Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma

Anna Maria Wirsing[1], Ida Korsnes Ervik[1], Marit Seppola[1], Lars Uhlin-Hansen[1,2], Sonja Eriksson Steigen[1,2] and Elin Hadler-Olsen[1,2]

[1]Department of Medical Biology, Faculty of Health Sciences, University of Tromsø – The Arctic University of Norway, 9037 Tromsø, Norway

[2]Department of Clinical Pathology, University Hospital of North Norway, 9038 Tromsø, Norway

Running title: The immune microenvironment in oral cancer

Corresponding author:

Elin Hadler-Olsen

Department of Medical Biology, Faculty of Health Sciences,

University of Tromsø – The Arctic University of Norway, 9037 Tromsø, Norway

Telephone: +47 77620912

E-mail: elin.hadler-olsen@uit.no

Abstract

Oral squamous cell carcinomas (OSCC) are associated with a poor prognosis, which may be partly due to functional impairment of the immune response. Lymphocyte recruitment to the tumor site is facilitated by high-endothelial venules (HEV), whereas expression of programmed-death ligand 1 (PD-L1) can impair T cell function. Thus, we hypothesize that these factors are important in shaping the immune response in OSCC. In the present study, we characterized the immune infiltrate in formalin-fixed, paraffin-embedded tumor samples from 75 OSCC patients. We used immunohistochemistry to determine the distribution of immune cell subsets, HEV and PD-L1, as well as quantitative real-time polymerase chain reaction to assess the expression of inflammatory cytokines and chemokines associated with lymphocyte trafficking. Finally, we calculated correlations between the presence of immune cell subsets, the gene expression patterns, HEV, PD-L1 and the clinicopathological parameters including patient survival. The presence of HEV correlated with increased number of CD3+ T cells and CD20+ B cells, higher levels of the chemokines CXCL12 and CCL21, and lower levels of CCL20, irrespective of the tumors' T-stage. In univariate analysis, high levels of CD20+ B cells and CD68+ macrophages, positive HEV-status, and low T- and N-stages predicted longer patient survival. However, only the presence of HEV and a low T-stage were independent positive prognosticators. This indicates that HEV are important mediators and a convenient marker of an antitumor immune response in OSCC. Our findings support HEV as a potential immunomodulatory target in OSCC. PD-L1 staining in tumor cells correlated with lower T-stage, increased infiltration of CD4+ cells and higher expression of several inflammation-related cytokines. Thus, OSCC tumors rich in CD4+ cells may preferentially respond to PD-1/PD-L1 blockade therapy.

The majority of oral cancers are squamous cell carcinomas (SCC), and their incidence is increasing in many Western countries (1). Oral (O) SCC are regarded as aggressive cancers, but tumors of the same stage show substantial heterogeneity in progression and response to treatment. Unlike many other cancers, such as breast-, lung- and colorectal cancer, there are no reliable biomarkers that predict the aggressiveness or treatment response of individual OSCC (2-4).

The immune system is an important and complex regulator of tumor evolution. Immune suppression promotes tumorigenesis in murine models (5); and likewise, a number of cancers occur with increased frequency and aggressiveness in immunocompromised patients (6). Mutated cell express altered proteins or tumor-specific antigens that usually evoke an immune response (7). However, the intensity and composition of the tumor-associated inflammation varies between patients and may affect prognosis, as demonstrated in melanoma, breast and ovarian cancer (8-10). For some cancers, including melanoma and head and neck cancer, an inflamed and a non-inflamed phenotype have been described (11, 12). Inflamed tumors are characterized by marked T cell infiltration and high expression of chemokines that can recruit effector T cells, and are generally associated with a favorable prognosis (13). Non-inflamed tumors, on the other hand may be linked to defective recruitment of immune cells to the tumor microenvironment, and predict a poor outcome. Lymphocyte extravasation can be regulated by high-endothelial venules (HEV), which are blood vessels expressing peripheral node addressin (PNAd) that binds L-selectin on circulating naïve lymphocytes (14). HEV are present in secondary lymphoid organs, and develop regularly in chronically inflamed tissue (15). We have previously reported that the presence of HEV is associated with improved survival in OSCC (16), which is in line with results from studies on melanoma and breast cancer (17, 18). However, HEV are plastic, and their remodeling into regular venules may impair lymphocyte recruitment and precede sentinel lymph node metastasis (19, 20).

Although OSCC are mostly of the inflamed phenotype suggesting effective immune surveillance (13), these cancers are generally associated with poor survival. This could be explained by immune editing mechanisms that impair infiltrating lymphocytes and allow tumor progression despite the apparent

immune response (21). Programmed-death ligand 1 (PD-L1) expressing cells can induce immune suppression by binding the PD 1 receptor (PD-1) on activated T cells and downregulate effector T cell functions (22-24). Reversing immunosuppression through blockade of the PD-1/PD-L1 pathway has shown clinical efficacy in various cancers, including OSCC (25-27). However, studies on the prevalence and prognostic role of PD-L1 expression in OSCC are limited, and conflicting results are reported (22, 28, 29). To date, it remains unclear why only a subset of patients respond to PD1/PD-L1 blockade treatment.

In a cohort of 75 OSCC patients, we recently demonstrated that advanced stage tumors with HEV present were associated with a more pronounced inflammatory response and longer patient survival than their HEV-negative counterpart (16). In the present study on the same patient cohort, we explored the hypothesis that HEV are master regulators of tumor immunity in OSCC by characterizing the immune cell infiltration and cytokine expression in relation to the HEV status. Furthermore, we analyzed the association between the tumors' PD-L1 expression and the immune response, and examined a potential relationship between HEV and PD-L1 statuses.

Materials and Methods

Patients and material

In this retrospective study, we included 75 patients diagnosed with primary OSCC in the period 1986-2002 from the archives of the Department of Clinical Pathology, University Hospital of North Norway (UNN). Follow-ups were continued until January 1st, 2012. Patients were included in the study if both clinical data and formalin-fixed, paraffin-embedded (FFPE) tumor specimens (biopsy or surgical resection) were available. Exclusion criteria were prior radiotherapy to the head and neck area as well as previous oral or oropharyngeal cancer. Sixty-eight percent of the patients received surgery in combination with radiotherapy, 15% concomitant radiotherapy and chemotherapy, 11% surgery in combination with local neck-dissection, five percent none or palliative therapy, and one percent were unknown. We

retrieved clinical and histopathologic information from the patients' hospital files, pathology reports and the Statistics of Norway, Cause of Death Registry, and have earlier published these data in studies analyzing the same patient cohort (16, 30). Pathological tumor staging was determined according to the most recent TNM classification at the time of diagnosis (31-33). There were no changes in the classification of cancers of the lip and oral cavity between the different editions used. We conducted the study in line with the REMARK guidelines for tumor marker prognostic studies (34), and following approval from the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007).

Immunohistochemistry

We used four-micrometer-thick sections of FFPE tumor specimens from OSCC patients for immunohistochemical analyses, and performed both automated and manual staining methods. Specifications of the primary antibodies as well as their incubation conditions are listed in Table 1. We used CD3 as a pan T-cell marker. Various subtypes of T helper and T regulatory (Treg) cells express CD4 strongly, whereas monocytes, macrophages and dendritic cells may show weak expression. Cytotoxic T cells, and to less extent, natural killer cells and subsets of dendritic cells express CD8. CD20 is a pan B cell marker, and CD68 is a pan macrophage marker, but it may also stain subsets of lymphocytes, fibroblasts and endothelial cells. Mature dendritic cells and, to some extent, pneumocytes express DC-lamp/CD208.

Automated staining. Staining for CD3, CD4. CD8, CD20, CD68 and PD-L1 was done in the automated slide stainer Ventana Benchmark, XT (Ventana, Tucson, AZ, USA) at the Diagnostic Clinic – Clinical Pathology, UNN, accredited according to the ISO/IEC 15189 standard for the respective stainings, as previously published (30). Briefly, for antigen retrieval, deparaffinized and blocked sections were heat-treated in 0.01 M sodium citrate buffer at pH 6.0. A cocktail of HRP labelled goat anti-mouse IgG/IgM and mouse anti-rabbit secondary antibodies (Ventana UltraView Universal DAB Detection Kit, #760-500, Roche) were used for visualization with diaminobenzidine. As these procedures are automatized, the

manufacturer controls the incubation time of secondary antibodies. In every run, one slide with known positivity for the different antibodies (tonsil or lymph node) was added as control. Automated staining runs for PD-L1 included in addition a negative control slide with rabbit monoclonal negative control Ig (#790-4795, Ventana) for each patient. DC-LAMP staining was performed in the Ventana Discovery ULTRA autostainer (Ventana). After dewaxing, cell conditioning-1 solution (CC1) (#950-124, Ventana) was applied for antigen retrieval for 32 minutes at 95°C. Endogenous peroxidase was blocked by discovery inhibitor CM (#760-4840, Ventana). After incubation with the DC-LAMP primary antibody, the pre-diluted secondary antibody (OmniMap anti-mouse HRP; #760-4310, Ventana) was loaded for 20 minutes followed by HRP amplification and visualization by ChromoMap DAB (#760-159; Ventana). Counterstaining was performed using the Hematoxylin II counterstain reagent (#790-2208, Ventana). Metastatic lymph node TMA slides of lung cancer were used as positive control. Giemsa staining was performed using BenchMark Special Stains, an automated slide stainer from Ventana. In every run, one slide with known positivity for the stain was added as control.

Manual staining. PNAd staining for the detection of HEV was performed as previously described (30). In brief, sections were deparaffinized, rehydrated, subjected to heat-induced antigen retrieval, blocked and incubated with the PNAd primary antibody. Next, the sections were incubated with HRP-labelled goat anti-rat light chain secondary antibody (#AP202P, Millipore, Temecula, CA, diluted 1:250, incubated 30 minutes) and diaminobenzidine (Dako EnVision + System-Horseradish Peroxidase, Dako,) before being counterstained with Harry's hematoxylin (Sigma-Aldrich, St. Louis, MO). Human lymph node specimens were used as positive control, and specimens with the primary antibody omitted served as negative control.

Immunohistochemical evaluation

Two trained, independent observers (either EHO and AMW, or SES and IKE) who were blinded to the clinical outcome evaluated the immunohistochemical stainings quantitatively and semi-quantitatively, as illustrated in the flow chart of Figure S1. We assessed inter-observer variations for all stainings except

DC-Lamp and PD-L1, and the results are listed in Table S1. Agreement was reached by reevaluation and discussion in case of differing scores. For DC-Lamp and PD-L1, the two investigators reached consensus when evaluating the slides together. Micrographs were taken with a Leica DFC 420 camera on a Leica DM2000 microscope (Leica, Wetzlar, Germany). Presence of HEV was assessed as earlier published (16). Briefly, we scanned the PNAd stained tumor-adjacent tissue at low power magnification (100×) to identify five areas with high density of HEV (hotspots). Micrographs of these hotspots were taken at high power magnification (400×), and the mean number of HEV per section was calculated for each patient by dividing the sum of HEV in the five hotspots by five. The median number of HEV per hotspot for all patients served as cutoff for positive and negative HEV count. Giemsa staining was used to identify mast cells and eosinophils as cells with round nuclei and blue/purple granules or cells with lobulated nuclei and bright orange/pink granules, respectively. For each of the cell types, five hotspots in the tumor stroma were identified at low power magnification, and micrographs were taken at 400× magnification. The total cell number in the hotspots was counted and the mean number calculated. The cutoff for high versus low count was defined as the median number for the patient cohort, which was 10 for eosinophils and 3 for mast cells. We scored the CD3+, CD4+, CD8+, CD20+ and CD68+ stainings semi-quantitatively, as it was impossible to apply a quantitative scoring system in densely stained areas. First, we identified the invasive margin of the tumor at low power magnification (100×), and micrographs were taken at 400× magnification, capturing every second visual field of this area. Thus, the number of microscope fields depended on the tumor size. We developed a four-degree scoring scale for the different cell subsets as illustrated in Figure S2, and assigned each micrograph a score: 0 = no or almost no infiltration; 1 = mild infiltration; 2 = moderate infiltration; 3 = heavy infiltration. In cases where evaluation was difficult because of weak staining or non-specific background staining, the positively stained cells were counted, and the scores determined according to the respective cutoff. We calculated a mean score for each section before dichotomizing as low or high if it was 0-1.49 or 1.5-3, respectively. PD-L1 expression was assessed based on the percentage of positively labeled tumor cells in each section, and was classified as follows: 0 (labelling in ≤5% of cells), 1 (labelling in >5% and ≤10% of cells), 2 (labelling in >10% and ≤

50% of cells), 3 (labelling in >50% of cells). Expression of DC-LAMP was found only in inflamed areas of the tumor stroma and was scored as not expressed (0), slightly expressed (1), moderately expressed (2) or strongly expressed (3) based on semi-quantitative evaluation. Both PD-L1 and DC-LAMP scores were subsequently grouped into 2 categories: low expression (0 or 1) and high expression (2 or 3).

RNA extraction and quality control

RNA extraction was performed to evaluate gene expression of cytokines. In cases with sufficient residual tumor material, we isolated total RNA from FFPE OSCC tissue blocks using the High Pure FFPET RNA Isolation Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. In brief, up to 4 consecutive, 5-10 micrometer thick sections from each block were deparaffinized and digested with proteinase K, followed by multiple silica based column purification steps and DNAse I treatment. The RNA on the column was washed several times before being eluted in 20-35 µl elution buffer. We used a mixture of RNA isolated from three different fresh frozen (in liquid nitrogen) human lymphoma specimens as a positive control for further qPCR analyses. The RNeasy Fibrous Tissue mini Kit (Qiagen, Hilden, Germany) was used for RNA isolation according to the manufacturer's protocol. Briefly, the tissue was homogenized using a TissueLyser (Qiagen, Hilden, Germany) before digestion with proteinase K and centrifugation. The supernatant was washed and treated with DNAse I on a miniature column, and the RNA eluated in 50 µl nuclease free water. We measured total RNA quantity and purity on the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and assessed RNA integrity using the Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, USA).

Real-time quantitative PCR (qPCR)

We used the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) to reverse transcribe 100-200 ng total RNA into cDNA, which was subsequently diluted 1:15 in nuclease-free water. QPCR was performed in duplicates or triplicates using the Light Cycler 96 instrument (Roche, Mannheim, Germany). Target cDNA was amplified through 40 cycles in 20 µl reactions containing 1× FastStart Essential DNA Green Master (Roche), 10 µl diluted cDNA (1:15), and 300nM primers (Table S2). Melting curve

analysis was used to verify the specificity of the primers. Controls with the reverse transcriptase omitted (no-RT) and non-template controls (NTC) were included to test for genomic DNA contamination and carry-over products, respectively. A positive control consisting of cDNA from three different fresh frozen lymphoid tissues was included in each run. The qPCR amplification efficiency for each gene was calculated from the slope and correlation coefficient (R2) of regression curves from 2-fold serially diluted cDNA. The ΔΔCt method (35) was used to calculate the relative amount of target mRNA normalized against the geometric mean of the reference genes elongation factor 1 alpha (eF1a), ribosomal protein L27 (RPL27), and ribosomal protein S13 (RPS13). We used geNorm analyses (36) to detect the three most stably expressed reference genes, B-actin, and Beta 2 macroglobulin were discarded. For association with HEV and PD-L1 statuses, we present the results as fold increase compared to the mean of the group with the lowest gene expression (+/- standard error of mean) For survival and correlation analyses, we dichotomized the results in low and high expression based on the median of fold increase as cutoff.

Statistical Analysis

We used SPSS software version 22.0 for Windows (IBM, Armonk, NY) and Microsoft Excel 2013 (Microsoft, Redmond, WA) for all calculations. Inter-observer variability for the various quantitative and semi quantitative cell counts was analyzed using the Spearman correlation test, and correlation between two variables was assessed by the Fisher's exact test. We used univariate Kaplan Meier analyses to calculate disease-specific death rates and plot disease-specific survival curves, and the log-rank test to evaluate the statistical significance. Multivariate analyses were done using a stepwise forward multiple Cox regression model. Linear regression analyses of standard curves derived from serially diluted cDNA were used to estimate qPCR amplification efficiency. The significance level was set at $P < 0.05$, and the borderline significance level at $P < 0.09$.

Results

HEV predict T and B cell infiltration into OSCC

We recently demonstrated a correlation between the presence and morphology of HEV and the intensity of the inflammatory infiltrate in OSCC (16). Now, to determine the composition of the immune infiltrate, we immunohistochemically stained tumor sections from 75 OSCC patients with antibodies against the immune cell markers CD3 (pan T-cell), CD4 (T-helper, Treg cells and macrophages) CD8 (cytotoxic T cell), CD20 (pan B-cell), CD68 (pan macrophage) and DC-lamp/CD208 (mature dendritic cell), and performed Giemsa staining to identify eosinophils and mast cells. The HEV-negative tumors were generally less infiltrated with immune cells compared to the HEV-positive tumors (Table 2). The differences were statistically significant for CD3+ (P=0.002) and CD20+ (P=0.038) cells, and borderline significant for CD8+ cells (P=0.061).

To determine cytokines associated with HEV, we conducted qRT-PCR analysis of 42 of the 75 OSCC tissue samples, of whom 36 were HEV-positive and 6 were HEV-negative (Fig. 1A). The lymphoid chemokines CCL19, CCL21 and CXCL12 were significantly higher expressed in the HEV-positive compared to the HEV-negative tumors (P=0.04, P=0.02, and P=0.001, respectively). The inflammatory chemokine CCL20, however, was significantly lower expressed in the HEV-positive than in the HEV-negative tumors (P=0.02). Taken together, our results shows that HEV-positive tumors are more heavily infiltrated with lymphocytes than HEV-negative, which may be promoted by the chemokines CCL19, CCL21 and CXCL12.

HEV positive OSCC retain an inflamed phenotype even at high T-stage

In the same OSCC patient cohort, we have earlier found that HEV were present in all T1/T2 tumors, compared to two thirds of the T3/T4 tumors (16), and hypothesized that absence of HEV in advanced tumor stages may suppress the immune reaction. To elaborate this theory, we compared the immune cell infiltrate and cytokine expression in the T1/T2 tumors with the T3/T4 tumors, as well as the T3/T4 tumors

subdivided into HEV-positive and -negative. We found a significantly higher amount of infiltrating CD3+ T cells, CD68+ macrophages and eosinophils in the T1/T2 tumors (all HEV-positive) compared to the T3/T4 tumors (HEV-positive and -negative) as presented in Table 2 (P=0.026, P=0.002 and P=0.035, respectively). Dividing the T3/T4 tumors into HEV-positive and HEV-negative revealed that the HEV-positive had an immune cell infiltrate that resembled the T1/T2 tumors, except for fewer CD68+ cells in the T3/T4 tumors (P=0.014). In contrast, the HEV-negative T3/T4 tumors showed lower scores than T1/T2 tumors for all immune cell subsets analyzed, except CD4+ cells and DC-lamp + dendritic cells that were not significantly different.

QRT-PCR analysis of 28 T1/T2 tumor samples (all HEV-positive) and 13 T3/T4 tumor samples (HEV-positive, n=7; HEV-negative, n=6) revealed higher levels of CCL21 and CXCL12, and lower levels of CCL20 in the T1/T2 compared to the T3/T4 tumors (P=0.04, P=0.02, and P=0.01, respectively; Fig. 1B). In accordance with the results from immune cell analyses, the HEV-positive T3/T4 tumors displayed no statistically significant or borderline significant differences to the T1/T2 tumors' cytokine expression (Fig. 1C). The HEV-negative T3/T4 tumors, however, had significantly lower expression of CCL21 and CXCL12 and higher expression of CCL20 (P=0.02; P=0.04; P=0.01; Fig. 1D) than the T1/T2 tumors. Together, our results suggest that the presence of HEV supports an inflamed phenotype even in large (T3/T4) tumors.

T cell infiltration predicts an immune cell rich tumor microenvironment and is associated with CCL19, CXCL12 and lymphotoxin (LT) b expression

Cytokines, chemokines and cells of the innate and adaptive immune system interact in complex attraction, activation and inhibition networks. To reveal putative regulatory mechanisms in the OSCC immune infiltrate, we performed correlation analyses between the expression level of cytokines, the various immune cell scores, and the clinicopathological variables. The immunohistochemical score for CD3+ T cells showed statistically significant, positive correlations with all other immune cells analyzed except for

mast cells (Fig. 2A). There were few significant correlations between the other immune cell subsets. With the exception of CXCL8, CCL20 and IL-1β, we found numerous positive, significant correlations between the expression levels of the different cytokines analyzed (Fig. 2B). Lymphocyte score (CD3+, CD4+, CD8+ and CD20+ cells) showed significant positive correlation with one or several of the cytokines CCL19, CXCL9, CXCL10, CXCL12, CXCL13 and LTb (Fig. 2C). None of the cytokines was significantly correlated with the number of CD 68+ macrophages, DC-lamp+ dendritic cells or mast cells, suggesting that these cells are regulated by mechanisms distinct from the other studied immune cell populations. Correlation data of the patients' clinicopathological variables with immune cell scores and cytokine expression levels are presented in Table S3. Of note, the group size for many of the variables is small, smoking history and alcohol consumption are patient reported data, and treatment choice is strongly influenced by tumor stage and co-morbidity. Altogether, our results show that high numbers of CD3+ T cells in OSCC is correlated with increased infiltration of a number of other immune cells, and suggest that the cytokines CCL19, CXCL12 and LTb may have a prominent role in promoting immune cell infiltration through their association with T cells.

PD-L1 expression in OSCC cells correlates with increased infiltration of CD4+ cells and small tumor size

Cancer cells may escape immune surveillance by expressing PD-L1, which induces apoptosis in PD-1 expressing T-lymphocytes, most notably cytotoxic T-cells. PD-1/PD-L1 checkpoint blockade has shown promising results in various types of cancer, but only subgroups of patients respond to the treatment. To increase the understanding of PD-L1 expression and its association with the immune microenvironment, we analyzed PD-L1 expression in 45 tumors, randomly chosen from the 75 OSCC patient cohort, and determined correlation to infiltrating immune cells, cytokine expression and the presence of HEV. Eighteen (40%) of the 45 tumor sections stained positive for PD-L1. The staining was either membranous and/or cytoplasmic as demonstrated in Figure S3. We did not assess PD-L1 positivity of stromal cells because of diffuse staining. Tumors with high PD-L1 expression showed increased infiltration of CD4+

and CD8+ cell compared to those with low PD-L1 expression (P=0.025 and P=0.066, respectively; Table 3). They also expressed higher levels of a number of cytokines including CCL19, CCL21, CXCL9, CXCL10, CXCL13, and LTb (P=0.006, P=0.010, P<0.001, P<0.001, P<0.001 and P=0.011, respectively; Fig. 3). PD-L1 score was not correlated with patients' survival (P=0.207; Table S4). The T3/T4 tumors had significantly lower PD-L1 immune score than the T1/T2 tumors (P=0.024; Table 2). Dividing the T3/T4 tumors into HEV-positive and HEV-negative revealed that all HEV-positive T3/T4 tumors had low PD-L1 expression, whereas the HEV-negative showed no statistically significant difference in PD-L1 expression compared to the T1/T2 tumors (P=0.010 and P=0.386, respectively). Our results demonstrate that PD-L1 expression in tumor cells is associated with a tumor microenvironment rich in CD4+ cells and inflammatory cytokines, and that HEV are negatively correlated with PD-L1 in large tumors (T3/T4).

HEV are more powerful prognosticators than the N-stage and separate components of the OSCC immune infiltrate

Many components of the immune infiltrate have been suggested as potential prognosticators for oral cancer, but study results are contradictory. Univariate Kaplan-Meier survival analyses of the immune cell scores and cytokine expression levels in our OSSC cohort showed that high numbers of CD20+ B cells and CD68+ macrophages were significant prognosticators for longer 5-year survival (P=0.002 and P=0.027, respectively; Fig. 4 and Table S4). We have previously reported that the HEV status, the tumor site as well as the T- and N-stages were significant prognostic factors in the same patient cohort (16). All variables that were significantly associated with disease-specific death in univariate analyses were entered into multivariate Cox regression analyses. The proportional hazards assumption was fulfilled for all variables (Fig. S4 and (16)). Only the T stage (P<0.001) and the HEV status (P=0.002) were independent predictors for disease-specific death (Table 4). These results suggest that HEV are more important prognostic factors than the N stage and the subsets of the immune infiltrate assessed in this study.

Discussion

In this study, we show that the presence of HEV is indicative of a favorable immune microenvironment in OSCC, and that these vessels seem to counter-act immunosuppressive mechanisms and improve patient survival irrespective of the tumors' T-stage. PD-L1 expressing tumor cells correlated positively to a tumor microenvironment rich in CD4+ cells, but had no prognostic significance. Our results suggest that HEV have a pivotal role in shaping an antitumor immune response in OSCC, and that PD-1/PD-L1 targeted immunotherapy might be specifically successful in patients with tumors rich in CD4+ cell. To the best of our knowledge, this is the first study to investigate jointly the role of PD-L1 and HEV in OSCC, two components of the immune infiltrate with important regulatory functions.

High density of tumor-associated HEV has earlier been associated with improved survival and inflammation in several cancers including melanoma, oral- and breast cancer (16, 17, 37), and in the present study, we aimed to determine the composition of the HEV associated immune infiltrate in OSCC. We found that the HEV-positive OSCC were heavily infiltrated by CD3+ T cells, CD8+ cytotoxic T cells and CD20+ B cells, which is in accordance with previous findings in melanoma and breast cancer (17, 18). Infiltration of CD8+ cytotoxic T cells, and especially a high CD8+/Treg ratio have been associated with a favorable prognosis in a variety of human solid tumors including ovarian, cervical and oral cancer (38-40). However, of the immune cell subsets analyzed in our study, only increased infiltration of CD20+ B cells and CD68+ macrophages were significantly correlated with improved patient outcome in univariate analyses. The presence of B cells has earlier been associated with positive outcome in head and neck cancer patients (41, 42). B cells mediate a humoral immune response by producing tumor-specific antibodies, and these cells often localize and cooperate with T cells to facilitate potent, long-term antitumor responses (43). In contrast to our results, previous studies have mainly found a negative association between macrophages and patient survival in oral cancer (44-46). The conflicting results may be due to differences in immunohistochemical procedures and scoring. Besides, tumor-associated macrophages appear in different functional states that may vary between tumors and within specific tumor areas (47, 48), and the pan-macrophage marker CD68 does not distinguish between these phenotypes. The

CD68 antibody, as well as several of the other antibodies used in this study, are not strictly specific for a single cell type. Accurate assessment of immune cell subsets requires multiple markers for each subtype, which is laborious and costly to incorporate in a day-to-day routine clinical practice. In the present study, only the HEV- and T-statuses were independent positive prognosticators for 5-year disease-specific survival. This indicates that HEV are more relevant as prognostic markers than other components of the tumor microenvironment analyzed, and strengthens their role as a potent surrogate marker of an effective antitumor immune response. Immunohistochemical detection of HEV is simple and reliable (16), and implementation in clinical pathology practice could thus be straightforward.

Chemokines in and around HEV are thought to be crucial for lymphocyte extravasation into lymphoid organs (49). In the present study, the gene expression of the lymphoid chemokines CXCL12, CCL19 and CCL21 was upregulated in the HEV-positive tumors. Expression of CXCL12 is an important attractant for naïve T and B cells (50, 51), and has a potential role in HEV-mediated T cell trafficking into lymph nodes *in vitro* (52). CCL19 and CCL21 interact with CCR7 on lymphocytes, and trigger efficient T cell homing. Thus, increased expression of CCL19, CCL21 and CXCL12 in HEV-positive tumors supports an important role for HEV in homing of naïve T-cells into OSCC. Beside their role in lymphocyte homing, CCL19 and CCL21 stimulate migration and maturation of dendritic cells (53). Through lymphotoxin (LT) expression, dendritic cells promote maintenance of a mature HEV phenotype (54). Therefore, downregulation of CCL21 in T3/T4 tumors may be indicative of a tumor microenvironment with less infiltrating mature dendritic cells, causing dedifferentiation of HEV into normal blood vessels (19, 20). However, we did not find a significant association between DC-lamp, a marker for mature dendritic cells, and HEV score. To further study the association between dendritic cells and HEV, we also performed HEV/dendritic cell double staining, but faced technical difficulties (data not shown). In future studies, assessing markers for tumor angiogenesis could be a valuable supplement to investigate HEV plasticity, and to shed light on HEV development and maintenance in advanced tumors.

HEV negative tumors expressed higher levels of the chemokine CCL20 than HEV positive tumors. CCL20 attracts cells expressing the CCR6 receptor, such as dendritic cells and memory and effector T-cells. Several studies have found that CCL20 is a chief attractant of Treg cells, a distinct lineage of CD4+ T cells that suppresses anti-tumor immune responses (55-57). Interestingly, tumor cells may also express CCR6, and accordingly, CCL20 have been associated with migration and metastases of cancer cells (58). We did not specifically stain for Treg cells nor CCR6, but speculate that the downregulation of CCL20 in HEV-positive OSCC could help sustain an anti-tumor immune response by avoiding recruitment of Treg cells. This would be interesting to investigate in future studies. In summary, we show that both the early and the advanced HEV-positive tumors displayed higher levels of tumor-suppressive components of the immune infiltrate than the HEV-negative. Along with the vessels' independent prediction of improved survival, this suggests that HEV promote a tumor-suppressive immune response irrespective of the tumors' T-stage.

Tumor progression often correlates with immune evasion. In several cancers, including OSCC, immune suppression may be facilitated by PD-L1 that inhibits T cell functions (22, 23). In the present study, tumors with high PD-L1 score showed a significant increase in CD4+ cell infiltration ($P=0.025$). Increased numbers of immunosuppressive CD4+ Treg, as well as dysfunctional T lymphocytes have previously been found to predict immunosuppressive properties in oral cancer patients (59-61). We also found a borderline significant association between PD-L1+ tumor cells and infiltrating CD8+ cells ($P=0.066$), as well as the expression level of a number of inflammation-related cytokines. This suggests that PD-L1 expression by itself does not lead to a non-inflamed tumor environment, but may influence the efficacy of the immune reaction. This is in accordance with several other studies that report PD-L1 expression to be associated with cytotoxic T cells that can induce PD-L1 expression in an interferon (IFN)-γ dependent manner (62-64). CXCL9 is an IFN-γ-inducible chemokine, and we found a perfect linear relationship between PD-L1 score and CXCL9 expression ($r=1$; data not shown). Interestingly, high PD-L1 expression was also correlated to a lower T-stage ($P=0.037$), and we found a significantly lower score of PD-L1 expressing

tumor cells in the HEV-positive T3/T4 compared to the T1/T2 tumors (P=0.010). This may suggest that HEV-positive large tumors have overcome PD-L1 mediated immunosuppression, which could contribute to the increased survival of patients with HEV-positive T3/T4 tumors. PD-1/PD-L1 blockade is a new immunotherapeutic approach in the combat against cancer, and the clinical success of this treatment correlates to some extent with the tumors' PD-L1 expression (25-27). It can be speculated that PD-L1 expressing T1/T2 tumors and HEV-negative T3/T4 tumors with marked T cell infiltration are good candidates for PD-1/PD-L1 checkpoint targeting therapies.

In conclusion, we show that HEV are markers of a favorable anti-tumor immune microenvironment in OSCC, and stronger prognosticators than other subsets of the immune infiltrate. As detection of HEV is easy and reliable, HEV status may serve as a valuable supplement to stratify OSCC patients for targeted therapeutic approaches. OSCC are generally immunosuppressive tumors with poor patient outcome. Understanding the mechanisms that drive immune cell recruitment and generate effective anti-tumor responses may provide opportunities to develop new immunomodulatory targets and thereby increase the consistently low survival rates of patients with oral cancer.

Acknowledgments

The study was supported by grants from The North Norwegian Health Authorities. The authors thank the technical staff at the Department of Clinical Pathology, University Hospital of North Norway (UNN), Bente Mortensen, Mehrdad Rakaee Khanehkenari and Kjersit Julin at the Department of Medical Biology, University of Tromsø – The Arctic University of Norway (UiT) for excellent technical help. We are also grateful for statistical advice from Professor Tom Wilsgård at the Department of Community Medicine, UiT.

Conflict of interest

The authors declare no conflicts of interest.

Supplementary information is available at Modern Pathology's website.

References

1. Annertz K, Anderson H, Palmer K, et al. The increase in incidence of cancer of the tongue in the Nordic countries continues into the twenty-first century. *Acta Otolaryngol* 2012;132:552-7.

2. Soland TM, Brusevold IJ. Prognostic molecular markers in cancer - quo vadis? *Histopathology* 2013;63:297-308.

3. Holdenrieder S. Biomarkers along the continuum of care in lung cancer. *Scand J Clin Lab Invest Suppl* 2016;245:S40-5.

4. Al-Hajeili M, Shields AF, Hwang JJ, et al. Molecular Testing to Optimize and Personalize Decision Making in the Management of Colorectal Cancer. *Oncology (Williston Park)* 2017;31:301-12.

5. Swann JB, Smyth MJ. Immune surveillance of tumors. *J Clin Invest* 2007;117:1137-46.

6. Krisl JC, Doan VP. Chemotherapy and Transplantation: The Role of Immunosuppression in Malignancy and a Review of Antineoplastic Agents in Solid Organ Transplant Recipients. *Am J Transplant* 2017;17:1974-91.

7. Marcus A, Gowen BG, Thompson TW, et al. Recognition of tumors by the innate immune system and natural killer cells. *Adv Immunol* 2014;122:91-128.

8. Marrogi AJ, Munshi A, Merogi AJ, et al. Study of tumor infiltrating lymphocytes and transforming growth factor-beta as prognostic factors in breast carcinoma. *Int J Cancer* 1997;74:492-501.

9. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003;348:203-13.

10. Clemente CG, Mihm MC, Jr., Bufalino R, et al. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 1996;77:1303-10.

11. Gajewski TF. The Next Hurdle in Cancer Immunotherapy: Overcoming the Non-T-Cell-Inflamed Tumor Microenvironment. *Semin Oncol* 2015;42:663-71.

12. Woo SR, Corrales L, Gajewski TF. The STING pathway and the T cell-inflamed tumor microenvironment. *Trends Immunol* 2015;36:250-6.

13. Mandal R, Senbabaoglu Y, Desrichard A, et al. The head and neck cancer immune landscape and its immunotherapeutic implications. *JCI Insight* 2016;1:e89829.

14. van Zante A, Rosen SD. Sulphated endothelial ligands for L-selectin in lymphocyte homing and inflammation. *Biochem Soc Trans* 2003;31:313-7.

15. Hayasaka H, Taniguchi K, Fukai S, et al. Neogenesis and development of the high endothelial venules that mediate lymphocyte trafficking. *Cancer Sci* 2010;101:2302-8.

16. Wirsing AM, Rikardsen OG, Steigen SE, et al. Presence of tumour high-endothelial venules is an independent positive prognostic factor and stratifies patients with advanced-stage oral squamous cell carcinoma. *Tumour Biol* 2016;37:2449-59.

17. Martinet L, Garrido I, Girard JP. Tumor high endothelial venules (HEVs) predict lymphocyte infiltration and favorable prognosis in breast cancer. *Oncoimmunology* 2012;1:789-90.

18. Martinet L, Le Guellec S, Filleron T, et al. High endothelial venules (HEVs) in human melanoma lesions: Major gateways for tumor-infiltrating lymphocytes. *Oncoimmunology* 2012;1:829-39.

19. Lee SY, Chao-Nan Q, Seng OA, et al. Changes in specialized blood vessels in lymph nodes and their role in cancer metastasis. *J Transl Med* 2012;10:206.

20. Qian CN, Berghuis B, Tsarfaty G, et al. Preparing the "soil": the primary tumor induces vasculature reorganization in the sentinel lymph node before the arrival of metastatic cancer cells. *Cancer Res* 2006;66:10365-76.

21. Tong CC, Kao J, Sikora AG. Recognizing and reversing the immunosuppressive tumor microenvironment of head and neck cancer. *Immunol Res* 2012;54:266-74.

22. Zandberg DP, Strome SE. The role of the PD-L1:PD-1 pathway in squamous cell carcinoma of the head and neck. *Oral Oncol* 2014;50:627-32.

23. Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. *Cancer Res* 2013;73:1733-41.

24. Keir ME, Butte MJ, Freeman GJ, et al. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;26:677-704.

25. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012;366:2455-65.

26. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2443-54.

27. Ibrahim R, Stewart R, Shalabi A. PD-L1 Blockade for Cancer Treatment: MEDI4736. *Semin Oncol* 2015;42:474-83.

28. Hsu MC, Hsiao JR, Chang KC, et al. Increase of programmed death-1-expressing intratumoral CD8 T cells predicts a poor prognosis for nasopharyngeal carcinoma. *Mod Pathol* 2010;23:1393-403.

29. Badoual C, Hans S, Merillon 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.

30. Wirsing AM, Rikardsen OG, Steigen SE, et al. Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma. *BMC Clin Pathol* 2014;14:38.

31. AJCC. Manual for Staging of Cancer. Fourth ed. Philadelphia: J.B. Lippincott Company; 1992.

32. AJCC. AJCC Cancer Staging Manual. Fifth ed. Philadelphia: Lippincott-Raven Publishers; 1997.

33. UICC. TNM Classification of Malignant Tumours. Fourth ed. Berlin; Heidelberg; New York; London; Paris; Tokyo: Springer-Verlag; 1987 1987.

34. Altman DG, McShane LM, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. *BMC Med* 2012;10:51.

35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-8.

36. Pattyn F, Speleman F, De Paepe A, et al. RTPrimerDB: the real-time PCR primer and probe database. *Nucleic Acids Res* 2003;31:122-3.

37. Martinet L, Garrido I, Filleron T, et al. Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. *Cancer Res* 2011;71:5678-87.

38. Sato E, Olson SH, Ahn J, 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 U S A* 2005;102:18538-43.

39. Jordanova ES, Gorter A, Ayachi O, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res* 2008;14:2028-35.

40. Watanabe Y, Katou F, Ohtani H, et al. Tumor-infiltrating lymphocytes, particularly the balance between CD8(+) T cells and CCR4(+) regulatory T cells, affect the survival of patients with oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010;109:744-52.

41. van Herpen CM, van der Voort R, van der Laak JA, et al. Intratumoral rhIL-12 administration in head and neck squamous cell carcinoma patients induces B cell activation. *Int J Cancer* 2008;123:2354-61.

42. Pretscher D, Distel LV, Grabenbauer GG, et al. Distribution of immune cells in head and neck cancer: CD8+ T-cells and CD20+ B-cells in metastatic lymph nodes are associated with favourable outcome in patients with oro- and hypopharyngeal carcinoma. *BMC Cancer* 2009;9:292.

43. Nelson BH. CD20+ B cells: the other tumor-infiltrating lymphocytes. *J Immunol* 2010;185:4977-82.

44. Fujii N, Shomori K, Shiomi T, et al. Cancer-associated fibroblasts and CD163-positive macrophages in oral squamous cell carcinoma: their clinicopathological and prognostic significance. *J Oral Pathol Med* 2012;41:444-51.

45. Marcus B, Arenberg D, Lee J, et al. Prognostic factors in oral cavity and oropharyngeal squamous cell carcinoma. *Cancer* 2004;101:2779-87.

46. Ni YH, Ding L, Huang XF, et al. Microlocalization of CD68+ tumor-associated macrophages in tumor stroma correlated with poor clinical outcomes in oral squamous cell carcinoma patients. *Tumour Biol* 2015;36:5291-8.

47. Mantovani A, Sozzani S, Locati M, et al. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002;23:549-55.

48. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 2006;66:605-12.

49. Miyasaka M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol* 2004;4:360-70.

50. Pablos JL, Amara A, Bouloc A, et al. Stromal-cell derived factor is expressed by dendritic cells and endothelium in human skin. *Am J Pathol* 1999;155:1577-86.

51. Campbell JJ, Hedrick J, Zlotnik A, et al. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* 1998;279:381-4.

52. Phillips R, Ager A. Activation of pertussis toxin-sensitive CXCL12 (SDF-1) receptors mediates transendothelial migration of T lymphocytes across lymph node high endothelial cells. *Eur J Immunol* 2002;32:837-47.

53. Marsland BJ, Battig P, Bauer M, et al. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. *Immunity* 2005;22:493-505.

54. Moussion C, Girard JP. Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature* 2011;479:542-6.

55. Liu JY, Li F, Wang LP, et al. CTL- vs Treg lymphocyte-attracting chemokines, CCL4 and CCL20, are strong reciprocal predictive markers for survival of patients with oesophageal squamous cell carcinoma. *Br J Cancer* 2015;113:747-55.

56. Lee JJ, Kao KC, Chiu YL, et al. Enrichment of Human CCR6+ Regulatory T Cells with Superior Suppressive Activity in Oral Cancer. *J Immunol* 2017;199:467-76.

57. Chen KJ, Lin SZ, Zhou L, et al. Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis. *PLoS One* 2011;6:e24671.

58. Frick VO, Rubie C, Keilholz U, et al. Chemokine/chemokine receptor pair CCL20/CCR6 in human colorectal malignancy: An overview. *World J Gastroenterol* 2016;22:833-41.

59. Reichert TE, Strauss L, Wagner EM, et al. Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. *Clin Cancer Res* 2002;8:3137-45.

60. Hoffmann TK, Dworacki G, Tsukihiro T, et al. Spontaneous apoptosis of circulating T lymphocytes in patients with head and neck cancer and its clinical importance. *Clin Cancer Res* 2002;8:2553-62.

61. Gasparoto TH, de Souza Malaspina TS, Benevides L, et al. Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment. *Cancer Immunol Immunother* 2010;59:819-28.

62. Spranger S, Spaapen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl Med* 2013;5:200ra116.

63. Iwai Y, Okazaki T, Nishimura H, et al. Microanatomical localization of PD-1 in human tonsils. *Immunol Lett* 2002;83:215-20.

64. Shi F, Shi M, Zeng Z, et al. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. *Int J Cancer* 2011;128:887-96.

Figure legends

Figure 1. Log2 fold gene expression of various chemokines in formalin-fixed paraffin-embedded oral squamous cell carcinoma tissue samples. Comparison of the following groups: A) high endothelial venules (HEV)+ versus HEV- tumors, B) T1/T2 versus T3/T4 tumors, C) T1/T2 versus T3/T4 tumors with HEV and D) T1/T2 versus T3/T4 tumors without HEV. All T1/T2 tumors were HEV positive. Error bars indicate +/- standard error of the mean, and * indicates $p < 0.05$. The P-value was calculated using two sample T-test.

Figure 2. Heatmaps of Pearson correlation coefficients. The heatmaps show results from bivariate correlation analyses of the following parameters in the oral squamous cell carcinomas: A) immune cell immunohistochemical scores, B) gene expression pattern, and C) gene expression pattern and immune cell immunohistochemical scores. White crosses in the heatmaps show significant correlation between the different variables with $p < 0.05$.

Figure 3. Comparison of log2 fold gene expression of various chemokines and cytokines in formalin-fixed paraffin-embedded oral squamous cell carcinoma tissue samples with high and low programmed-death ligand 1 (PD-L1) score. Error bars indicate +/- standard error of the mean, and * indicates $p < 0.05$. The P-value was calculated using two sample T-test.

Figure 4. Kaplan-Meier analysis of 5-year disease-specific survival for patients with oral squamous cell carcinoma. High counts of A) CD20+ B cells and B) CD68+ macrophages were associated with improved survival ($P = 0.002$ and $P = 0.027$, respectively). The Kaplan-Meier curves show a 5-year

disease-specific survival rate of 81.4% for CD20 high versus 50% for CD20 low tumors (A), and 80.6% for CD68 high versus 56.4% for CD68 low tumors (B). The P-value was calculated using the log-rank test.