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Immunological aspects of head and neck cancer in relation to etiology

Imunologické aspekty maligních nádorů hlavy a krku ve vztahu k etiologii

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Abstrakt

Nádorová imunologie je progresivně se rozvíjející multidisciplinární vědní obor. Výsledky základního výzkumu již navíc byly úspěšně přeneseny do klinické praxe a okamžitý úspěch nových imunoterapeutických přípravků, především blokátorů kontrolních bodů imunitního systému, dále vede k podpoře a růstu výzkumu v této problematice. V případě karcinomů hlavy a krku (HNSCC) byly identifikovány některé složky imunitní odpovědi, jako například $CD8^+$ T lymfocyty, které hrají významnou roli v průběhu onemocnění. HNSCC je navíc rozmanitá skupina nádorů, kdy významná část z nich je indukována vysoce rizikovými kmeny lidského papilomaviru (HPV). HPV-asociované (HPV^+) nádory mají lepší odpověď na standardní léčbu a právě imunitní odpověď byla identifikována jako jeden z hlavních faktorů zodpovědných za tento jev. V naší práci jsme se zaměřili na analýzu fenotypu, funkčních vlastností a prognostické hodnoty tumor-infiltrujících leukocytů u pacientů s HNSCC se zohledněním HPV statutu tumoru. Detekovali jsme $CD8^+$ tumor-infiltrující T lymfocyty reaktivní vůči antigenům HPV16 u většiny HPV^+ orofaryngeálních karcinomů. Tyto T lymfocyty byly schopny po specifické stimulaci HPV-derivovanými peptidy produkovat důležité prozánětlivé cytokiny, IFNγ a TNFα. Produkce cytokinů pak byla umocněna kombinovanou blokádou dvou důležitých kontrolních bodů imunitního systému - receptorů PD-1 a TIM-3. Tyto výsledky tak podporují myšlenku kombinované imunoterapie u těchto nádorů. V naší imunohistochemické studii jsme identifikovali silnou pozitivní prognostickou hodnotu tumor-infiltrujících B lymfocytů (TIL-Bs) u pacientů s karcinomy orofaryngu. Ještě silnějším prognostickým faktorem se pak ukázaly být membránové interakce mezi TIL-Bs a $CD8^+$ T lymfocyty. V naší poslední práci jsme analyzovali funkční kapacitu tumor-infiltrujících plasmacytoidních dendritických buněk (pDCs) u HNSCC, podskupiny dendritických buněk hrající zásadní roli v protivirové imunitě. Zjistili jsme, že narozdíl od nádorů asociovaných s HPV inhibuje cytokinové prostředí HPV-negativních tumorů produkci IFNα plasmacytoidními DC a takto suprimované pDCs navíc indukují proliferaci regulačních T lymfocytů v nádorovém mikroprostředí. Našimi poznatky jsme významně přispěli ke znalostem o imunologické diverzitě nádorů hlavy a krku a identifikovali B lymfocyty jako důležitý biomarker s klinickým potenciálem.

Klíčová slova: dlaždicobuněčný karcinom hlavy a krku, lidský papilomavirus, nádorová imunologie, tumor-infiltrující lymfocyty, plasmacytoidní DC

Abstract

Tumor immunology is a progressively developing, multidisciplinary branch of biology. Results of basic research have already been successfully translated to clinical practice. The immediate success of new immunotherapeutic drugs, especially immune checkpoint inhibitors, has further supported the expansion of basic and clinical research in this field. In the case of head and neck squamous cell carcinoma (HNSCC), some immune system elements, such as $CD8^+$ T cells, were shown to play an important role in the progression of the disease. Importantly, HNSCC is a diverse group of diseases, and a significant number of the tumors are induced by high-risk strains of human papillomavirus (HPV). HPV-associated (HPV^+) tumors respond better to standard therapy, and the immune system was shown to be one of the crucial factors in this phenomenon. We focused on the analysis of phenotype, function, and prognostic value in tumor-infiltrating immune cells in HNSCC patients regarding the HPV status of the tumor. We were able to detect $CD8^+$ tumor-infiltrating T cells reacting to HPV16 antigens in the majority of HPV^+ oropharyngeal cancers. Moreover, activity of these T cells was enhanced after blockade of both PD-1 and TIM-3 immune-checkpoint pathways, supporting a concept of combined immunotherapy. In our immunohistochemical analysis, we identified a strong prognostic role of tumor-infiltrating B lymphocytes (TIL-Bs) in oropharyngeal cancer patients. Furthermore, visible cell-to-cell interactions between TIL-Bs and $CD8^+$ T cells were a superior prognostic marker. Finally, we analyzed the functional capacity of tumor-infiltrating plasmacytoid dendritic cells (pDCs) in HNSCC, a subset of DCs that is essential for antiviral immunity. We showed that, compared with HPV-associated HNSCC, the cytokine milieu of HPV-negative tumors significantly impacted production of IFNα by pDCs and favored induction of regulatory T cells in the tumor microenvironment. We significantly contributed to the knowledge of the HNSCC immunological diversity and described B cells as an important new biomarker with translational potential.

Key words: head and neck squamous cell carcinoma, human papillomavirus, tumor immunology, tumor-infiltrating lymphocytes, plasmacytoid dendritic cells

1. Introduction

Over the last few years, we have had a chance to witness a rise of a new cancer treatment strategy that is based on the activation of the patient's immune system. The success of new immunotherapeutic drugs, especially immune checkpoint inhibitors (ICIs), provided a dose of optimism for tumor immunology, which has become one of the leading topics in cancer research.

1.1 Head and neck cancer overview

Head and neck carcinomas are burdened with high morbidity and mortality; however, they are exciting from an immunological point of view. More than 90% of these tumors are squamous cell carcinomas (HNSCCs) whose progression can differ significantly according to the anatomic locality and etiology. The annual incidence of HNSCC was reported to be 890 000 cases worldwide in 2018 [Bray et al. 2018], with more than 450 000 cases of cancer-associated death [Bray et al. 2018]. In the Czech Republic, the incidence of head and neck cancer in 2017 was around 2 200 cases. Originally, well-established dispositions for the development of HNSCC were heavy tobacco smoking and alcohol intake. The incidence of tobacco-related tumors is decreasing slightly in developed countries, and the rise of oropharyngeal cancer, which we can observe in the Czech Republic, is due to the carcinogenic action of high-risk strains of human papillomavirus (HPV), especially HPV16. Importantly, patients with HPV-positive (HPV^+) tumors show markedly better response to standard treatment protocols, regardless of the chosen modality, and significantly improved overall survival [Ang et al. 2010, Fakhry et al. 2008]. Nevertheless, based on current knowledge, it is evident that HPV^+ patients are not a uniform group of patients and there are individuals with highly aggressive disease and unfavorable prognosis. In 2022, there was still no valid, generally accepted biomarker that could further stratify HPV^+ patients.

1.2 Carcinogenic effect of human papillomaviruses

To date, 14 high-risk strains of HPV that can lead to cancer development in case of persistent infection have been identified. The reasons why a persistent infection will stay productive or become cell-transforming are not yet fully elucidated. The HPV16 subtype is the most prevalent high-risk strain that is responsible for the majority of both cervical and oropharyngeal cancers (OPSCC); it makes HPV one of the most important human carcinogens. Sexual intercourse,

penetrative or non-penetrative, represents a method of HPV transmission. HPV infects the basal layer of the stratified epithelium, and the key elements in sustained viral replication and in acquisition of malignant potential are the oncogenes E6 and E7. The E6 protein can bind directly to p53 and prevent its activity, or it can mobilize E3 ubiquitin ligase E6-associated protein that forms a complex with p53 and causes its degradation [Thomas et al. 1999]. The E7 protein binds to retinoblastoma (RB) tumor-suppressor protein, which is an important regulator of the E2F family of transcription factors [Boyer et al. 1996]. Via a negative feedback mechanism, CDK inhibitor 2A (CDKN2A), which encodes the $p16^{INK4A}$ protein, is upregulated, and as a result we can observe p16 overexpression, one of the essential, but not specific, markers of HPV infection via immunohistochemical staining.

1.3 Immune microenvironment of head and neck cancer

Immuno-oncology research is predominantly focused on the study of local immune response and analysis of the processes in the tumor microenvironment (TME). The local immune response shows a more specific reflection of the immunity setting in case of a malignant tumor compared with peripheral blood, which could be affected by many additional factors.

1.3.1 T lymphocytes

Current immuno-oncologic research and immunotherapeutic approaches (e.g., checkpoint inhibitors, adoptive cellular transfer, CAR T cells) are mainly focused on the most important effector cells of adaptive immunity: T lymphocytes. $CD8^+$ cytotoxic T cells are an especially valuable target because of their cell-killing capacity and their increased abundance in the HNSCC TME has often been reported as a positive prognostic marker [Balermpas et al. 2016, Solomon et al. 2018]. HPV-induced HNSCCs generally have higher levels of tumor-infiltrating T lymphocytes [Chen et al. 2018, Gameiro et al. 2018]. T cells specific for HPV antigens E6 and E7 have previously been detected in OPSCC tissues and represent a promising therapeutic target [Welters et al. 2018, Heusinkveld et al. 2012]. One subtype of $CD4^+$ T cells, T regulatory cells (Tregs), has been highly studied and discussed in cancer immunology. These cells mediate self-tolerance, silencing of over-reactive immune responses, and prevention of autoimmunity. Important mechanism of Treg induction in the TME is expression of indoleamine-2,3-dioxygenase (IDO) by DCs that promote differentiation of naïve $CD4^+$ T cells into Tregs [Munn et al. 2007]. We can assume their protumoral effect in HNSCC; however, there are confronting results showing both the positive and negative prognostic value of Tregs [Sun et al. 2012,

Seminerio et al. 2019]. One of the explanations is that higher Treg counts could reflect higher T-cell infiltration in general and that the ratio of $FoxP3^+$ Tregs to other effector immune cells, especially $CD8^+$ T cells, signifies the true setting of the TME [Partlova et al. 2015, Chen et al. 2018].

1.3.2 Immune-checkpoint molecules

Immune-checkpoint molecules belong to the most important group of factors that regulate the immune response. Their constant activation in the TME leads to diminished antitumor activity of the immune machinery and subsequently to cancer progression. The first-described and the most studied immune checkpoints are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell-death protein 1 (PD-1). Inhibition of these molecules has become the fourth pillar of cancer therapy. Following CTLA-4 and PD-1, other immune checkpoints with therapeutic potential were discovered. Among the newly emerging TIM-3 was reported as an important factor mediating and signifying immunosuppression in the TME of HNSCC [Oweida et al. 2018, Shayan et al. 2017]. Furthermore, recent data have shown that higher TIM-3 expression correlates with worse survival in HNSCC patients [Yang et al. 2021].

1.3.3 B lymphocytes

Until recently, the role of B lymphocytes in cancer has been underestimated. However, in recent years, the significant prognostic role of tumor-infiltrating B cells (TIL-Bs) was reported in a wide range of solid tumors. Importantly, B cells have been shown to be a predictor of response to checkpoint inhibitors [Griss et al. 2019]. Despite these optimistic reports, both the pro- and anti-tumoral effects of B cells were shown in different studies. In HNSCC, increased B-cell infiltration was reported in HPV-induced tumors based on both immunohistochemical and flow cytometry studies [Lechner et al. 2019, Russell et al. 2013]. There are few studies reporting the prognostic importance of TIL-Bs. Distel et al. reported that the survival of a low-risk group of HNSCC patients showed a positive correlation with $CD20^+$ infiltration; in contrast, high-risk patients with low $CD20^+$ counts had significantly better survival [Distel et al. 2009]. Furthermore, Zhou et al. reported a negative correlation between the level of $IL10^+$ Breg infiltration and survival of patients with tongue cancer [Zhou et al. 2016].

1.3.4 Plasmacytoid dendritic cells

The main function of plasmacytoid dendritic cells (pDCs) is the production of IFNα upon TLR7/9 stimulation, although they also possess antigen-presenting properties [Koucky et al.

2019]. pDCs are essential players in antiviral immunity; however, their important role in cancer immunology, both pro- and anti-tumorigenic, has also been reported [Koucky et al. 2019]. A decreased capacity of pDCs to produce IFNα was observed in many tumors, including ovarian and breast cancer, where the levels of pDCs correlated with worse prognosis [Sisirak et al. 2012, Labidi-Galy et al. 2011]. An important pro-tumorigenic effect of pDCs is their capability of inducing Tregs *in situ* through the ICOS/ICOS-L pathway and inducing IDO expression under the influence of the TME [Chen et al. 2008]. As in other malignancies, pDCs were reported to have a diminished capacity to produce IFNα in HNSCC [Hartmann et al. 2003]. Moreover, Han et al. observed a correlation between a high density of tumor-infiltrating pDCs with poor prognosis in patients with oral cancer [Han et al. 2017]. However, the functional capacity of pDCs in HPV^+ HNSCC has not yet been evaluated.

1.4 Head and neck cancer immunotherapy

For several reasons HNSCC is an attractive target for immunotherapy. Many reports show the prevalent immunosuppressive setting of the HNSCC TME that supports tumor growth. Furthermore, a high mutational burden in HNSCC predicts the existence of a wide spectrum of neoantigens. Finally, a significant portion of HNSCCs are associated with oncoviruses (HPV in the case of oropharyngeal cancer and Epstein-Barr virus in the case of nasopharyngeal cancer) that can provide additional antigen-specific stimulation. ICIs are the only approved immunotherapy for HNSCC. Although inhibitors of PD-1 and other checkpoint molecules revolutionized treatment and prognosis in melanoma and lung cancer, response rates in HNSCC are low and its application is reserved for recurrent and/or metastatic HNSCC (R/M HNSCC). Cancer vaccines might be another promising strategy, both in HPV^- and HPV^+ HNSCC, especially in combination with other immune-based agents that can help to overlap immunosuppressive mechanisms. In HPV^+ HNSCC, it is possible to design vaccines based on the well-defined HPV antigens E6 and E7. Clinical trial NCT02426892 combining the HPV vaccine with nivolumab showed a better response rate (33%) than ICI alone in a group of 24 patients with incurable HPV-induced cancer [Massarelli et al. 2019].

2. Aims of the study and hypotheses

Immune system response is one of the major factors influencing HNSCC patient prognosis. Indeed, new immune-targeted drugs already showed their high efficacy in cancer treatment, exceeding classic treatment modalities in some tumor types. Our research focuses on profound analysis of immune infiltration in HNSCC in relation to HPV carcinogenic influence. In our studies we analyzed three tumor-infiltrating leukocyte subgroups: T lymphocytes, B lymphocytes and pDCs. The aim of the studies was to describe phenotype and functional characteristics of these immune cells and find their possible prognostic and therapeutic value in HNSCC patients. Aims and hypotheses of each of the project are stated below.

2.1 Study of HPV-specific tumor-infiltrating T cells in oropharyngeal cancer (Study 1)

- Expression of immune checkpoint molecules and a process of adaptive resistance negatively affects anti-tumor immune response mediated by HPV-specific tumor-infiltrating lymphocytes with exhausted phenotype

2.2 Study of tumor-infiltrating B cells in oropharyngeal cancer (Study 2)

- Tumor-infiltrating B lymphocytes have a prognostic role in OPSCC patients
- Tumor-infiltrating B lymphocytes support antigen-specific $CD8^+$ T cell-mediated anti-tumor immune response in HPV^+ OPSCC

2.3 Study of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer (Study 3)

- Plasmacytoid dendritic cells infiltrating HNSCC have negative prognostic impact on overall survival of the patients
- Tumor microenvironment affects function of tumor-infiltrating plasmacytoid dendritic cells.

3. Materials and methods

3.1 Patient cohorts

All the patient native tumor tissue, control and blood samples were obtained at the Department of Otorhinolaryngology, Head and Neck Surgery, First Medical Faculty, Motol University Hospital, between 2015 – 2020. For the Study 1 we obtained samples of OPSCC from 51 patients, for the Study 2 we collected 21 primary fresh OPSCC tissues and for the Study 3 we collected fresh tumor samples from 76 HNSCC patients. Formalin-fixed paraffin embedded (FFPE) samples for immunohistochemistry staining were obtained from the Department of Pathology and Molecular Medicine, Second Medical Faculty, Motol University Hospital and the Fingerland Department of Pathology, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové.

3.2 Tumor tissue processing

Fresh tumor tissue samples were mechanically minced and enzymatically digested to form single cell suspension. Subsequently, cell counts were determined using Trypan blue staining and Bürker chamber. The same protocol was used for control tonsillar tissues, healthy oral mucosa and peritumoral mucosa. Further, cell suspensions were used for flow cytometric analysis, cytokine/chemokine detection and/or quantitative real-time PCR.

3.3 Flow cytometric analysis

Single cell suspensions derived from tumor tissues were labeled with different panels of fluorescent-marked monoclonal antibodies. Afterwards the cells were analyzed on a BD LSR Fortessa (BD Biosciences). For final evaluation FlowJo software (TreeStar) was used.

3.4 Immunohistochemistry

Sections were deparaffinized and prepared for staining with diluted primary antibodies against desired antigens. After 1h incubation, polymer detection kits were used (ImPress AP, Vector Laboratories; VisUcyte, RD Systems). After 30min incubation, depending on the antibody used, corresponding chromogens were added followed by either of Mayer's hematoxylin (Dako) or Nuclear Fast Red (Vector Laboratories) counterstaining. The images were acquired using an Aperio AT2 scanner (Leica).

3.5 Quantitative real-time PCR

Bulk HNSCC-derived cells suspensions or magnetically isolated tumor-infiltrating $CD8^+$ cells were used for RNA extraction. At least 1 x 10^6 cells per specimen were lysed in RLT buffer. For the quantitative real-time PCR itself, 2ul of synthesized cDNA and selected forward and reversed primers were pipetted to the 96-well plate together with Kappa Fast qPCR Master Mix (Kapabiosystems).

3.6 Cytokine and chemokine detection

For detection of cytokines and chemokines in tumor-derived supernatants, ELISA and Luminex techniques were performed.

3.7 HPV detection

For determination of HPV etiology of the HNSCC we did both p16 protein immunohistochemical staining and PCR for detection of HPV DNA or 16E6*I mRNA. Only the samples positive for 16E6*I mRNA expression in case of Study 1 or samples positive for both HPV DNA and p16 in Study 2 and Study 3 were considered as HPV-induced tumors.

3.9 Statistical analysis and graph editing

Statistical analyses were performed with Statistica® 10.0 software (StatSoft) and Graphpad Prism 8 (GraphPad Software). Statistical significance of all the results was considered when $p < 0.05$.

4. Results

4.1 Study of HPV-specific tumor-infiltrating T cells in oropharyngeal cancer (Study 1)

The study was focused on the detection of HPV-specific tumor-infiltrating T cells in native samples of OPSCC. Further, a functional state of T cells that are able to react to HPV-derived antigens, oncoproteins E6 and E7, was evaluated according to the expression of important immune checkpoints on their surface, PD-1 and TIM-3 specifically. We were able to detect HPV-specific T cells in 73.1 % of HPV^+ OPSCC samples but none in (HPV-negative) HPV^- tumors. Moreover, T cells able to react by IFNγ and TNFα production upon specific stimulation by HPV peptides presented on autologous $CD14^+$ monocytes were mostly $PD\text{-}1^+TIM\text{-}3^-$ and $PD\text{-}1^-TIM\text{-}3^-$ (55.1±11.0% and 29.7±13.6% from all IFNγ producing cells, respectively). TIM-

3, but not PD-1 expression, proved to be the possible crucial marker of T-cell dysfunction even after blockade of TIM-3 and PD-1 pathways. Only the blockade of both of these pathways led to an increase of pro-inflammatory cytokine production by tumor-infiltrating T cells (36% increase in $CD8^+$ T cell IFNγ response). In addition, we observed increase in TIM-3 expression (+ 60% proportional change in TIM3 expression) after selective PD-1 blockade in freshly isolated TILs. This upregulation of TIM-3 was decreased when specific stimulation by HPV peptides was used (+ 10% proportional change in TIM-3 expression) suggesting important role of specific antigen stimulation in the prevention of the development of adaptive resistance to checkpoint blockade.

Author of the thesis performed management of the patients, collection of tissue samples, evaluation of the clinical data of patients and preparation of single cell suspension and flow cytometry analysis of the samples. Author further participated on the final data analysis and preparation of the manuscript.

4.2 Study of tumor-infiltrating B cells in oropharyngeal cancer (Study 2)

The aim of the study was to evaluate a prognostic value and functional capacity of TIL-Bs in OPSCC. Using immunohistochemical staining of $CD20^+$ cells we found a strong positive prognostic value of TIL-Bs no matter the HPV status of the tumor ($p<0.001$). Moreover, for the first time we described small non-organized aggregates of $CD20^+$ B cells/$CD8^+$ T cells with even stronger positive prognostic value compared to TIL-Bs and T cells alone both in case of intratumoral and stromal tissue ($p = 0.001$ and $p = 0.009$, respectively). Further, we wanted to evaluate whether the interactions between $CD20^+$ B cells and $CD8^+$ T cells could be associated with the specific anti-HPV T-cell response. We described a significant positive correlation between $CD20^+$ B cell/$CD8^+$ T cell interaction density in tumor nests and proportions of HPV16 E6/E7-specific $CD8^+$ T cells. The main phenotype of TIL-Bs was a memory subtype (IgD^-CD38^- cell) in samples showing both high (> 0.5% B cells from all measured cells) and low B cell infiltration. To evaluate mechanisms behind B-T cell interactions and their impact on patient survival we evaluated TIL-Bs phenotype. B cells from B^{high} oropharyngeal samples expressed higher levels of co-stimulatory molecules (CD40, CD86, HLA-ABC and HLA-DR), suggesting their role in antigen presentation and T cell stimulation. Moreover, when we depleted $CD19^+$ cells from tumor-derived suspensions, the T cell survival was significantly decreased compared to bulk samples ($15.1 \pm 7.8\%$ vs. $11.0 \pm 4.5\%$ for $CD4^+$ T cells; $p = 0.068$

and 22.4 ± 10.6% vs. 14.4 ± 8.4% for $CD8^+$ T cells; p = 0.068), presuming the essential role of B cells for effector T cell sustainability in the tumor tissue.

Author of the thesis performed management of the patients, collection of tissue samples and evaluation of the clinical data. In the experimental part of the study the author primarily arranged prospective part of the study using native tissue samples and flow cytometry and performed all the functional cell experiments. The author participated on the final data analysis and preparation of the manuscript.

4.3 Study of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer (Study 3)

In this study we analyzed phenotype and functional capacity of plasmacytoid dendritic cells infiltrating HNSCC with respect to HPV status of the tumor, using native tumor tissue. We found similar proportions of pDCs in HPV^+ and HPV^- tumors (0.13±0.23% and 0.11±0.14%, respectively); however, the pDCs in HPV^+ tumors were able to produce higher amounts of IFNα in response to TLR7 stimulation by CpG based on mean fluorescent intenstity (4137±3346 vs. 7879±4272 for HPV^- and HPV^+ samples, respectively) albeit the proportions of IFNα-producing cells were similar (3.2±5.4 vs. 3.0±3.1%). We confirmed the result by comparison of IFNα levels in tumor-derived cell culture supernatants after stimulation by CpG. Again, the levels of IFNα were significantly higher in HPV^+ samples (240.7±380.8 pg/ml for HPV^- cultures vs. 971.8±1461 pg/ml for HPV^+ cultures). Although we did not find any correlation of pDC counts to clinical and histo-pathological characteristics of the patients, when we compared survival of the HPV^+ patients according to the IFNα levels in cell culture supernatants, we found lower amounts of IFNα in samples from patients that deceased. Further, we identified different composition of cytokine environment as the main factor affecting pDC functional capacity. After 24h of culture of healthy donor blood-derived pDCs, IFNα levels were significantly lower in samples incubated with HPV^- tumor-derived cell culture supernatants compared to HPV^+ samples, suggesting important role of cytokine milieu in pDC activity. After identification of the cytokines with significantly different levels in HPV^+ and HPV^- supernatants, we tested the effect of IL-10, IL-6 and TNFα on healthy donor blood-derived pDCs. High levels of IL-10 and TNFα in culture supernatants derived from HPV^- tumor cell suspensions were shown to suppress the IFNα production in pDCs. We confirmed the observed inhibitory effect of IL10, TNFα and IL-6 by co-cultivation of healthy donor blood-derived pDCs with HPV^- tumor-derived supernatants or IL6, IL-10 and TNFα with addition of corresponding neutralizing antibodies. The neutralization of TNFα restored pDC ability to

produce IFNα to 101 ± 5.9% in pDC cultures with recombinant cytokines and to 82.8 ± 13.5% in pDC cultures with HPV^- cell culture supernatants. Neutralization of IL-10 restored the IFNα production to 44.2 ± 9.4 and 47.3 ± 19.9%, respectively. Using immunohistochemistry staining of HNSCC HPV^+ and HPV^- FFPE tissue sections we showed that tumor cells seemed to be a very important source of the mentioned cytokines. Moreover, proportions of pDCs in the tumor tissue of HPV^-, but not HPV^+ tumors, correlated to Tregs ($CD4^+CD127^{-/lo}CD25^{hi}FoxP3^+$ cells) proportions. Indeed, pDCs influenced by HPV^- HNSCC supernatants generated significantly higher numbers of Tregs compared to control pDCs and pDCs cultured with HPV^+ HNSCC supernatants.

Author of the thesis performed management of the patients, collection of tissue samples, evaluation of the clinical data and great majority of laboratory experiments. Author significantly participated on data analysis and wrote the publication.

5. Discussion

HNSCC is one of the major groups of malignant tumors affecting the global population. Current treatment protocols produce promising results in cases of limited disease; however, many patients are diagnosed in advanced stages with significantly decreased chances for complete recovery. Recently, modern immunotherapy was introduced for use in recurrent or metastatic HNSCC, with a rather low response rate of 13–18% (Seiwert, 2016, Ferris, 2016). Studies on the differences in composition of the TME and functional properties of immune elements between HPV^- and HPV^+ HNSCCs may elucidate the key mechanisms of the anti-tumor immune response in HNSCC and improve treatment results.

In Study 1, we showed that in 73.1% of HPV^+ OPSCC samples, $CD8^+$ T cells were able to react against HPV16 E6 and E7 by producing of IFN. The results confirmed the findings of Heusinkveld et al. using larger sample cohort [Heusinkveld et al. 2012] . Similarly, Welters et al. detected HPV16-specific $CD4^+$ and $CD8^+$ T cells mostly producing IFNγ and IL-17 in 64% of HPV-driven tumors [Welters et al. 2018]. Moreover, the authors reported a positive association between their abundance and lower stage and better overall patient survival, signifying the important role of the activated local immune response and potential for its artificial support [Welters et al. 2018]. We found that, upon stimulation with HPV oncoproteins, the IFNγ-producing T cells were mostly PD-1^+ TIM-3^- and PD-1^- $TIM3^-$, suggesting that TIM-

3 is a better marker of T-cell exhaustion than PD-1 in OPSCC. Badoual et al. showed that an increased density of intratumoral T cells expressing PD-1 correlated with better prognosis, and PD-1 expression alone instead reflected a state of activation [Badoual et al. 2013]. Kim et al. also found prolonged OS in HNSCC patients with higher expression of PD-1 regardless of HPV positivity [Kim et al. 2016]. In line with our results, Fourcade et al. reported that TIM-3 expression on $PD1^+$ T cells marked the most dysfunctional subset in melanoma patients [Fourcade et al. 2010]. Shayan et al. reported a similar finding in an HPV^+ HNSCC mouse model [Shayan et al. 2017]. Therefore, we tested dynamic changes in TIM-3 expression and IFNγ production in TILs following anti-PD-1 treatment with nivolumab. Consistent with previous reports, we observed a significant increase in the proportion of IFNγ-producing T cells following blockade of both PD-1 and TIM-3 pathways compared with single-agent treatment. Importantly, the phenomenon of TIM-3 overexpression on TILs was markedly lower in cells that underwent stimulation by the HPV16 oncoproteins E6 and E7. The results strongly suggest that specific stimulation of effector T cells using vaccination may circumvent adaptive TIM-3 overexpression and be a promising complementary treatment for combined immune checkpoint blockade.

In Study 2, we thoroughly studied intratumoral B lymphocytes in HPV^+ and HPV^- oropharyngeal cancer. We reported a significant correlation between the abundance of E6/E7 HPV-specific T cells and the cell-to-cell interactions of B and T lymphocytes. Localization of B cells and their potential interactions with other leukocytes mainly occur in tertiary lymphoid structures (TLS) in the tumor stroma. Ruffin et al. found that higher TLS frequency was observed in HPV^+ [Ruffin et al. 2021]. The authors also observed a strong positive correlation between the density of TLS with germinal centers and the OS of HNSCC patients, regardless of HPV status. In contrast, we observed typical TLS with germinal centers only in 29.8% and 25% of HPV^+ and HPV^- OPSCC samples, respectively, with no significant difference between the groups. However, we described small, well-defined cell aggregates of B cells and $CD8^+$ T cells with clear membrane interactions. We confirmed B/T-cell interactions, both in the tumor nests and tumor stroma, as a positive prognostic factor using univariate and multivariate survival analysis. The significance of this biomarker was even stronger than HPV status. Importantly, the presence of high densities of T/B-cell interactions were statistically stronger prognostic factors than T- and B-cell counts alone. Publications on the association of the abundance of TIL-Bs and patient prognosis are contradictory. The differences might have been caused by insufficient phenotyping of the B-cell population, which could have revealed

potentially different functionality of the B-cell subsets present in the TME. We showed that, based on the expression of CD38 and IgD, TIL-Bs in HNSCC were mainly $CD38^{+}IgD^{-}$ memory cells. Importantly, in B^{hi} (>0.5% of total cell count) tumor samples, TIL-Bs expressed higher levels of CD40, CD86, HLA-ABC, and HLA-DR, indicating an activated phenotype. The activated phenotype, co-localization of T and B cells, and the correlation of such interactions with the abundance of E6/E7 HPV-specific T cells suggest that B cells may serve as APCs that could support T cell-mediated immunity and prolong the survival of T cells in the tumor tissue. CD40, expressed in high levels on B cells from B^{hi} samples, could interact with CD40L on T cells, resulting in "licensing" of B cells to become APCs. The activated APCs upregulate other co-stimulatory molecules that provide promoting signals to other T-cell receptors, such as CD27 and OX40, and enhance T cell-mediated antitumoral activity. The CD27-CD70 pathway has often been reported to positively affect T-cell survival and clonal expansion both in an IL-2-dependent and independent manner [Carr et al. 2006, Peperzak et al. 2010]. We observed that depletion of B cells from tumor-derived cell cultures resulted in significantly decreased survival of both $CD4^{+}$ and $CD8^{+}$ T cells. Nevertheless, whether prolonged T-cell survival is caused mainly by direct T/B-cell interactions or by secreted B-cell products requires further evaluation.

Although myeloid DCs are responsible for the initiation of antigen-specific T-cell activity, we hypothesized that additional antigenic stimuli that might be presented by B cells to effector T cells could play a crucial role in sustaining the antitumor immune response. A smaller subgroup of DCs, pDCs, was also shown to support adaptive immunity both via production of type I IFN and through direct cell-to-cell contact. In Study 3, we found similar proportions of pDCs in HPV^{+} and HPV^{-} HNSCC and no correlations between pDC density and the clinical and pathological characteristics of the patients. The finding is in contrast with those of Han et al., who reported a negative prognostic role of pDCs in a cohort of oral squamous cell carcinoma patients [Han et al. 2017]. Nevertheless, our cohort consisted of HNSCC of various sublocations and a more specific identification of pDCs with BDCA-2 compared with CD123 staining only. Importantly, we observed a diminished capability of pDCs to produce IFNα upon TLR stimulation by imiquimod and CpG in HPV^{-} tumors only. Interestingly, the proportions of pDCs that were able to produce IFNα were similar between HPV^{+} samples, HPV^{-} samples, and control tissue; however, the amount of IFNα differed significantly based on mean fluorescence intensity values. We confirmed the result of decreased IFNα production in HPV^{-} tumors by measuring IFNα levels in supernatants of CpG-stimulated tumor-derived single-cell suspensions. Reduced IFNα production by pDCs derived from HNSCC compared with blood-

derived pDCs was also observed by Hartmann et al. [Hartmann et al. 2003]; however, healthy tonsillar tissue and peritumoral mucosa were used as controls in our study following the finding that sample processing markedly influenced the capacity of pDCs to produce IFNα (not published). To explain the difference in IFNα production by pDCs between HPV^+ and HPV^- samples, we evaluated the expression of functional markers associated with IFNα secretion. We did not find any difference in expression of TLR7, TL9, CD28, TIM-3, TRAIL, IDO, or granzyme B on a protein level. We further tested the influence of the tumor cytokine microenvironment on pDC functional capacity. In accordance with our aforementioned results, only HPV^- tumor-derived culture supernatants significantly inhibited IFNα production in blood-derived pDCs from healthy donors. Similar results were previously reported in relation to cervical, breast, ovarian cancer, and HNSCC, where IL-6, IL-10, HMGB-1, TNFα and TNFβ were identified as the main factors directly impairing pDC function [Labidi-Galy et al. 2011, Sisirak et al. 2013, Demoulin et al. 2015, Bruchhage et al. 2018]. In our study, we selected cytokines with the highest difference in levels between the most suppressive HPV^- supernatants and non-suppressive HPV^+ supernatants, and we tested their direct effect on pDCs. IL-6, IL-10, and TNFα were present at significantly higher levels in HPV^- supernatants. When the recombinant forms of the selected cytokines were applied to blood-derived pDCs from healthy donors at the concentrations detected in tumor supernatants, IL-10 and TNFα caused a significant decrease in IFNα secretion. Further, our immunohistochemical analysis showed tumor cells as the main source of the cytokines. The second most discussed mechanism of pDC-mediated immunosuppression is peripheral induction of Tregs, mainly through the ICOS/ICOS-L pathway [Conrad et al. 2012, Aspord et al. 2013]. Moreover, Tregs produce IL-10 that could intensify pDCs impairment, as demonstrated previously, and could potentially contribute to a vicious cycle of immunosuppression in the TME. In the current study, we did not observe any significant differences in Treg counts between HPV^+ and HPV^- tumors. However, we found a significant positive correlation between pDC proportion and Tregs in HPV^- but not in HPV^+ HNSCC. Interestingly, blood-derived pDCs from healthy donors exposed to HPV^- tumor-derived supernatants were able to induce Treg expansion, whereas HPV^+ tumor-derived supernatants did not have this effect.

6. Conclusions

Following the approval of ICIs, immunotherapy has become a fourth pillar of cancer treatment. HNSCCs are a large group of tumors contributing significantly to worldwide cancer morbidity and mortality. Fortunately, based on current knowledge, HNSCC is a promising target for various immunotherapeutic approaches. Despite the success of ICIs, there remains a significant group of non-responders that require a different therapeutic approach. Complex study of the TME and insight into the processes that contribute to tumor-mediated evasion of the immune system has one of the highest priorities in the cancer-related research. Results presented in the thesis represent a small but significant piece of the puzzle and deepen the knowledge of the HNSCC immune microenvironment. We described the functional capacity of HPV-specific tumor-infiltrating T cells in HPV-induced OPSCC in relation to the expression of immune checkpoint molecules. Our findings support the combined use of ICIs in oropharyngeal cancer with therapeutic HPV vaccines. Indeed, combined immunotherapy is the main theme of current clinical trials. Although most of the focus in basic and clinical research is devoted to T cells, we evaluated the prognostic role and functional properties of other, less-studied TIL populations. We identified the positive prognostic role of TIL-Bs in oropharyngeal carcinoma and, more importantly, we were the first team to describe B/T-cell interactions that showed even stronger prognostic impact. We showed that B cells might affect T-cell survival and potentially their antitumor function. The results identified B cells as a valuable biomarker for patient stratification and as a promising target for future immunotherapy. Finally, we studied the differences of pDCs infiltrating HPV-related and nonrelated HNSCCs. We were the first team to publish the functional characteristics of pDCs in HNSCC, based on the HPV status of the disease. We showed that pDCs significantly supported immunosuppression in the TME of HPV^- HNSCC and had preserved functional capacity in HPV^+ tumors. In conclusion, the thesis partially clarifies the important relationships and mechanisms in the TME of HNSCC; at the same time, the results emphasize the diversity and complexity of the immunological network. Further research on the cancer–immune system relationship is needed; nevertheless, the current data imply that widely effective and long-lasting cancer immunotherapy must target more than one of the immune system components by utilizing multiple approaches in combination.

7. References

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8. List of publications

1. publications *in extenso*, in relation to the topic of the thesis

a) With IF

Koucky, V., K. Hladikova, E. Taborska, J. Boucek, M. Grega, et al., The cytokine milieu compromises functional capacity of tumor-infiltrating plasmacytoid dendritic cells in HPV-negative but not in HPV-positive HNSCC. Cancer Immunol Immunother, 2021. 70(9): p. 2545-2557.

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IF 5.738

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IF 5.972

b) without IF

Koucký, V., J. Bouček, J. Plzák, A. Fialová.Význam imunitního infiltrátu v nádorovém mikroprostředí karcinomů hlavy a krku. Onkologie (Solen), 2021: 15(2): 67-72

2. publications *in extenso*, with no relation to the topic of the thesis

a) with IF

Koucky, V., D. Kalfert, D. Kodetova Novakova and J. Plzak, Low-grade fibromyxoid sarcoma of the maxillary sinus. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2021. 165(3): p. 342-345.

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IF 3.236

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IF 0.411

b) without IF

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