trautmann F, Polishchuk L, Telgeev GD, Dubrovskaya A. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. *Onco Targets Ther* 2013; 6:1347-61; PMID:24124379; <http://dx.doi.org/10.2147/OTT.S36109>
 39. Alferink J, Lieberam I, Reindl W, Behrens A, Weiss S, Huser N, Gerauer K, Ross R, Reske-Kunz AB, Ahmad-Nejad P et al. Compartmentalized production of CCL17 *in vivo*: strong inducibility in peripheral dendritic cells contrasts selective absence from the spleen. *J Exp Med* 2003; 197:585-99; PMID:12615900; <http://dx.doi.org/10.1084/jem.20021859>
 40. Gupta RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* 2001; 1:11-21; PMID:11900248; <http://dx.doi.org/10.1038/35094017>
 41. Howe LR, Subbaramaiah K, Brown AM, Dannenberg AJ. Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. *Endocr Relat Cancer* 2001; 8:97-114; PMID:11397667; <http://dx.doi.org/10.1677/erc.0.0080097>
 42. Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res* 2002; 62:632-5; PMID:11830510
 43. Melero I, Hervas-Stubbs S, Glennie M, Pardoll DM, Chen L. Immunostimulatory monoclonal antibodies for cancer therapy. *Nat Rev Cancer* 2007; 7:95-106; PMID:17251916; <http://dx.doi.org/10.1038/nrc2051>
 44. Mueller SN, Ahmed R. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA* 2009; 106:8623-8; PMID:19433785; <http://dx.doi.org/10.1073/pnas.0808918106>
 45. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 2010; 129:474-81; PMID:20201977; <http://dx.doi.org/10.1111/j.1365-2567.2010.03255.x>
 46. Wherry EJ. T cell exhaustion. *Nat Immunol* 2011; 12:492-9; PMID:21739672; <http://dx.doi.org/10.1038/ni.2035>
 47. Gao X, Zhu Y, Li G, Huang H, Zhang G, Wang F, Sun J, Yang Q, Zhang X, Lu B. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. *PLoS One* 2012; 7:e30676; PMID:22363469; <http://dx.doi.org/10.1371/journal.pone.0030676>
 48. Yang ZZ, Grote DM, Ziesmer SC, Niki T, Hirashima M, Novak AJ, Witzig TE, Ansell SM. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J Clin Invest* 2012; 122:1271-82; PMID:22426209; <http://dx.doi.org/10.1172/JCI59806>
 49. Koslabova E, Hamsikova E, Salakova M, Klozar J, Foltynova E, Salkova E, Rotnaglova E, Ludvikova V, Tachezy R. Markers of HPV infection and survival in patients with head and neck tumors. *Int J Cancer* 2013; 133:1832-9; PMID:23564321; <http://dx.doi.org/10.1002/ijc.28194>
 50. Rotnaglova E, Tachezy R, Salakova M, Prochazka B, Koslabova E, Vesela E, Ludvikova V, Hamsikova E, Klozar J. HPV involvement in tonsillar cancer: prognostic significance and clinically relevant markers. *Int J Cancer* 2011; 129:101-10; PMID:21190188; <http://dx.doi.org/10.1002/ijc.25889>
 51. Klozar J, Kratochvil V, Salakova M, Smahelova J, Vesela E, Hamsikova E, Betka J, Tachezy R. HPV status and regional metastasis in the prognosis of oral and oropharyngeal cancer. *Eur Arch Otorhinolaryngol* 2008; 265 Suppl 1:S75-82; PMID:18094985; <http://dx.doi.org/10.1007/s00405-007-0557-9>
 52. Tachezy R, Klozar J, Salakova M, Smith E, Turek L, Betka J, Kodet R, Hamsikova E. HPV and other risk factors of oral cavity/oropharyngeal cancer in the Czech Republic. *Oral Dis* 2005; 11:181-5; PMID:15888110; <http://dx.doi.org/10.1111/j.1601-0825.2005.01112.x>
 53. van den Brule AJ, Pol R, Franssen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. GP5+6+PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* 2002; 40:779-87; PMID:11880393; <http://dx.doi.org/10.1128/JCM.40.3.779-787.2002>
 54. Gravit PE, Peyton C, Wheeler C, Apple R, Higuchi R, Shah KV. Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays. *J Virol Methods* 2003; 112:23-33; PMID:12951209; [http://dx.doi.org/10.1016/S0166-0934\(03\)00186-1](http://dx.doi.org/10.1016/S0166-0934(03)00186-1)
 55. Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, Meijer CJ, Braakhuis BJ, Leemans CR, Brakenhoff RH. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer* 2007; 121:2465-72; PMID:17680565; <http://dx.doi.org/10.1002/ijc.22980>