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Título de la tesis:

Estudio de las poblaciones macrofágicas en el microambiente tumoral de los carcinomas escamosos orales.

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Estudio de las poblaciones macrofágicas en el microambiente tumoral de los carcinomas escamosos orales.	Inglés: The study of macrophages in the Tumor Microenvironment of Oral Squamous Cell Carcinoma.

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RESUMEN (en español)

La compleja interacción entre los diferentes componentes celulares en el microambiente tumoral (TME) modula la respuesta inmune antitumoral. Más allá de las alteraciones genéticas de la célula tumoral, la demostración de un ecosistema alterado, con sus interconexiones a nivel sistémico, abre una nueva perspectiva de la biología y del comportamiento del cáncer. Los macrófagos asociados a tumores (TAMs) muestran una polarización en dos subtipos, categorizados como macrófagos activados por vía clásica M1 antitumoral y vía alternativa M2 protumoral e inmunosupresora. La relevancia clínica y factor pronóstico tanto de los TILs (Linfocitos infiltrantes de tumor), linfocitos By macrófagos en el carcinoma de células escamosas oral (OSCC), fueron investigados en este estudio mediante técnicas de inmunohistoquímica y anticuerpos anti-$CD4^+$, $CD8^+$, $FOXP3^+$, $CD68^+$ y $CD163^+$, así como sus relaciones potenciales con la expresión PD-L1 (ligando 1 de muerte programada) tumoral y marcadores de células troncales del cáncer (CSC) (NANOG, SOX2, OCT4, Nestina y Podoplanina (PDPN)). Todos estos marcadores han sido evaluados tanto en los nidos tumorales como en el estroma circundante.

Sobre estas premisas hemos investigado aplicando un estudio retrospectivo de una serie de 125 pacientes diagnosticados de OSCC, obteniendo los resultados mediante estudio óptico cuantitativo en muestras de tejido teñidas con H&E.

La infiltración del estroma de macrófagos marcados con $CD163^+$ se asoció significativamente con el tumor localizado en la lengua. Los TAMs estromales/intratumorales, marcados con $CD68^+$ y $CD163^+$, fueron más abundantes en no fumadores y no bebedores. En nuestro estudio se manifiesta una relación inversa entre los TAMs $CD68^+$ y $CD163^+$ y la expresión de marcadores de CSC en el OSCC. La alta densidad de TAMs $CD163^+$, tanto en el tumor como en el estroma, se correlacionó fuerte y significativamente con la ausencia de expresión de NANOG. Además, la infiltración de TAMs, tanto $CD68^+$ como $CD163^+$, fue también significativa y asociada con una alta expresión tumoral de PD-L1. Los TILs $CD4^+$ y $CD8^+$ se asocian significativamente con los hábitos de fumar y alcohol. Los TILs $CD4^+$ y $CD8^+$ muestran una relación inversa con la expresión de NANOG y SOX2, y los TILs $FOXP3^+$ se correlacionan significativamente con la expresión de Nestina y PDPN. La alta infiltración de TILs $CD4^+$ y $CD8^+$ y una alta proporción tumoral $CD8^+/FOXP3^+$ se relacionan con tumores que albergan expresión de PD-L1. La infiltración de TILs $FOXP3^+$ estromales/tumorales y una baja proporción de $CD8^+/FOXP3^+$ estromales revierten significativamente en una mejor supervivencia específica de la enfermedad. El análisis multivariante revela que la proporción de TILs $CD8^+/FOXP3^+$ estromales es un factor pronóstico independiente significativo. En cuanto a los TILs $CD20^+$ se correlacionaron inversamente con la expresión NANOG/SOX2. Los TILs $CD20^+$ estromales se asociaron significativamente con la clasificación T y segundos tumores primarios. Un análisis estratificado de supervivencia mostró que los TILs $CD20^+$ tumorales se asociaron significativamente con el pronóstico en pacientes jóvenes, masculinos, consumidores de alcohol y tabaco, densidad de TILs $CD8^+$ tumorales altos, macrófagos $CD68^+$ tumorales bajos, expresión PD-L1 positiva y NANOG/SOX2 negativa. En conclusión, nuestros resultados sugieren que hay un vínculo entre la infiltración de TAMs y el escape inmunológico en pacientes con OSCC. En cuanto a la supervivencia de estos pacientes, el cociente TILs $CD8^+/FOXP3^+$ es un factor pronóstico independiente. Los TILs pueden actuar como biomarcadores y posibles dianas terapéuticas en pacientes con OSCC. La alta densidad de TILs $CD20^+$ surge como un factor de buen pronóstico independiente en los pacientes con OSCC, lo que sugiere un papel relevante en la inmunidad antitumoral. En nuestro estudio también se descubrió una correlación inversa entre los TILs $CD20^+$ y la expresión de marcadores de CSC.

RESUMEN (en Inglés)

The complex interaction between the different cellular components in the tumor microenvironment (TME) modulates the antitumor immune response. Beyond the genetic alterations of the tumor cell, the demonstration of an altered ecosystem, with its interconnections at the systemic level, opens a new perspective on the biology and behavior of cancer. Tumor-associated macrophages (TAMs) show a polarization in two subtypes categorized as classically activated macrophages M1 antitumor and alternative pathway M2protumoral and immunosuppressive. The clinical relevance and prognostic factor of both TILs (tumor infiltrating lymphocytes), B lymphocytes and macrophages in oral squamous cell carcinoma (OSCC) were investigated in this study using immunohistochemical techniques and anti-CD4 $^{+}$, CD8 $^{+}$, FOXP3^{+}antibodies, CD68^{+} and CD163^{+}, as well as their potential relationships with tumor PD-L1 (programmed death ligand 1) expression and cancer stem cell markers (CSC) (NANOG, SOX2, OCT4, Nestin and Podoplanin (PDPN)). All these markers were evaluated both in the tumor nests and in the surrounding stroma.

On this premise we have investigated under a retrospective study of a series of 125 patients diagnosed with OSCC, obtaining the results by quantitative optical study in tissue samples stained with H&E.

Infiltration of the stroma of macrophages marked with CD163^{+} was significantly associated with the tumor located on the tongue, stromal / intratumoral TAMs marked with CD68^{+} and CD163^{+} were more abundant in non-smokers and non-drinkers. In our study, an inverse relationship was observed between CD68^{+} and CD163^{+} TAMs and the expression of CSC markers in the OSCC. Higher density of CD163^{+} TAMs both in the tumor and in the stroma was strongly and significantly correlated with the absence of NANOG expression. Furthermore, the infiltration of both CD68^{+} and CD163^{+} TAMs was also significantly associated with a high tumor expression of PD-L1 (1). CD4^{+} and CD8^{+} TILs are significantly associated with smoking and alcohol habits. The CD4^{+} and CD8^{+} TILs show an inverse relationship with the expression of NANOG and SOX2, and the FOXP3^{+} TILs significantly correlate with the expression of Nestin and PDPN. High infiltration of CD4^{+} and CD8^{+} TILs and a high CD8^{+} / FOXP3^{+} tumor ratio is significantly associated with tumors harboring positive expression of PD-L1. Infiltration of stromal / tumor FOXP3^{+} TILs and a low CD8^{+} / FOXP3^{+} stromal ratio is significantly associated with better disease-specific survival. Multivariate analysis reveals that the ratio of stromal CD8^{+} / FOXP3^{+} TILs is a significant independent prognostic factor. As for the CD20^{+} TILs, they were inversely correlated with the NANOG / SOX2 expression. Stromal CD20^{+} TILs were significantly associated with T classification and second primary tumors. A stratified survival analysis showed that tumor CD20^{+} TILs were significantly associated with prognosis in young, male, alcohol and tobacco users, high tumor CD8^{+} TIL density, low tumor CD68^{+} macrophages, positive PD-L1 expression, and NANOG / SOX2 negative.

In conclusion, our results suggest that there is a link between infiltration of TAMs and immune escape in patients with OSCC. Regarding the survival of these patients, the CD8^{+} / FOXP3^{+} TILs ratio is an independent prognosticfactor. TILs can act as biomarkers and potential therapeutic targets in patients with OSCC. The high density of CD20^{+} TILs emerges as an independent good prognostic factor in patients with OSCC, suggesting a role in antitumor immunity. In our study, an inverse correlation was also found between CD20^{+} TILs and the expression of CSC markers.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO
EN _____

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Título de la tesis:

ESTUDIO DE LAS POBLACIONES MACROFÁGICAS EN EL MICROAMBIENTE TUMORAL DE LOS CARCINOMAS ESCAMOSOS ORALES.

Nombre del Autor:

JULIÁN SUÁREZ CANTO
2022

Director/Directores:

Prof. Dr. D. Juan Carlos de Vicente Rodríguez

PRESENTACIÓN:

La presente tesis doctoral sigue las directrices de la normativa para la presentación de tesis doctorales como un compendio de publicaciones –normativa establecida con el acuerdo del Consejo de Gobierno de la Universidad de Oviedo del 20 de Julio de 2018, por el que se aprueba el Reglamento de los Estudios de Doctorado–. Los tres artículos incluidos en esta tesis están encuadrados en una misma unidad temática y han sido publicados en revistas JCR del primer cuartil:

Artículo 1:

Suárez-Sánchez FJ, Lequerica-Fernández P, Suárez-Canto J, Rodrigo JP, Rodríguez-Santamarta T, Domínguez-Iglesias F, García-Pedrero JM, de Vicente JC. Macrophages in Oral Carcinomas: Relationship with Cancer Stem Cell Markers and PD-L1 Expression. Cancers (Basel). 2020 Jul 2;12(7):1764. DOI: 10.3390/12071764.

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Artículo 2:

Suárez-Sánchez FJ, Lequerica-Fernández P, Rodrigo JP, Hermida-Prado F, Suárez-Canto J, Rodríguez-Santamarta T, Domínguez-Iglesias F, García-Pedrero JM, de Vicente JC. Tumor-Infiltrating $CD20^{+}$ B Lymphocytes: Significance and Prognostic Implications in Oral Cancer Microenvironment. Cancers (Basel). 2021 Jan 21;13(3):395.
DOI: 10.3390/cancers13030395.

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Artículo 3:

Lequerica-Fernández P, Suárez-Canto J, Rodríguez-Santamarta T, Rodrigo JP, Suárez-Sánchez FJ, Blanco-Lorenzo V, Domínguez-Iglesias F, García-Pedrero JM, de Vicente JC. Prognostic Relevance of $CD4^{+}$, $CD8^{+}$ and $FOXP3^{+}$ TILs in Oral Squamous Cell Carcinoma and Correlations with PD-L1 and Cancer Stem Cell Markers. Biomedicines. 2021 Jun 8;9(6):653.
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LISTADO DE ABREVIATURAS:

AJCC	American Joint Commitee on Cancer
APC	Antigen presenting cell
AUC	Area under curve
Bregs	Regulatory B Lymphocytes
CEIC Asturias	Comité Ética de la Investigación del Principado de
CSC	Cancer Stem Cell
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cell
DEO	Displasiaepitelial oral
DFS	Disease free survival
DSS	Disease specific survival
FOXP3	Forkhead box P3
HIT	Heterogeneidad intratumoral
HNSCC	Head and Neck Squamous Cell Carcinoma
HRs	Hazardrisks
H&E	Hematoxilina y Eosina
IC del 95%	Intervalo de confianzadel 95%
IFN-γ	InterferónGamma
IHC	Immunohistochemistry
M1	Macrófagos fenotipo 1
M2	Macrófagos fenotipo 2
MDSCs	Myeloid-derived suppressor cells
NK	Natural Killer
OSCC	Oral Squamous Cell Carcinoma
OS	Overall survival
PD-1	Programmed death 1 receptor
PD-L1	Programmed death-ligand 1

PDPN	Podoplanina
PKD2	Protein Kinase D2
ROC	Característica operativa del receptor
SD	Standard deviation
SNC	Sistema nervioso central
TAMs	Tumor Associated Macrophages
Th1	T helper 1 Lymphocytes
TILs	Tumor Infiltrating Lymphocytes
TLS	Tertiary lymphoid structures
TMA	Tissue Microarray
TME	TumorMicro-environment
TNM	Classification of Malignant Tumors

TABLA DE CONTENIDO:

1. INTRODUCCIÓN

El carcinoma oral de células escamosas (OSCC) se engloba en el grupo de enfermedades a las que nos referiremos genéricamente como *cáncer* por compartir una determinada estructura biológica. Esta estaría conformada por un ecosistema complejo, en el cual se han alterado las relaciones intercelulares, el desarrollo y la función tisular. Diversas características del tumor, como su morfología, clasificación, agresividad clínica, pronóstico y respuesta al tratamiento abren una nueva perspectiva de la biología y del comportamiento del cáncer, cuya progresión compleja y dinámica podría ser el resultado de múltiples alteraciones genéticas y epigenéticas entre distintos tipos de células dentro del tumor, así como en el microambiente tumoral circundante [1]. El OSCC es el cáncer más frecuente en el área de la cabeza y el cuello, representando el 90% de los tumores malignos orales [2]. El 10% restante le correspondería a melanomas, sarcomas, carcinomas de glándulas salivales y metástasis, entre otros.

A pesar de los avances en la comprensión de la biología del cáncer, apoyándonos en los estudios de la teoría de la mutación somática en el cáncer –vigente desde hace más de 100 años, y hoy completada por estudios genéticos y moleculares de esta enfermedad y muchas otras áreas de conocimiento emergente incluyendo diagnóstico y tratamientos del OSCC [3]–, la tasa de supervivencia a los 5 años no ha logrado incrementarse y se ha mantenido por debajo del 50% en los últimos 30 años [4].

El microambiente tumoral (TME) es un sistema complejo donde las células tumorales se reprograman interaccionando con los diferentes componentes del microambiente. Esto origina una simbiosis célula-microambiente de la que resulta un comportamiento celular aberrante, favoreciendo la tumorigénesis, progresión del cáncer e invasión de tejidos adyacentes.

El TME está constituido por una diversidad de células, tanto tumorales como no tumorales, entre las que se encuentran células estromales (fibroblastos asociados a tumor, células endoteliales y pericitos), células inflamatorias, macrófagos asociados a tumor (TAMs), linfocitos infiltrantes de tumor (TILs), incluidos los linfocitos B. A diferencia de las células tumorales, todas estas células no tienen mutaciones, aunque su comportamiento se ve modulado por varias citoquinas [5].

Los TAMs representan las células inflamatorias más abundantes e importantes en el TME [6]. Los macrófagos desempeñan un papel de interfaz entre la inmunidad innata y la adquirida, y participan tanto en el control antitumoral fenotipos M1 (clásico) como en la progresión tumoral fenotipo M2 (alternativo) [7]. En condiciones fisiológicas, los macrófagos se diferencian con características de fenotipo M1 con funciones de respuesta inflamatoria, eliminación de patógenos e inmunidad antitumoral. Sin embargo, las células tumorales actúan como potente factor de polarización, generando macrófagos fenotipo M2 por varias vías CCL-2, IL-1, IL-4, IL-6, IL-8, IL-10, IL-13, CSF-1, PD-1/PD-L1, CD47/SIRP, TGF-β [5,7, 8]. A su vez, los macrófagos M2, secretan altos niveles de citoquinas, enzimas y factores de crecimiento como VEGF, PDGF, TGF-13, FGF-13, FGF y varios enzimas proteolíticos (metaloproteinasas, catepsinas) la mayoría de ellas codificadas por genes que son dianas transcripcionales de las vías de señalización NF-KB, NFAT y STAT3 [8], lo que aumenta la inflamación y promueve la progresión del tumor, y, también, inmunosupresión, angiogénesis, migración, metástasis y resistencia al tratamiento [5, 7]. Inmunohistoquímicamente se han empleado diferentes marcadores para identificar los TAMs: CD68, una proteína asociada a lisosomas, que se expresa en macrófagos independientemente de su fenotipo, y CD163, que es un receptor transmembrana ampliamente utilizado como marcador de los macrófagos M2 [7]. CD163 tiene un papel esencial en la eliminación de los complejos hemoglobina/haptoglobina y en la inducción de la respuesta inmune innata [9].

La relevancia clínica de las subpoblaciones de macrófagos en el cáncer no se ha establecido de manera clara. En varios tipos de cánceres como linfomas, glioma, cáncer de pulmón, adenocarcinoma gástrico, carcinoma de tiroides, mama y riñón, la mayor expresión de CD163 en los TAMs se ha asociado con un peor pronóstico [10,11,12]. Sin embargo, en el carcinoma de células escamosas de cabeza y cuello (HNSCC) se ha demostrado una asociación entre los macrófagos $CD163^{+}$ y un mal pronóstico en el análisis de supervivencia univariante, pero no multivariante [12]. Además, cuando la expresión de CD163 fue analizada por subgrupos se evidenció que el impacto de la supervivencia estaba específica y significativamente asociado con la expresión estromal, pero no intratumoral [12].

La respuesta inmunitaria del huésped es un factor clave que determina el TME y que influye en la evolución de la propia enfermedad [13]. Los estudios de las células

inmunes que infiltran el tumor indican que las acumulaciones de linfocitos T, macrófagos, células dendríticas (DC), células mieloides supresoras (MDSCs) y células asesinas naturales (NK) pueden determinar campos de información determinantes de comportamiento biológico, pronóstico y respuesta al tratamiento del tumor.

Los linfocitos $CD20^+$, además de su papel relevante en la inmunidad humoral, poseen otras funciones de modulación de la respuesta de activación de linfocitos T a través de señales, actuando cómo células colaboradoras [14].

Los TILs representan una importante población del microambiente inmune tumoral de la mayoría de los tumores sólidos. Su cuantificación está relacionada con la inmunogenicidad del tumor y con una mayor producción de citoquinas por parte de las células tumorales. La respuesta inmune adaptativa o específica esta mediada por los linfocitos T y B. Los linfocitos T presentan subpoblaciones $CD4^+$ (*helper*) y $CD8^+$ (citotóxicas) [15]. Altos niveles de TILs se han relacionado con mejor supervivencia libre de enfermedad (DFS) y supervivencia global (OS).

Los linfocitos T citotóxicos $CD8^+$ (CTL), generalmente apoyados por linfocitos T auxiliares (Th1), se consideran las principales células inmunitarias efectoras dirigidas contra las células tumorales [16, 17]. Específicamente en el HNSCC, altas densidades de linfocitos T $CD8^+$ se han relacionado con un pronóstico favorable, aunque pocos estudios han investigado la relevancia pronóstica de los linfocitos T $CD8^+$ en el OSCC [18, 19, 20]. Sin embargo, altas tasas de infiltración tanto de $CD4^+$ como de $CD8^+$ no se asociaron significativamente con una mejor supervivencia para los pacientes con cáncer oral o faríngeo, lo que en términos de resultado clínico en cánceres de cabeza y cuello [21, 22] indica que la localización del tumor podría ser un factor más discriminatorio que el recuento de TILs.

Dentro de las ya enumeradas en el TME, otra subpoblación de linfocitos T $CD4^+$ son los linfocitos T reguladores (*tregs*) que expresan el factor de transcripción *forkhead box* P3 (FOXP3). Por lo general, representan una fracción más pequeña de TILs, pero pueden tener una influencia significativa en el desarrollo de los tumores. Su potencial para inhibir las respuestas Th1 al interferir con funciones antitumorales de linfocitos T efectores, contribuyen a la tolerancia inmune, promoviendo así la evasión inmune del cáncer [23]. Por consiguiente, el reclutamiento de los *tregs* FOXP3 se ha asociado con un peor pronóstico en algunos tumores [24, 25, 26, 27, 28], mientras que se ha

correlacionado con un buen pronóstico en los cánceres de cabeza y cuello, esófago y colorrectal [29, 30]. Por lo tanto, las implicaciones pronósticas de las subpoblaciones de los linfocitos T $CD4^+$, $CD8^+$ y $FOXP3^+$ en el OSCC siguen sin ser concluyentes [31, 32, 33].

La cooperación entre linfocitos B y TILs conduce a una mayor supervivencia de los pacientes, seguramente porque los linfocitos B, que sirven como célula presentadora de antígeno (APC), secretan citoquinas polarizantes, mejorando así la inmunidad antitumoral [14].

El ligando 1 de muerte celular programada (PD-L1) es una glicoproteína de punto de control inmunitario (*checkpoint*) expresada en células inmunitarias activadas y células tumorales. Estas actúan como un factor inhibitorio que se une a los receptores de muerte celular programada 1 (PD-1) presente en la superficie celular de los linfocitos T activados. Esto induce anergia y apoptosis regulando la respuesta inmunitaria y la respuesta autoinmune [34, 35]. Mizoguchi et al. [36] han descrito una subpoblación de linfocitos B reguladores (*Bregs*) que manifiestan efectos protumorales por su capacidad para suprimir las respuestas de los linfocitos T a través de la secreción de citoquinas como IL-10 y TGF-β, así como para regular al alza ligandos inmunitarios como PD-L1, induciendo la muerte de los linfocitos T $CD4^+$.

La heterogeneidad intratumoral (HIT), hace referencia a la coexistencia en un mismo tumor de diferentes subpoblaciones de células neoplásicas. Estas subpoblaciones difieren en sus características genéticas, fenotípicas o de comportamiento, fomentando tanto el desarrollo del propio tumor como su resistencia a pautas de tratamiento. Entre ellas se encuentran las células troncales cancerosas (CSC), que constituyen el 0,01-10% de las células intratumorales [16] y que se caracterizan por sus propiedades de autorreplicación, división asimétrica y persistencia clonal a largo plazo [37], siendo significativa en el proceso la interacción con el TME [28]. Las CSC desempeñan un papel crucial en la iniciación, progresión, agresividad, heterogeneidad, metástasis, recurrencia y resistencia al tratamiento del tumor [17, 26, 38]. También tienen propiedades de troncalidad respaldadas por factores de transcripción nuclear, como son NANOG, SOX2 y OCT4 [39] –que se han implicado en la carcinogénesis oral–, pobre diferenciación y mal pronóstico [40, 41, 42, 43, 44, 45, 46, 47]. NANOG y SOX2 han sido identificados como predictores de progresión tumoral a cáncer en pacientes con lesiones precancerosas orales [40, 41]. Nestina es una proteína de filamento intermedio

de clase VI, identificada como marcador de célula troncal neural durante la embriogénesis. Su expresión en tejidos adultos normales está limitada al sistema nervioso central (SNC), estando sus aplicaciones diagnósticas relacionadas con tumores del SNC [48]. Además, se expresa también en otros tumores, incluido el OSCC, actuando como reguladores del ciclo celular, apoptosis, invasión y metástasis en las CSCs [49]. La podoplanina (PDPN) es una glicoproteína transmembrana que se expresa en una amplia variedad de tejidos, siendo en el endotelio linfático donde alcanza su máxima importancia. La PDPN es muy utilizada como marcador de las patologías de vasos linfáticos. También regula las células troncales en los tejidos normales y tumorales [50]. En leucoplasias orales actúa como un biomarcador valioso para la evaluación de riesgo de malignidad [51].

El propósito de esta tesis es evaluar e investigar la relevancia clínica y el valor pronóstico de la infiltración de TAMs, Linfocitos B $CD20^{+}$ y TILs en el OSCC. Asimismo, hemos llevado a cabo un estudio de las posibles relaciones entre las células del TME antes referenciadas con PDL1 y marcadores de CSC en cuanto a implicaciones pronósticas y análisis de supervivencia [52, 53] en una serie homogénea de 125 pacientes con OSCC.

Estos estudios se realizaron con métodos inmunohistoquímicos, empleando diferentes anticuerpos: CD68 y CD163 para las poblaciones de macrófagos M1 y M2, CD20 para linfocitos B, CD4, CD8 y FOXP3 para subpoblaciones de linfocitos T, PDPN para determinar la puntuación inmunorreactiva (IRS) de células tumorales [51, 54]. En nuestro estudio, el sistema de gradación del cáncer se realizó bajo los criterios de clasificación de la *American Joint Commitee Cancer* (AJCC) [55].

2. HIPOTESIS Y OBJETIVOS

Las hipótesis contrastadas en este estudio han sido las siguientes:

1. La infiltración tumoral y/o estromal por macrófagos M1 y M2 ¿se relaciona con el pronóstico de pacientes aquejados de cáncer de la cavidad oral?
2. Los macrófagos asociados a tumores ¿se relacionan con las células troncales tumorales y con la regulación del eje PD-1/ PD-L1 en cáncer oral?
3. Los linfocitos T y B infiltrantes tumorales ¿se asocian con las características clínicas y el pronóstico de pacientes con cáncer oral?
4. Los linfocitos T y B infiltrantes tumorales ¿se relacionan con las células troncales tumorales y con la regulación del eje PD-1/ PD-L1 en cáncer oral?

Los objetivos del estudio son:

1. Cuantificar los macrófagos M1 y M2, así como diversas subpoblaciones de linfocitos T y B en pacientes con OSCC.
2. Estudiar las asociaciones entre las densidades de las células inmunes infiltrantes de los tumores y de sus estromas y los rasgos clinicopatológicos y pronósticos de los pacientes con OSCC.
3. Estudiar la correlación entre las distintas poblaciones de células inmunes infiltrantes de OSCCs.

3. MATERIAL Y MÉTODOS. RESULTADOS

Tanto la descripción del material y método como de los resultados de los estudios se han publicado como artículos científicos independientes:

Trabajo 1:

Suárez-Sánchez FJ, Lequerica-Fernández P, Suárez-Canto J, Rodrigo JP, Rodríguez-Santamarta T, Domínguez-Iglesias F, García-Pedrero JM, de Vicente JC. Macrophages in Oral Carcinomas: Relationship with Cancer Stem Cell Markers and PD-L1 Expression. Cancers (Basel). 2020 Jul 2;12(7):1764. DOI: 10.3390/12071764.

PMID: 32630659; PMCID: PMC7408350.

Trabajo 2:

Suárez-Sánchez FJ, Lequerica-Fernández P, Rodrigo JP, Hermida-Prado F, Suárez-Canto J, Rodríguez-Santamarta T, Domínguez-Iglesias F, García-Pedrero JM, de Vicente JC. Tumor-Infiltrating $CD20^+$ B Lymphocytes: Significance and Prognostic Implications in Oral Cancer Microenvironment. Cancers (Basel). 2021 Jan 21;13(3):395.
DOI: 10.3390/cancers13030395.

PMID: 33494389; PMCID: PMC7865920.

Trabajo 3:

Lequerica-Fernández P, Suárez-Canto J, Rodríguez-Santamarta T, Rodrigo JP, Suárez-Sánchez FJ, Blanco-Lorenzo V, Domínguez-Iglesias F, García-Pedrero JM, de Vicente JC. Prognostic Relevance of $CD4^+$, $CD8^+$ and $FOXP3^+$ TILs in Oral Squamous Cell Carcinoma and Correlations with PD-L1 and Cancer Stem Cell Markers. Biomedicines. 2021 Jun 8;9(6):653.
DOI: 10.3390/biomedicines9060653.

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Macrophages in Oral Carcinomas: Relationship with Cancer Stem Cell Markers and PD-L1 Expression

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Abstract: Tumor-associated macrophages (TAMs) can be polarized into antitumoral M1 and protumoral and immunosuppressive M2 macrophages. This study investigated the clinical relevance of TAM infiltration in oral squamous cell carcinoma (OSCC), evaluating CD68 (M1 and M2 macrophage marker) and CD163 expression (M2 macrophage marker) in the tumor nests and surrounding stroma. Immunohistochemical analysis of both stromal/tumoral $CD68^+$ and $CD163^+$ TAMs was performed in paraffin-embedded tissue specimens from 125 OSCC patients, and correlated with clinical data. Potential relationships with the expression of cancer stem cell (CSC) markers and PD-L1 in the tumors were also assessed. Stromal $CD163^+$ infiltration was significantly associated with the tumor location in the tongue, and stromal and tumoral $CD68^+$ and $CD163^+$-infiltrating TAMs were more abundant in nonsmokers and non-alcohol-drinkers. Strikingly, this study uncovers an inverse relationship between $CD68^+$ and $CD163^+$ TAMs and CSC marker expression (NANOG and SOX2) in OSCC. High infiltration of $CD163^+$ TAMs in both tumor and stroma was strongly and significantly correlated with the absence of NANOG expression. Moreover, infiltration of both $CD68^+$ and $CD163^+$ TAMs was also significantly associated with high tumor expression of PD-L1. Our results suggest that there is a link between TAM infiltration and immune escape in OSCC.

Keywords: oral squamous cell carcinoma; tumor-associated macrophages; tumor microenvironment; CD68; CD163; NANOG; PD-L1

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent cancer of the head and neck area [1]. In spite of recent advances in understanding the molecular biology, diagnosis, and treatment of

the OSCC, including microsurgical reconstruction and advances in multimodal tumor therapy, the five-year survival rate has remained under 50% over the past 30 years [2], mainly due to local incontrollable recurrence or metastasis. In 1889, Paget described by first time the "seed and soil" hypothesis, wherein carcinomas induce changes in adjacent stromal cells, which contribute to neoplastic tissue invasion [3,4]. The tumor microenvironment (TME) is a complex system where tumor cells reprogram surrounding stromal cells in order to support tumorigenesis, cancer progression, and invasion of adjacent tissues [5]. TME is composed by a variety of stromal cells, such as fibroblasts, endothelial cells, pericytes, and immune cells. Unlike tumor cells, all these cells do not have mutations, although their behavior is modulated by several cytokines. Tumor-associated macrophages (TAMs) are the most abundant and important immune cells in the TME. In solid tumors, 5% to 40% of the tumor mass consists of macrophages [6]. Macrophages play a role as an interface between innate and acquired immunity, and can be polarized into M1 and M2 phenotypes, based on the expression of cytokines, receptors, and effector molecules [7]. Under physiological conditions, macrophages are polarized into proinflammatory and antitumor M1 phenotype; however, tumor cells can switch macrophages to alternatively activated M2 phenotype via several pathways (CCL-2, IL-1, IL-4, IL-6, IL-8, IL-10, IL-13, CSF-1, PD-1/PD-L1, CD47/SIRPα, TGFβ) [5,7,8]. M2 macrophages, in turn, secrete high levels of cytokines, chemokines, enzymes, and growth factors, such as VEGF, PDGF, TGF-β, FGF, uPA, and several matrix metalloproteinases, most of them encoded by genes that are transcriptional targets of NF-κB, NFAT, and STAT3 signaling pathways [9], thereby increasing inflammation as well as promoting tumor progression, immunosuppression, angiogenesis, migration, metastasis, and treatment resistance [5,7]. Furthermore, in vitro studies have shown that transformation from M1 to M2 can reverse their polarization depending on the chemokine stimuli [10]. TAMs are a mixed population of macrophages harboring both M1 and M2, but mainly composed by M2 macrophages, recruited and educated by cancer cells [6]. Immunohistochemistry has been widely employed to identify TAMs. Antibodies against CD68, a pan-macrophage marker, allows to identify all macrophages regardless of their phenotype, while the CD163 marker is a transmembrane scavenger receptor for haptoglobin–hemoglobin, highly expressed by M2 macrophages, and as such it has been widely recognized and used as marker for M2 macrophages [7]. CD163 has an essential role in eliminating hemoglobin–haptoglobin complexes and inducing the innate immune response [11].

Cancer stem cells (CSCs), or cancer-initiating cells, are a small population of cancer cells capable of self-renewal and multipotency. Macrophages are the most important ancillary cells regulating CSC activities [1]. However, the functional role of TAMs in OSCC is poorly understood, and the possible relationship between TAM infiltration and CSC phenotypes has not been investigated yet in oral carcinogenesis [11]. Among the numerous CSCs markers, NANOG and SOX2 have recently been identified as predictors of oral cancer risk in patients with oral precancerous lesions [12,13].

The host immune response is a key factor shaping the TME, and immune escape has been recognized as an important hallmark of cancer. Thus, compelling evidence indicates that immune tolerance in the TME is involved in tumor progression [8]. The axis of immune checkpoint inhibitors is represented by programmed-death ligand 1 (PD-L1) expressed in tumor cells, which binds to the programmed-death 1 receptor (PD-1) on activated T cells, delivering an inhibitory signal to those T cells that prevents tumor elimination from the immune system [14]. OSCC suppresses antitumor immunity by the induction of PD-L1 expression on M2 macrophages, causing T cell apoptosis [15].

The clinical relevance of macrophage subpopulations in cancer has not been clearly established. In several types of cancers, such as lymphoma, glioma, lung, gastric, thyroid, breast, and kidney cancers, higher CD163 expression on TAMs has been associated with a worse prognosis [8,16,17]. In head and neck squamous cell carcinoma, an association between CD163-positive macrophages and poor prognosis has been reported in univariate but not multivariate survival analysis [17]. Moreover, when the CD163 expression was analyzed by subgroups, it was revealed that the impact on patient

survival was specifically and significantly associated with stromal, but not intratumoral CD163 expression [17].

In this study we aimed to investigate the clinical relevance and prognostic value of TAM infiltration in a homogeneous series of 125 OSCC patients, by means of immunohistochemical analysis of CD68 and CD163. Stromal/tumoral expression of CD68 and CD163 was evaluated, as well as possible relationships with the expression of CSCs markers and tumor PD-L1.

2. Materials and Methods

2.1. Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Regional Ethical Committee from Principado de Asturias for the project PI19/01255 (136/19)) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2. Patients and Tissue Specimens

This retrospective study was approved by the Institutional Ethics Committee of the Hospital Universitario Central de Asturias and by the Regional CEIC from Principado de Asturias. Written informed consent was obtained from the patients involved in the study. Tissue specimens from 125 patients with OSCC who underwent surgical treatment with curative purposes at the Hospital Universitario Central de Asturias between 1996 and 2007 were sourced from archival tissue blocks provided by the Principado de Asturias BioBank (PT17/0015/0023), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures. The histological diagnosis was confirmed by an experienced pathologist (FDI). Clinicopathologic data were collected from medical records. Patients with OSCC in this study (82 male and 43 female) ranged in age from 28 to 91 years (mean 58.69, standard deviation 14.34 years). Cancers were located in the tongue (n = 51, 41%), floor of the mouth (n = 37, 30%), gingiva (n = 22, 18%), buccal mucosa (n = 7, 6%), retromolar area (n = 6, 4%), and palate (n = 2, 1%). Smoking habit and alcohol consumption were seen in 84 (67%) and 69 (55%) patients, respectively. AJCC [18] stage T1 was detected in 27 (22%) patients, T2 in 54 (43%) patients, T3 in 16 (13%) patients, and T4 in 28 (22%) patients. Stage pN0 was observed in 76 (61%), pN1 in 25 (20%), and pN2 in 24 (19%) patients. According to overall AJCC stages, 20 (16%) had stage I, 32 (26%) had stage II, 26 (20%) had stage III, and finally, 47 (38%) had stage IV. Regarding histopathologic degree of differentiation, 80 (64%) OSCCs were well-differentiated, 41 (33%) moderately, and 4 (3%) poorly-differentiated. During the follow-up period (6 to 230 months) 54 (43%) patients had local or regional recurrence, 19 (15%) suffer from a second primary cancer, and 53 (42%) died of OSCC.

2.3. Tissue Microarray (TMA) Construction

Three morphological representative areas were selected from each individual paraffin tumor block, including both the invasive border as well as the center of tumor sheets or islands without necrotic areas. In addition, each TMA contained morphologically normal oral mucosa samples from nononcological patients undergoing oral surgery as internal negative controls. In order to check the histopathological diagnosis and the adequacy of tissue sampling, a section from each microarray was stained with hematoxylin and eosin and examined by light microscopy.

2.4. Immunohistochemistry (IHC)

The TMAs were cut into 3 μm sections and dried on Flex IHC microscope slides (DakoCytomation, Glostrup, Denmark). The sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed by heating the sections with Envision Flex Target Retrieval solution, high pH (Dako, Glostrup, Denmark). Staining was done at room temperature on

an automatic staining workstation (Dako Autostainer Plus, Dako). The following primary antibodies were used: anti-CD68 (Agilent-Dako, clone KP1, prediluted), anti-CD163 (Biocare Medical, Pacheco, CA, USA; clone 10D6, 1:100 dilution), mouse monoclonal PD-L1 antibody (clone 22C3, PD-L1 IHC 22C3 pharmDx, Dako SK006; 1:200 dilution), NANOG (D73G4) XP® rabbit monoclonal antibody (Cell Signaling technology, Inc., Leiden, The Netherlands; 1:200 dilution), and anti-SOX2 rabbit polyclonal antibody (AB5603, Merck Millipore, Darmstadt, Germany; 1:1000 dilution) by using the Dako EnVision Flex + Visualization System (Dako Autostainer) and diaminobenzidine chromogen as substrate. Negative controls were prepared by omitting the primary antibody. Positive controls were prepared using appropriate positive control slides. Counterstaining with hematoxylin was the final step. The IHC results were independently evaluated by four observers (FDI, FJSS, JPR, and JMG-P) blinded to clinical data. The number of CD163-positive cells was counted in each 1 mm^2 area from three independent high-power representative microscopic fields (HPFs, 400×; 0.0625 μm^2), in both the tumor nests and the surrounding stroma. In the survival analysis, all the specimens were divided into low and high groups on the basis of the number of positive cells/mm^2, using cut-off values of 25, 50 (median), and 75 percentiles, both for CD68- and CD163-positive cells.

PD-L1 immunostainings was semiquantitatively scored into five different categories according to the percentage of stained tumor cells (0, negative; 1, 1–10%; 2, 10–25%; 3, 26–50%; and 4, more than 50% of stained cells). Since PD-L1 expression in more than 10% of tumor cells was associated with a poorer survival [19], this was established as a cut-off point for subsequent analyses. Accordingly, for analytical purposes, OSCC patients were dichotomized into two groups: relevant versus nonrelevant PD-L1 expression, based on the established cut-off point of 10% of stained tumor cells. NANOG expression was scored as negative (absence of staining, score 0), weak to moderate (some cytoplasmic staining in dysplastic areas, score 1), and strong protein expression (intense and homogeneous cytoplasmic staining in dysplastic areas, score 2), and SOX2 expression was evaluated as the percentage of tumor cells with positive nuclear staining, following previously described methods [12,13]. For statistical purposes, NANOG staining was dichotomized as negative (score 0) versus positive expression (scores 1–2). SOX2 staining scores were classified as negative or positive expression on the basis of values below or above the median value of 10%, respectively. A sample of testicular seminoma (a tumor known to express NANOG and SOX2) was used as a positive control.

2.5. Statistical Analysis

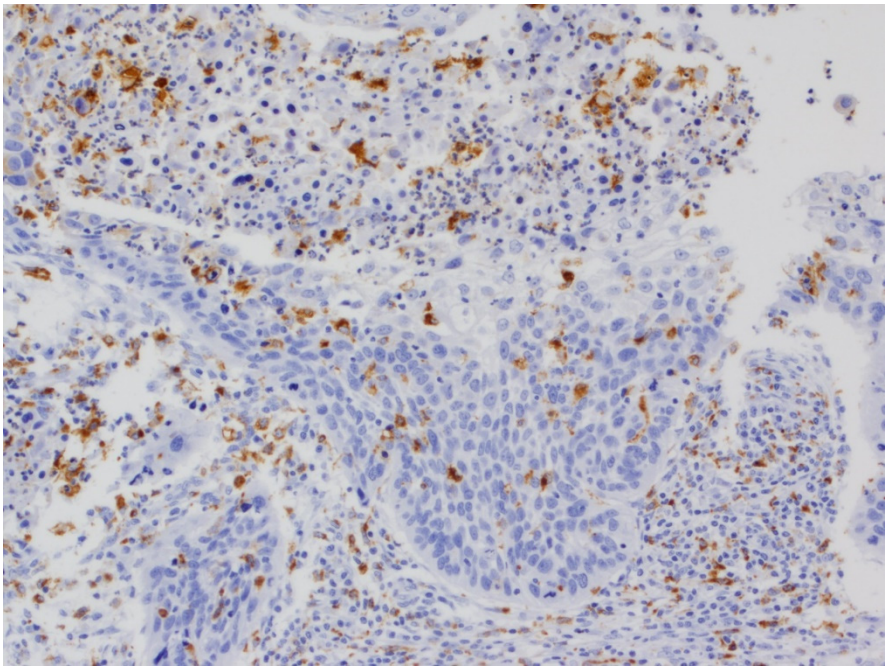
χ^2 and Fisher's exact tests were used for comparison between categorical variables. Comparison of mean values was performed with a *t* test. Disease-specific survival (DSS) was determined as the time from the initial treatment completion to the date of death due to the tumor or the presence of a nontreatable recurrence. For time-to-event analysis, survival curves were plotted using the Kaplan–Meier method. Differences between survival times of subgroups of patients were compared by using Mantel's log-rank test. All *p* values were based on the two-sided statistical analysis, and a *p* value less than 0.05 was considered as statistically significant. All statistical analyses were performed using SPSS version 18 (IBM Co., Armonk, NY, USA).

3. Results

3.1. Expression of CD68 and CD163 in OSCC Tissue Specimens

To investigate the density and distribution of TAMs in OSCC, CD68 and CD163 expression was analyzed by immunohistochemistry in 125 OSCC patients, using tissue microarrays. The mean number of $CD68^+$ macrophages in the tumor nests and the surrounding stroma was 51.11 ± 45.08 per mm^2 (range: 0.00 to 194.00) and 122.72 ± 82.49 per mm^2 (range: 9.67 to 488.67), respectively (Figure 1). CD163 staining was primarily detected in the cytoplasm of macrophages (Figure 2). The mean CD163 expression scores in the tumor nests and the tumor stroma were 31.116 ± 28.91 (range: 0.00 to 187.33) and 168.146 ± 97.591 (range: 14.33 to 476.67) cells per mm^2, respectively.

A



B

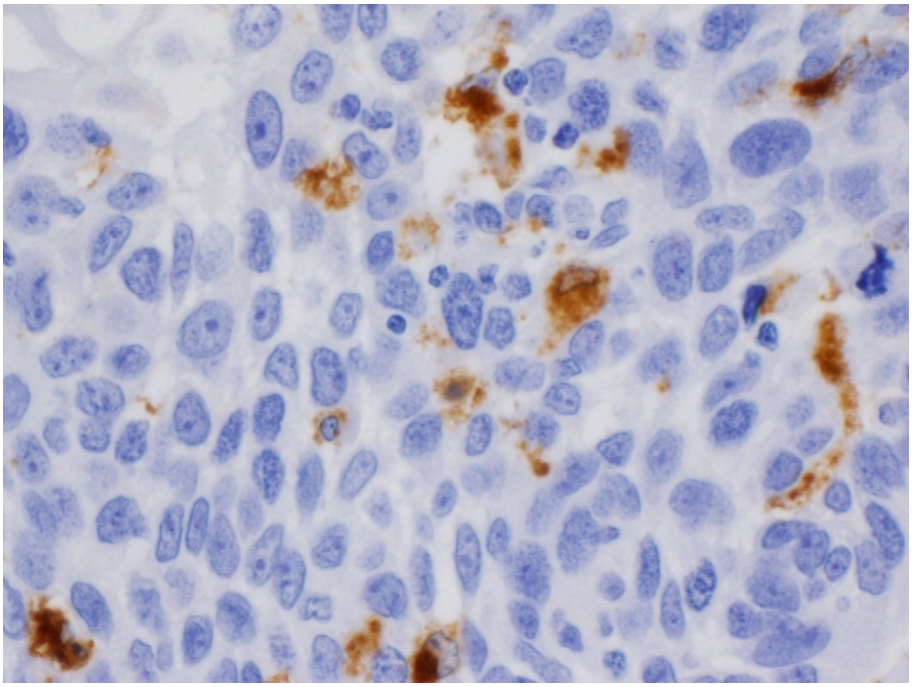
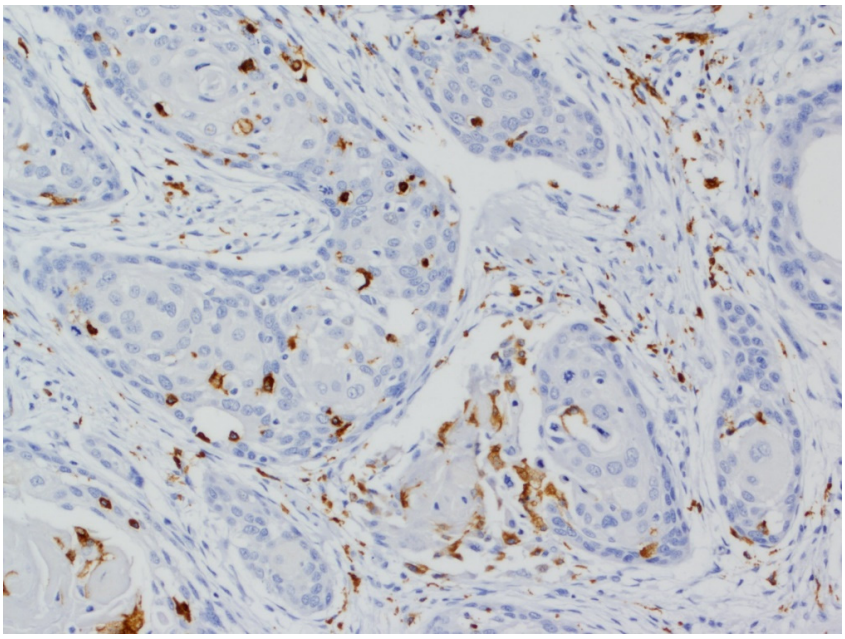


Figure 1. Immunohistochemical analysis of CD68 expression in OSCC tissue specimens. (**A**) Representative example of positive CD68 expression in tumor-associated macrophages (TAMs) both in tumor nests and in the stroma (original magnification ×100). (**B**) CD68 cytoplasmic staining in cells identified as TAMs (original magnification ×400).

A



B

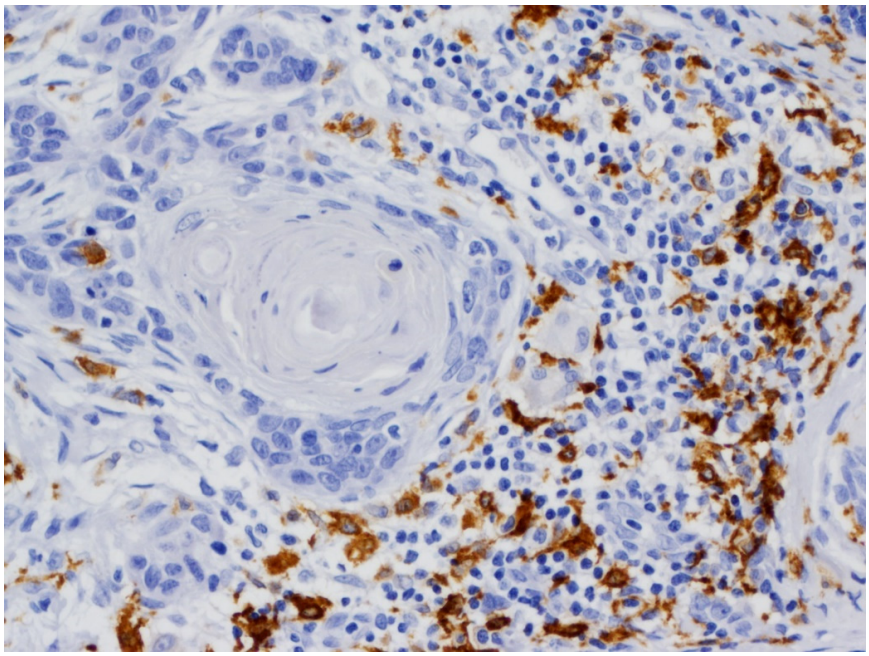


Figure 2. Immunohistochemical analysis of CD163 expression in OSCC tissue specimens (**A**) CD163 expression both in stromal and tumoral TAMs (original magnification ×100). (**B**) CD163 expression in stromal TAMs (original magnification ×400).

3.2. Associations between CD68 and CD163 Expression and Clinicopathological Variables

$CD68^+$ TAM infiltration (either categorized as mean number of positive cells or staining intensity) showed no significant associations with any of the clinicopathological parameters studied (age, gender, tobacco or alcohol consumption, TNM classification, differentiation grade, tumor location, recurrence or second primary tumors) (Table 1).

Table 1. Correlations of $CD68^+$ and $CD163^+$ macrophages with clinicopathological parameters in oral squamous cell carcinoma (OSCC) patients.

Variable	Number (%)	Stromal CD68 Mean (SD)	*p*	Tumoral CD68 Mean (SD)	*p*	Stromal CD163 Mean (SD)	*p*	Tumoral CD163 Mean (SD)	*p*
Age (Years)									
<65	77 (62)	124.34 (88.26)	0.78	49.08 (46.59)	0.56	169.10 (105.50)	0.89	28.91 (30.74)	0.28
≥65	48 (38)	120.13 (73.13)		52.80 (46.75)		166.60 (84.40)		34.64 (25.62)	
Gender									
Female	43 (34)	125.78 (96.54)	0.76	47.93 (29.74)	0.82	159.06 (90.40)	0.45	30.31 (29.74)	0.82
Male	82 (66)	121.12 (74.69)		31.53 (28.64)		172.90 (101.36)		31.53 (28.64)	
Tobacco									
No	41 (33)	134.04 (80.06)	0.28	62.22 (50.03)	0.05	182.44 (96.12)	0.25	36.12 (28.52)	0.17
Yes	84 (67)	117.20 (83.57)		45.62 (41.67)		161.16 (98.10)		28.67 (28.96)	
Alcohol Consumption									
No	56 (45)	136.11 (85.67)	0.10	57.72 (46.32)	0.13	177.82 (91.55)	0.32	35.77 (25.93)	0.10
Yes	69 (55)	111.86 (78.77)		45.67 (43.63)		160.28 (102.21)		27.33 (30.79)	
T									
T1 + 2	81 (65)	124.53 (80.99)	0.74	47.44 (40.39)	0.25	173.53 (100.19)	0.40	29.50 (25.21)	0.44
T3 + 4	44 (35)	119.40 (86.04)		57.79 (52.41)		158.22 (92.91)		34.08 (34.84)	
N									
N0	76 (61)	123.26 (80.08)	0.92	52.62 (43.72)	0.64	160.67 (84.64)	0.32	31.83 (32.04)	0.72
N+	49 (39)	121.89 (86.94)		48.80 (47.47)		179.72 (114.82)		29.99 (23.53)	
Stage									
I + II	52 (42)	125.50 (87.57)	0.75	45.62 (40.87)	0.25	166.00 (93.61)	0.83	27.16 (25.73)	0.19
III + IV	73 (58)	120.74 (79.24)		54.95 (47.71)		169.67 (100.94)		33.92 (30.84)	
Grade									
Well	80 (64)	124.05 (86.15)	0.81	46.05 (38.52)	0.13	164.89 (97.59)	0.62	29.28 (28.68)	0.34
Moderate + Poor	45 (36)	120.37 (76.44)		60.00 (54.08)		173.92 (98.41)		34.37 (29.35)	
Site									
Tongue	51 (41)	133.69 (86.47)	0.21	51.46 (46.43)	0.94	193.00 (117.24)	0.02	27.50 (22.38)	0.24
Rest	74 (59)	115.16 (79.35)		50.88 (44.47)		151.01 (77.67)		33.60 (32.58)	
Second Primary Tumor									
No	106 (85)	125.19 (87.02)	0.43	51.14 (44.08)	0.98	167.69 (98.51)	0.90	32.43 (29.68)	0.22
Yes	19 (15)	108.98 (49.70)		50.93 (52.02)		170.68 (94.83)		23.73 (23.52)	

Stromal CD163$^+$ infiltration was significantly associated with the tumor location, and higher in the tongue (p = 0.02) (Table 2). Even though no other significant associations were found with all the remaining clinicopathological variables, it is worth noting that higher stromal and tumoral CD163$^+$-infiltrating cells were observed in nonsmokers and non-alcohol-drinkers. Similarly, stromal and tumoral CD68$^+$ infiltration was higher in those patients without tobacco/alcohol habits (Table 1). Moreover, higher stromal CD68$^+$ and stromal CD163$^+$-infiltrating cells were observed in bigger tumors (T3–T4), advanced III–IV stages and moderate/poorly differentiated tumors, although differences did not attain statistical significance (Table 1).

Table 2. Correlation between CD68$^+$ and CD163$^+$ macrophages and the expression of cancer stem cell (CSC) markers in OSCC.

Factor	SOX2 Expression		p	NANOG Expression		p
	Negative	Positive		Negative	Positive	
	(n = 72, 60%)	(n = 49, 40%)		(n = 83, 68%)	(n = 39, 32%)	
Stromal CD68 (Mean, SD)	129.55 (85.06)	113.71 (75.68)	0.29	133.97 (89.65)	103.43 (61.67)	0.05
Tumoral CD68 (Mean, SD)	54.00 (44.79)	50.87 (45.56)	0.70	55.85 (47.50)	42.05 (39.40)	0.11
Stromal CD163 (Mean, SD)	179.84 (98.14)	155.55 (95.18)	0.17	183.79 (106.56)	133.53 (55.78)	0.001
Tumoral CD163 (Mean, SD)	33.65 (31.25)	29.82 (25.07)	0.47	34.42 (31.64)	24.16 (20.98)	0.03

3.3. Correlations between the Expression of CD68, CD163, and CSC Markers

The mean number of CD68$^+$-TAM infiltration in both the tumor and the surrounding stroma was higher in tumors harboring negative expression of NANOG and SOX2, although these differences did not reach statistical significance (Table 2).

Concordantly, the mean number of stromal/tumoral CD163$^+$ cells was also higher in tumors with negative expression of NANOG and SOX2. Noteworthy, CD163$^+$ TAM infiltration was inversely and significantly correlated with NANOG expression, in both the stroma (p = 0.001) and tumor nests (p = 0.03) (Table 2).

3.4. Correlation between TAM Infiltration and PD-L1 Expression

Positive PD-L1 expression in more than 10% of tumor cells was found in 18 (14.4%) in our series of OSCC samples, as previously reported [19]. CD68$^+$-infiltrating cells in both the stroma and the tumor nests was significantly associated with PD-L1-positive tumors (p = 0.04 and $p < 0.0001$, respectively; Table 3). Similarly, stromal and tumoral CD163$^+$ TAM infiltration was consistently and significantly correlated with positive PD-L1 expression (above 10% of tumor cells) (p = 0.01 and $p < 0.0001$, respectively; Table 3).

Table 3. Correlation between CD68$^+$ and CD163$^+$ macrophages and PD-L1 expression (clone 22C3) in OSCC.

Factor	Tumor PD-L1		p
	≤10%	>10%	
	(n = 104, 85%)	(n = 18, 15%)	
Stromal CD68 (Mean, SD)	114.31 (66.89)	183.59 (130.68)	0.04
Tumoral CD68 (Mean, SD)	46.39 (40.98)	86.03 (52.99)	<0.0001
Stromal CD163 (Mean, SD)	161.28 (91.29)	224.83 (114.23)	0.01
Tumoral CD163 (Mean, SD)	27.28 (23.66)	58.20 (41.02)	<0.0001

3.5. Impact of TAM Infiltration on the Survival of OSCC Patients

Follow-up information was available for 121 patients with OSCC, ranging from 6 to 230 months with a mean of 74.10 (SD: 57.08) and a median of 61 months. The median follow-up for all patients was 61 months, and for survivors was 113 months. At the end of this study, 17 (14%) patients were lost during the follow-up period, 53 (44%) patients were alive and free of recurrence, and finally, 51 (42%) of them died of cancer or showed a nontreatable recurrence.

Correlations between TAM infiltration (as mean numbers of $CD68^+$ and $CD163^+$ cells) and the disease-specific survival of OSCC patients were assessed by using different cut-off values (based on 25, 50 (median), and 75 percentiles), and survival rates calculated using the Kaplan–Meier method. No significant associations were observed between the number of $CD68^+$ and $CD163^+$-infiltrating cells and patient survival regardless the different cut-off values used (Tables S1 and S2).

4. Discussion

We herein investigated the density and tissue distribution of TAMs infiltration by immunohistochemically evaluating CD68 and CD163 staining in 125 OSCC samples, and potential relationships with the expression of NANOG and SOX2 as major CSC markers, and PD-L1 expression. CD68 is a marker of M1 and M2 activated TAMs [17]. The analysis of $CD68^+$ TAM infiltration both in tumor nests and the surrounding stroma showed no associations with clinicopathological variables and survival in our cohort of 125 OSCC patients. Lin et al. [20] studied TAMs infiltration by assessing CD68 expression using immunohistochemistry in 84 laryngeal carcinomas found to significantly correlate with tumor recurrence and poor survival. Contrasting this, Troiano et al. [17] recently conducted a meta-analysis in HNSCC, and reported no association between tumor or stromal expression of $CD68^+$ macrophages and survival. In the present study, further analysis of CD163 expression, a highly specific marker for M2 macrophages, was also performed. M2 macrophages infiltrating the tumor nests and the tumor stroma were separately scored. The number of $CD163^+$-infiltrating TAMs was not significantly associated with any clinicopathological parameters or prognosis in none of these two compartments. These results were consistent with those reported in other OSCC studies [2,16,21]. However, it has also been reported that a high number of $CD163^+$ macrophages was significantly correlated with a worse survival [5,17,22,23]. Interestingly, in some studies, only stromal expression of CD163 resulted to be significantly associated with survival, whereas no significance was detected for intratumoral expression [17]. Hence, the association between the expression of CD163 and clinical prognosis in OSCC patients remains controversial. Surprisingly, high density of TAMs in colorectal cancer seems to be associated with a better survival, while no significant correlation was observed with survival in some esophageal cancer patients [24]. The explanation to these inconsistencies may be related to differences in tumor biology, the methodologies used to assess macrophage infiltration (e.g., systemic counting or hot spot counting, counting tumoral or stromal macrophages, or both), immunohistochemical evaluation, as well as differences in the number of clinical cases among studies, tumor extension, tumor stage, and the as of yet not well understood role of M2-macrophages in tumor biology.

Cancer stem cells (CSCs) are defined as a small subpopulation of cells in the tumors that possess the ability to initiate neoplasms and sustain tumor self-renewal. It has been suggested that CSCs rely on a TME niche, demonstrated using knockdown experiments that CD163 promoted cell proliferation and stemness [11], thereby contributing to tumorigenesis. This could be, at least partially explained, because CD163 contributes to regulating the transcription of cyclin D1, CD133, ALDH1A1, NANOG, and OCT4 [11]. However, here, we consistently found higher infiltration of $CD163^+$ TAM in tumors with negative expression of the CSC markers NANOG and SOX2. In particular, stromal and tumoral $CD163^+$ TAMs were inversely and significantly associated with NANOG expression, thereby suggesting an inverse correlation between TAM infiltration and stemness in OSCC. This relationship could suggest a potential role for the CSC niche in immune evasion and OSCC progression, plausibly through paracrine signals to control TAMs infiltration in the tumor microenvironment. NANOG is

a transcription factor that is a key regulator of pluripotency in stratified epithelia [25], and mediates tumor cell proliferation, epithelial–mesenchymal transition, and escape from immune system [26]. A feedback system between CSCs and TAMs has been described, based on the recruitment of TAMs through the blood vasculature, and chemokine release by the TAMs to maintain CSCs quiescence [1]. TAMs have been traditionally considered as blood monocytes recruited from the tumor vasculature by tumor-derived signals [27], but more recently it has been shown that most tissue macrophages arise from yolk sac progenitors, with some exceptions such as those from intestines [28]. This fact raises the possibility that TAMs could have a CSCs origin in the tumor microenvironment [29]. NANOG and SOX2 maintain embryonic stem cells (ESCs) properties [30,31], as well as CSC. We herein unveil an unprecedented relationship between TAM infiltration and the oral CSC niche, perhaps reciprocally regulated by paracrine signals. Nevertheless, the precise role of TAMs in regulating CSCs and the molecular mechanisms underlying these interactions in the context of OSCC are not known. In the interplay between CSCs and TAMs, a putative role for the signal transducer and activator of transcription 3 (STAT3) pathway has been demonstrated in breast cancer models via a novel paracrine EGFR/Stat3/Sox-2 axis [32]. In the TME, hypoxia stimulates the expression of interleukin-6, which in turn induces STAT3 in CSCs, and M2 macrophage polarization [33,34]. While the ability of CSCs to induce macrophage polarization is well-established, the reciprocal effect of macrophages on the CSC phenotype remains unclear. Nusblat et al. [34] showed that in the presence of conditioned media from M2 macrophages, CSCs increased migration and collagen degradation capacities. In addition, CSCs may exert a strong immune suppressive effect by suppressing the Major Histocompatibility Complex (MHC) by macrophages in the TME, releasing the exosomal miRNAs miR-9 and miR-21, or by secreting VEGF that plays a pivotal role in tumor angiogenesis and suppression of T-cell maturation [35]. Mazzoldi et al. [36] found that, the c-Kit ligand Stem Cell Factor (SCF), produced by M2 macrophages, may favor the immune escape of tumor cells in ovarian cancer. Similarly, ovarian CSCs and macrophages reciprocally interact through the WNT/β-catenin signaling, thereby contributing to tumorigenesis, invasiveness, and immune suppression [37]. Our study demonstrated for the first time a strong inverse correlation between both stromal and tumoral $CD163^+$ TAMs and NANOG expression. According to these findings, we hypothesize that at least M2 TAM infiltration does not seem to be related to CSC niche maintenance, and perhaps could play a fundamental role in the tumor immune evasion. Nevertheless, further studies are needed to fully depict the functions, underlying mechanisms, and specific contexts linking TAMs and stemness.

Recent studies reported that $CD163^+$ macrophages were associated with PD-L1 expression on tumor cells in several human cancers [38–40]. In good agreement, we found that both stromal and tumor-infiltrating M2 macrophages were associated with PD-L1 expression, which was previously associated with poor prognosis in OSCC [19]. Experimental studies have reported that tumor cells can induce M2 macrophage phenotype increasing the expression of PD-L1 [41]. In fact, PD-L1 expression is induced after exposure to interferon-γ (IFN-γ) released by T effector cells, as well as after other signals such as TNF-α, VEGF, and CXCL8, ultimately promoting lung cancer progression [42–45]. Yagyuu et al. [46] revealed that the increase in the number of subepithelial $CD163^+$ cells was significantly correlated with the presence of high-grade oral epithelial dysplasia, and the coexpression of PD-L1 and CD163 was found in 16.6% of subepithelial cells. In hepatocellular carcinomas [47], in HPV-associated tonsil squamous cell carcinomas [48], and in gastric adenocarcinoma [38], the infiltration rates of CD163-positive cells were significantly higher in tumors that expressed PD-L1, suggesting that M2 macrophage infiltration could be used as a predictive marker for PD-L1 expression. Nevertheless, the relationship between M2 TAM and PD-L1 has not been completely clarified. Tyro3, Axl, and Mertk, collectively called TAM receptors, can activate the expression of PD-L1 in tumor cells [49], and additionally, IFN-γ secreted by inflammatory cells in the TME is associated with macrophage differentiation. Interestingly, IFN-γ induces the expression of PD-L1 in tumor cells and also the protein kinase D isoform 2 (PKD2), an important regulator of PD-L1 in OSCC [50]. Furthermore, Fujita et al. [2] reported the possibility that IL-8 produced by cancer cells stimulates $CD163^+$ M2 macrophages to

produce IL-10, which, in turn, leads to the phosphorylation of STAT3, and then IL-10/STAT3 signaling induces PD-L1 overexpression [51]. STAT3 signaling is constitutively activated in various tumors and is also involved in the regulation of monocyte-chemotactic cytokines [9]. We speculate that this relationship between CD163 and PD-L1 expression could be the link between inflammation and immune escape in oral carcinogenesis. OSCC can be promoted by chronic inflammation, such as that which occurs in periodontitis [52]. Furthermore, it has been shown that a trauma, including an incisional biopsy, can provide a microenvironmental stimulus that affects macrophage polarization influencing tumor biology, leading to a worse prognosis and increasing the risk of developing lymph node metastasis in OSCC [53]. Weber et al. [53] demonstrated a shift in macrophage polarization towards the M2 phenotype in the time interval between diagnostic incisional biopsy and definitive tumor resection in OSCC.

5. Limitations

A possible limitation of this study is that we used CD163 as an M2 macrophage marker, which is the most currently used in the literature [5]; however, it is worth noting that M1 and M2 phenotypes are the extremes of a continuous spectrum of macrophage polarization [54], and macrophages display high plasticity in response to different stimuli. Consequently, some authors [55] consider that the single staining with CD163 is not sufficient for allocating macrophages towards M2 polarization. Another potential limitation is that this is a retrospective study that cannot exclude potential selection bias.

6. Conclusions

This study thoroughly investigated the clinical relevance of TAM infiltration in OSCC, jointly evaluating CD68 and CD163 expression in both the tumor nests and surrounding stroma. Results consistently showed that stromal/tumoral TAM infiltration had no impact on patient prognosis and disease progression/outcome. Interestingly, this study uncovers an inverse relationship between $CD68^+$ and $CD163^+$ TAMs and CSC marker expression in OSCC. In particular, high infiltration of $CD163^+$ TAMs was strongly and significantly correlated with the absence of NANOG expression. Moreover, infiltration of both $CD68^+$ and $CD163^+$ TAMs was also significantly associated with a high expression of PD-L1 in the tumors, a PD-1 ligand that negatively regulates local immunity, suggesting a link between TAM infiltration and immune escape in OSCC. A more profound elucidation of the role of TAMs could lead to the identification of novel macrophage-therapeutic targets in OSCC.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/7/1764/s1, Table S1: Univariate disease-specific survival analysis according to CD68 expression in 121 OSCC patients, Table S2: Univariate disease-specific survival analysis according to CD163 expression in 121 OSCC patients.

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References

1. He, K.F.; Zhang, L.; Huang, C.F.; Ma, S.R.; Wang, Y.F.; Wang, W.M.; Zhao, Z.L.; Liu, B.; Zhao, Y.F.; Zhang, W.F.; et al. CD163+ tumor-associated macrophages correlated with poor prognosis and cancer stem cells in oral squamous cell carcinoma. *Biomed Res. Int.* **2014**, *2014*, 838632. [CrossRef]
2. Fujita, Y.; Okamoto, M.; Goda, H.; Tano, T.; Nakashiro, K.; Sugita, A.; Fujita, T.; Koido, S.; Homma, S.; Kawakami, Y.; et al. Prognostic significance of interleukin-8 and CD163-positive cell-infiltration in tumor tissues in patients with oral squamous cell carcinoma. *PLoS ONE* **2014**, *9*, e110378. [CrossRef] [PubMed]
3. Paget, S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev.* **1989**, *8*, 98–101.
4. Kumar, A.T.; Knops, A.; Swendseid, B.; Martinez-Outschoom, U.; Harshyne, L.; Philp, N.; Rodeck, U.; Luginbuhl, A.; Cognetti, D.; Johnson, J.; et al. Prognostic Significance of Tumor-Associated Macrophage Content in Head and Neck Squamous Cell Carcinoma: A Meta-Analysis. *Front. Oncol.* **2019**, *9*, 656. [CrossRef]
5. Alves, A.M.; Diel, L.F.; Lamers, M.L. Macrophages and prognosis of oral squamous cell carcinoma: A systematic review. *J. Oral Pathol. Med.* **2018**, *47*, 460–467. [CrossRef] [PubMed]
6. Xiao, M.; Zhang, J.; Chen, W.; Chen, W. M1-like tumor-associated macrophages activated by exosome-transferred THBS1 promote malignant migration in oral squamous cell carcinoma. *J. Exp. Clin. Cancer Res.* **2018**, *37*, 143. [CrossRef] [PubMed]
7. Evrard, D.; Szturz, P.; Tijeras-Raballand, A.; Astorgues-Xerri, L.; Abitbol, C.; Paradis, V.; Raymond, E.; Albert, S.; Barry, B.; Faivre, S. Macrophages in the microenvironment of head and neck cancer: Potential targets for cancer therapy. *Oral Oncol.* **2019**, *88*, 29–38. [CrossRef]
8. Kubota, K.; Moriyama, M.; Furukawa, S.; Rafiul, H.A.S.M.; Maruse, Y.; Jinno, T.; Tanaka, A.; Ohta, M.; Ishiguro, N.; Yamauchi, M.; et al. $CD163^{+}CD204^{+}$ tumor-associated macrophages contribute to T cell regulation via interleukin-10 and PD-L1 production in oral squamous cell carcinoma. *Sci. Rep.* **2017**, *7*, 1755. [CrossRef] [PubMed]
9. Mou, W.; Xu, Y.; Ye, Y.; Chen, S.; Li, X.; Gong, K.; Liu, Y.; Chen, Y.; Li, X.; Tian, Y.; et al. Expression of Sox2 in breast cancer cells promotes the recruitment of M2 macrophages to tumor microenvironment. *Cancer Lett.* **2015**, *358*, 115–123. [CrossRef]
10. Davis, M.J.; Tsang, T.M.; Qiu, Y.; Dayrit, J.K.; Freij, J.B.; Huffnagle, G.B.; Olszewski, M.A. Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in *Cryptococcus neoformans* infection. *mBio* **2013**, *4*, e00264-13. [CrossRef]
11. Chen, T.; Chen, J.; Zhu, Y.; Li, Y.; Wang, Y.; Chen, H.; Wang, J.; Li, X.; Liu, Y.; Li, B.; et al. CD163, a novel therapeutic target, regulates the proliferation and stemness of glioma cells via casein kinase 2. *Oncogene* **2019**, *38*, 1183–1199. [CrossRef] [PubMed]
12. De Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Allonca, E.; Vallina, A.; Singhania, A.; Donate-Pérez Del Molino, P.; García-Pedrero, J.M. The Emerging Role of NANOG as an Early Cancer Risk Biomarker in Patients with Oral Potentially Malignant Disorders. *J. Clin. Med.* **2019**, *8*, 1376. [CrossRef] [PubMed]
13. De Vicente, J.C.; Donate-Pérez Del Molino, P.; Rodrigo, J.P.; Allonca, E.; Hermida-Prado, F.; Granda-Díaz, R.; Rodríguez Santamarta, T.; García-Pedrero, J.M. SOX2 Expression is an Independent Predictor of Oral Cancer Progression. *J. Clin. Med.* **2019**, *8*, 1744. [CrossRef]
14. Zou, W.; Chen, L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat. Rev. Immunol.* **2008**, *8*, 467–477. [CrossRef]
15. Jiang, C.; Yuan, F.; Wang, J.; Wu, L. Oral squamous cell carcinoma suppressed antitumor immunity through induction of PD-L1 expression on tumor-associated macrophages. *Immunobiology* **2017**, *222*, 651–657. [CrossRef]
16. Haque, A.S.M.R.; Moriyama, M.; Kubota, K.; Ishiguro, N.; Sakamoto, M.; Chinju, A.; Mochizuki, K.; Sakamoto, T.; Kaneko, N.; Munemura, R.; et al. $CD206^{+}$ tumor-associated macrophages promote proliferation and invasion in oral squamous cell carcinoma via EGF production. *Sci. Rep.* **2019**, *9*, 14611. [CrossRef]
17. Troiano, G.; Caponio, V.C.A.; Adipietro, I.; Tepedino, M.; Santoro, R.; Laino, L.; Lo Russo, L.; Cirillo, N.; Lo Muzio, L. Prognostic significance of $CD68^{+}$ and $CD163^{+}$ tumor associated macrophages in head and neck squamous cell carcinoma: A systematic review and meta-analysis. *Oral Oncol.* **2019**, *93*, 66–75. [CrossRef]

18. Brierley, J.D.; Gospodarowicz, M.K.; Wittekind, C. *TNM Classification of Malignant Tumors*, 8th ed.; Wiley Blackwell: Pondicherry, India, 2017; pp. 17–21.
19. De Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Blanco-Lorenzo, V.; Allonca, E.; García-Pedrero, J.M. PD-L1 Expression in Tumor Cells is an Independent Unfavorable Prognostic Factor in Oral Squamous Cell Carcinoma. *Cancer Epidemiol. Biomarkers Prev.* **2019**, *28*, 546–554. [CrossRef]
20. Lin, J.Y.; Li, X.Y.; Tadashi, N.; Dong, P. Clinical significance of tumor-associated macrophage infiltration in supraglottic laryngeal carcinoma. *Chin. J. Cancer* **2011**, *30*, 280–286. [CrossRef] [PubMed]
21. Kouketsu, A.; Sato, I.; Oikawa, M.; Shimizu, Y.; Saito, H.; Tashiro, K.; Yamashita, Y.; Takahashi, T.; Kumamoto, H. Regulatory T cells and M2-polarized tumour-associated macrophages are associated with the oncogenesis and progression of oral squamous cell carcinoma. *Int. J. Oral Maxillofac. Surg.* **2019**, *48*, 1279–1288. [CrossRef]
22. Lu, C.F.; Huang, C.S.; Tjiu, J.W.; Chiang, C.P. Infiltrating macrophage count: A significant predictor for the progression and prognosis of oral squamous cell carcinomas in Taiwan. *Head Neck* **2010**, *32*, 18–25. [CrossRef]
23. Hadler-Olsen, E.; Wirsing, A.M. Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma: A systematic review and meta-analysis. *Br. J. Cancer* **2019**, *120*, 714–727. [CrossRef]
24. Zhang, Q.W.; Liu, L.; Gong, C.Y.; Shi, H.S.; Zeng, Y.H.; Wang, X.Z.; Zhao, Y.W.; Wei, Y.Q. Prognostic significance of tumor-associated macrophages in solid tumor: A meta-analysis of the literature. *PLoS ONE* **2012**, *7*, e50946. [CrossRef]
25. Piazzolla, D.; Palla, A.R.; Pantoja, C.; Pantoja, C.; Cañamero, M.; de Castro, I.P.; Ortega, S.; Gómez-López, G.; Dominguez, O.; Megías, D.; et al. Lineage-restricted function of the pluripotency factor NANOG in stratified epithelia. *Nat. Commun.* **2014**, *5*, 4226. [CrossRef]
26. Wang, M.L.; Chiou, S.H.; Wu, C.W. Targeting cancer stem cells: Emerging role of Nanog transcription factor. *Onco Targets Ther.* **2013**, *6*, 1207–1220. [CrossRef] [PubMed]
27. Owen, J.L.; Mohamadzadeh, M. Macrophages and chemokines as mediators of angiogenesis. *Front. Physiol.* **2013**, *4*, 159. [CrossRef]
28. Hoeffel, G.; Ginhoux, F. Ontogeny of tissue-resident macrophages. *Front. Immunol.* **2015**, *6*, 486. [CrossRef]
29. Osman, A.; Afify, S.M.; Hassan, G.; Fu, X.; Seno, A.; Seno, M. Revisiting Cancer Stem Cells as the Origin of Cancer-Associated Cells in the Tumor Microenvironment: A Hypothetical View from the Potential of iPSCs. *Cancers* **2020**, *12*, 879. [CrossRef]
30. Chew, J.L.; Loh, Y.H.; Zhang, W.; Chen, X.; Tam, W.L.; Yeap, L.S.; Li, P.; Ang, Y.S.; Lim, B.; Robson, P.; et al. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol. Cell. Biol.* **2005**, *25*, 6031–6046. [CrossRef]
31. Rodda, D.J.; Chew, J.L.; Lim, L.H.; Loh, Y.H.; Wang, B.; Ng, H.H.; Robson, P. Transcriptional Regulation of Nanog by OCT4 and SOX2. *J. Biol. Chem.* **2005**, *280*, 24731–24737. [CrossRef]
32. Yang, J.; Liao, D.; Chen, C.; Liu, Y.; Chuang, T.H.; Xiang, R.; Markowitz, D.; Reisfeld, R.A.; Luo, Y. Tumor-associated macrophages regulate murine breast cancer stem cells through a novel paracrine EGFR/Stat3/Sox-2 signaling pathway. *Stem Cells* **2013**, *31*, 248–258. [CrossRef]
33. Wu, A.; Wei, J.; Kong, L.Y.; Wang, Y.; Priebe, W.; Qiao, W.; Sawaya, R.; Heimberger, A.B. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.* **2010**, *12*, 1113–1125. [CrossRef] [PubMed]
34. Nusblat, L.M.; Carroll, M.J.; Roth, C.M. Crosstalk between M2 macrophages and glioma stem cells. *Cell Oncol* **2017**, *40*, 471–482. [CrossRef] [PubMed]
35. Ganguli, P.; Sarkar, R.R. Exploring immuno-regulatory mechanisms in the tumor microenvironment: Model and design of protocols for cancer remission. *PLoS ONE* **2018**, *13*, e0203030. [CrossRef] [PubMed]
36. Mazzoldi, E.L.; Pavan, S.; Pilotto, G.; Leone, K.; Pagotto, A.; Frezzini, S.; Nicoletto, M.O.; Amadori, A.; Pastò, A. A juxtacrine/paracrine loop between C-Kit and stem cell factor promotes cancer stem cell survival in epithelial ovarian cancer. *Cell Death Dis.* **2019**, *10*, 412. [CrossRef] [PubMed]
37. Raghavan, S.; Mehta, P.; Xie, Y.; Lei, Y.L.; Mehta, G. Ovarian cancer stem cells and macrophages reciprocally interact through the WNT pathway to promote pro-tumoral and malignant phenotypes in 3D engineered microenvironments. *J. Immunother. Cancer* **2019**, *7*, 190. [CrossRef]
38. Harada, K.; Dong, X.; Estrella, J.S.; Correa, A.M.; Xu, Y.; Hofstetter, W.L.; Sudo, K.; Onodera, H.; Suzuki, K.; Suzuki, A.; et al. Tumor-associated macrophage infiltration is highly associated with PD-L1 expression in gastric adenocarcinoma. *Gastric Cancer* **2018**, *21*, 31–40. [CrossRef]

39. Ojalvo, L.S.; Thompson, E.D.; Wang, T.L.; Meeker, A.K.; Shih, I.M.; Fader, A.N.; Cimino-Mathews, A.; Emens, L.A. Tumor-associated macrophages and the tumor immune microenvironment of primary and recurrent epithelial ovarian cancer. *Hum. Pathol.* **2018**, *74*, 135–147. [CrossRef]
40. Sumitomo, R.; Hirai, T.; Fujita, M.; Murakami, H.; Otake, Y.; Huang, C.L. PD-L1 expression on tumor-infiltrating immune cells is highly associated with M2 TAM and aggressive malignant potential in patients with resected non-small cell lung cancer. *Lung Cancer* **2019**, *136*, 136–144. [CrossRef]
41. Wen, Z.F.; Liu, H.; Gao, R.; Zhou, M.; Ma, J.; Zhang, Y.; Zhao, J.; Chen, Y.; Zhang, T.; Huang, F.; et al. Tumor cell-released autophagosomes (TRAPs) promote immunosuppression through induction of M2-like macrophages with increased expression of PD-L1. *J. Immunother. Cancer* **2018**, *6*, 151. [CrossRef]
42. Zhang, X.; Zeng, Y.; Qu, Q.; Zhu, J.; Liu, Z.; Ning, W.; Zeng, H.; Zhang, N.; Du, W.; Chen, C.; et al. PD-L1 induced by IFN-γ from tumor-associated macrophages via the JAK/STAT3 and PI3K/AKT signaling pathways promoted progression of lung cancer. *Int. J. Clin. Oncol.* **2017**, *22*, 1026–1033. [CrossRef] [PubMed]
43. Tsukamoto, M.; Imai, K.; Ishimoto, T.; Komohara, Y.; Yamashita, Y.I.; Nakagawa, S.; Umezaki, N.; Yamao, T.; Kitano, Y.; Miyata, T.; et al. PD-L1 expression enhancement by infiltrating macrophage-derived tumor necrosis factor-α leads to poor pancreatic cancer prognosis. *Cancer Sci.* **2019**, *110*, 310–320. [CrossRef]
44. Lai, Y.S.; Wahyuningtyas, R.; Aui, S.P.; Chang, K.T. Autocrine VEGF signalling on M2 macrophages regulates PD-L1 expression for immunomodulation of T cells. *J. Cell. Mol. Med.* **2019**, *23*, 1257–1267. [CrossRef]
45. Lin, C.; He, H.; Liu, H.; Li, R.; Chen, Y.; Qi, Y.; Jiang, Q.; Chen, L.; Zhang, P.; Zhang, H.; et al. Tumour-associated macrophages-derived CXCL8 determines immune evasion through autonomous PD-L1 expression in gastric cancer. *Gut* **2019**, *68*, 1764–1773. [CrossRef] [PubMed]
46. Yagyuu, T.; Hatakeyama, K.; Imada, M.; Kurihara, M.; Matsusue, Y.; Yamamoto, K.; Obayashi, C.; Kirita, T. Programmed death ligand 1 (PD-L1) expression and tumor microenvironment: Implications for patients with oral precancerous lesions. *Oral Oncol.* **2017**, *68*, 36–43. [CrossRef] [PubMed]
47. Chen, J.; Li, G.; Meng, H.; Kurihara, M.; Matsusue, Y.; Yamamoto, K.; Obayashi, C.; Kirita, T. Upregulation of B7-H1 expression is associated with macrophage infiltration in hepatocellular carcinomas. *Cancer Immunol. Immunother.* **2012**, *61*, 101–108. [CrossRef]
48. Lyford-Pike, S.; Peng, S.; Young, G.D.; Taube, J.M.; Westra, W.H.; Akpeng, B.; Bruno, T.C.; Richmon, J.D.; Wang, H.; Bishop, J.A.; 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*, 1733–1741. [CrossRef]
49. Kasikara, C.; Kumar, S.; Kimani, S.; Tsou, W.I.; Geng, K.; Davra, V.; Sriram, G.; Devoe, C.; Nguyen, K.N.; Antes, A.; et al. Phosphatidylserine Sensing by TAM Receptors Regulates AKT-Dependent Chemoresistance and PD-L1 Expression. *Mol. Cancer Res.* **2017**, *15*, 753–764. [CrossRef]
50. Chen, J.; Feng, Y.; Lu, L.; Wang, H.; Dai, L.; Li, Y.; Zhang, P. Interferon-γ-induced PD-L1 surface expression on human oral squamous carcinoma via PKD2 signal pathway. *Immunobiology* **2012**, *217*, 385–393. [CrossRef]
51. Wölfle, S.J.; Strebovsky, J.; Bartz, H.; Sähr, A.; Arnold, C.; Kaiser, C.; Dalpke, A.H.; Heeg, K. PD-L1 expression on tolerogenic APCs is controlled by STAT-3. *Eur. J. Immunol.* **2011**, *41*, 413–424. [CrossRef]
52. Kondoh, N.; Mizuno-Kamiya, M.; Umemura, N.; Takayama, E.; Kawaki, H.; Mitsudo, K.; Muramatsu, Y.; Sumitomo, S. Immunomodulatory aspects in the progression and treatment of oral malignancy. *Jpn. Dent. Sci. Rev.* **2019**, *55*, 113–120. [CrossRef]
53. Weber, M.; Moebius, P.; Büttner-Herold, M.; Amann, K.; Preidl, R.; Neukam, F.W.; Wehrhan, F. Macrophage polarisation changes within the time between diagnostic biopsy and tumour resection in oral squamous cell carcinomas—An immunohistochemical study. *Br. J. Cancer* **2015**, *113*, 510–519. [CrossRef] [PubMed]
54. Mosser, D.M.; Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **2008**, *8*, 958–969. [CrossRef] [PubMed]
55. Barros, M.H.; Hauck, F.; Dreyer, J.H.; Kempkes, B.; Niedobitek, G. Macrophage polarisation: An immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS ONE* **2013**, *8*, e80908. [CrossRef] [PubMed]

Supplementary Materials

Macrophages in Oral Carcinomas: Relationship with Cancer Stem Cell Markers and PD-L1 Expression

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Table S1. Univariate disease-specific survival analysis according to CD68 expression in 121 OSCC patients.

CD68 Percentile	No. Cases	Censored Patients (%)	Disease-Specific Survival (95% CI)	*p*
Stromal CD68				
< P25	30	16 (53)	110.50 (80.02–140.97)	0.68
≥ P25	95	56 (59)	135.15 (112.95–157.34)	
Tumoral CD68				
< P25	31	15 (48)	117.17 (82.60–151.73)	0.48
≥ P25	94	57 (61)	139.06 (116.49–161.64)	
Stromal CD68				
< P50	63	36 (57)	134.67 (108.04–161.30)	0.88
≥ P50	62	36 (58)	129.00 (102.98–155.03)	
Tumoral CD68				
< P50	63	31 (49)	117.89 (91.90–143.88)	0.12
≥ P50	62	41 (66)	125.25 (105.02–145.48)	
Stromal CD68				
< P75	94	57 (61)	139.77 (117.03–162.50)	0.18
≥ P75	31	15 (48)	94.65 (67.23–122.07)	
Tumoral CD68				
< P75	93	50 (54)	125.12 (102.80–147.44)	0.19
≥ P75	32	22 (69)	129.73 (102.30–157.15)	

Table S2. Univariate disease-specific survival analysis according to CD163 expression in 121 OSCC patients.

CD163 Percentile	No. Cases	Censored Patients (%)	Disease-Specific Survival (95% CI)	*p*
Stromal CD163				
< P25	31	15 (48)	101.64 (71.20–132.08)	0.13
≥ P25	90	55 (61)	142.31 (120.14–164.48)	
Tumoral CD163				
< P25	29	15 (52)	126.47 (91.23–161.72)	0.73
≥ P25	92	55 (60)	138.55 (115.94–161.16)	
Stromal CD163				
< P50	60	32 (53)	110.95 (89.28–132.62)	0.29
≥ P50	61	38 (62)	142.93 (116.16–169.71)	
Tumoral CD163				
< P50	61	36 (59)	138.64 (111.79–165.49)	0.71
≥ P50	60	34 (57)	105.36 (86.33–124.38)	
Stromal CD163				
< P75	89	50 (56)	134.57 (112.24–156.89)	0.34
≥ P75	32	20 (62)	144.42 (112.05–176.79)	
Tumoral CD163				
< P75	92	50 (54)	128.50 (106.53–150.47)	0.31
≥ P75	29	20 (69)	122.14 (95.17–149.12)	

p values were estimated using the log-rank test. 95% CI: 95% Confidence Interval.

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Article

Tumor-Infiltrating CD20$^+$ B Lymphocytes: Significance and Prognostic Implications in Oral Cancer Microenvironment

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Simple Summary: The complex interplay between the different cellular components in the tumor microenvironment (TME) dynamically modulates the antitumor immune response. This study investigates the prognostic relevance of CD20$^+$ tumor-infiltrating B lymphocytes in oral squamous cell carcinoma (OSCC), and also possible relationships with other immune subtypes and key players within the oral TME.

Abstract: Immunohistochemical analysis of stromal/tumoral CD20$^+$ B lymphocytes was performed in 125 OSCC patients. Correlations with immune profiles CD4$^+$, CD8$^+$, and FOXP3$^+$ tumor-infiltrating lymphocytes (TILs), tumoral PD-L1, and stem-related factors NANOG and SOX2 were assessed, and also associations with clinical data and patient survival. There was a strong positive correlation between the infiltration of CD20$^+$ B lymphocytes and other immune profiles (i.e., CD4$^+$, CD8$^+$, and FOXP3$^+$ TILs, and CD68$^+$ and CD163$^+$ macrophages) both in stroma and tumor nests. Strikingly, CD20$^+$ TILs were inversely correlated with NANOG/SOX2 expression. Stromal CD20$^+$ TILs were significantly associated with T classification and second primary tumors. A stratified survival analysis showed that tumoral CD20$^+$ TILs were significantly associated with prognosis in male and younger patients, with tobacco or alcohol consumption, high tumoral CD8$^+$ TILs, low tumoral infiltration by CD68$^+$ macrophages, positive PD-L1 expression, and negative NANOG/SOX2. Multivariate Cox analysis further revealed clinical stage and tumoral CD20$^+$ TILs independently associated with disease-specific survival (HR = 2.42, $p = 0.003$; and HR = 0.57, $p = 0.04$, respectively). In conclusion, high CD20$^+$ TIL density emerges as an independent good prognostic factor in OSCC, suggesting a role in antitumor immunity. This study also uncovered an inverse correlation between CD20$^+$ TILs and CSC marker expression.

Keywords: CD20; tumor-infiltrating B lymphocytes; prognosis; oral squamous cell carcinoma; immunohistochemistry

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a heterogeneous malignant disease whose complex and dynamic progression could be the result of multiple genetic or epigenetic alterations among distinct cell types within the tumor as well as the surrounding tumor microenvironment (TME) [1,2]. In the TME, besides stromal cells, there are various populations of tumor-infiltrating lymphocytes (TILs), including B cells which may account for up to 25% to 40% of all cells in different tumor types [3]. These data suggest that B cells may play crucial roles in antitumor immunity in cooperation with other immune cells, such as T lymphocytes and macrophages [4]. Tumor-infiltrating lymphocytes in the TME may alter tumor biology, and the type, density, and location of these cells could influence tumor progression. The number of TILs in the TME may be explained by three possibilities. Firstly, the number of immune cells is associated with the immunogenicity of a specific tumor which may induce local activation and proliferation of immune cells. Secondly, an increase in B lymphocytes during tumorigenesis and tumor progression may be due to an increase in the load of associated antigens on tumor cells, antigens that can induce local activation and proliferation of the immunocytes. Thirdly, the increase in TIL number may reflect enhanced cytokine production by the tumor cells [5]. Cells of innate immunity, mainly tumor-associated macrophages (TAMs) can be polarized into antitumoral M1 macrophages, expressing CD68, and protumoral and immunosuppressive M2 macrophages, characterized by coexpression of CD68 and CD163 [6]. At the same time, the adaptive immune response is orchestrated by T and B lymphocytes. T cells are divided into $CD8^+$ T cells and $CD4^+$ T helper (Th) cells [7]. $CD8^+$ cytotoxic T lymphocytes (CTLs), usually supported by $CD4^+$ T helper 1 (Th1) cells [8], are considered the major effector immune cells directed against tumor cells [9]. On the contrary, regulatory T cells (Tregs), a subset of Th cells that express the transcription factor FOXP3, take part in the immune tolerance by suppressing self-antigen reactive T cells, thereby promoting the immune evasion of cancer [10].

Cancer cells express modified proteins or tumor-specific antigens that usually elicit an immune response [11]. Upon binding of an antigen, and supported by cytokines released by Th cells, B cells can differentiate into plasma cells and produce antibodies specific to the antigen that trigger their differentiation [7]. Beyond their role in humoral immunity, B cells can also modulate T cell responses to antigens [12]. In the T-helper 1 (Th1)/Th2 paradigm, the activity of cytotoxic T cells is supported by the Th1 lineage and M1 macrophages, while contrarily Tregs, B lymphocytes, and M2 macrophages are more closely related to the tumor-promoting Th2 response [13]. Programmed cell death ligand-1 (PD-L1) is a cell-surface glycoprotein that induces T-cell anergy and apoptosis by activating the PD-1 receptor on T lymphocytes [14,15]. Mizoguchi et al., [16] have described a subpopulation of B cells called regulatory B cells (Bregs) that exhibit tumor-promoting effects by their ability to suppress T cell responses through the secretion of cytokines such as IL-10 and TGF-β as well as to upregulate immune-regulatory ligands such as PD-L1, inducing $CD4^+$ T cell death through the expression of FasL [17–19].

According to the cancer stem cells (CSCs) model, tumors are composed of heterogeneous cellular components including a rare subpopulation (0.01–10% of cells within the tumor [18]) exhibiting CSC self-renewal and plasticity. This subset of CSC pluripotent cells has been demonstrated to play crucial roles in tumor initiation, progression, aggressiveness, heterogeneity, metastasis, recurrence, and treatment resistance [19–21]. CSCs bear stemness properties supported by gene master regulators, such as NANOG and SOX2 [22], involved in OSCC tumorigenesis, poor differentiation, and bad prognosis [23–25]. Furthermore, CSCs possess immunoregulatory properties [26] and can inhibit $CD8^+$ T cells or induce Tregs and myeloid-derived suppressor cells (MDSCs) [27].

From a prognostic point of view, stromal expression of $CD163^+$ macrophages in head and neck squamous cell carcinomas (HNSCCs) [6], and infiltrating T lymphocytes in OSCC have been consistently relevant [28,29]. However, the role of infiltrating B lymphocytes in these carcinomas has not been fully clarified [30], as both positive and negative impacts of B cells on tumor progression and prognosis have been reported [31,32].

CD20, a pan B-cell marker, is a membrane-embedded phosphoprotein, encoded by the *MS4A1* gene, expressed in B lymphocytes, and typically lost when B cells become plasma cells. This marker has been extensively and widely used for the evaluation of inflammatory infiltrate in different solid tumors [33]. We herein investigated the clinical relevance of infiltrating B lymphocytes, by means of CD20 immunohistochemical evaluation in both the stroma and tumor nests using a large homogeneous cohort of 125 OSCC specimens. Correlations with clinicopathological features and impact on patient prognosis were assessed, and also possible relationships with other important cellular components in the oral TME, such as immune subtypes (i.e., T cells, macrophages), as well as tumoral PD-L1 expression and two important CSCs-related factors NANOG and SOX2.

2. Results

2.1. Immunohistochemical Analysis of CD20⁺ TILs in OSCC Tissue Specimens and Associations with Other Immune Subtypes

The mean number of $CD20^+$ B cells in the tumor nests and the surrounding stroma was 18.67 ± 1.63 per mm^2 (range: 0.00 to 18.67) and 42.47 ± 78.63 per mm^2 (range: 0.00 to 426.67), respectively. Representative images of stromal and tumoral $CD20^+$ TILs detected in OSCC specimens are shown in Figure 1. The mean numbers for other immune subtypes, such as $CD4^+$ and $CD8^+$ TILs, $FOXP3^+$ Tregs, $CD68^+$, and $CD163^+$ macrophages are summarized in Table S1.

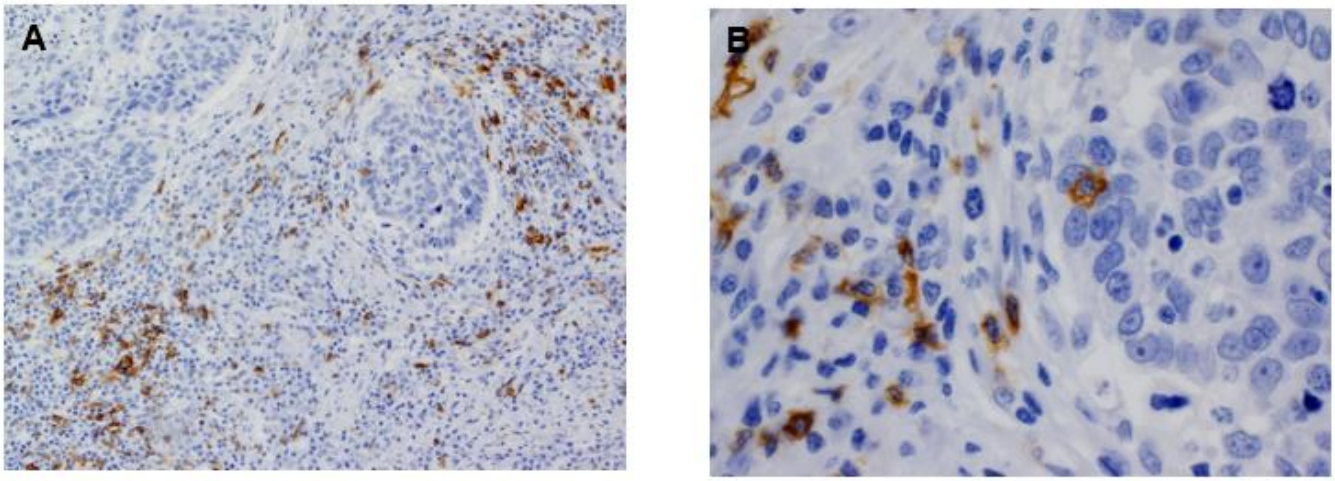
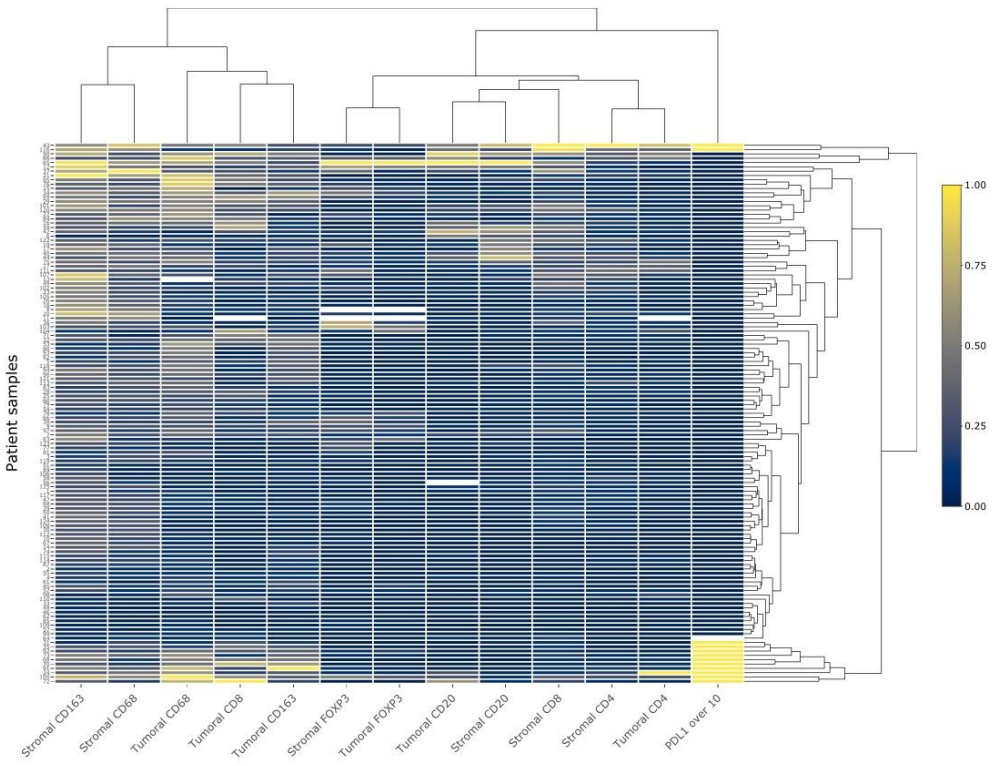


Figure 1. Immunohistochemical analysis of $CD20^+$ B lymphocytes in oral squamous cell carcinoma (OSCC) tissue specimens. (Original magnification × 200 in panel (**A**), × 400 Figure in panel (**B**)).

There was a strong positive correlation between the infiltration of $CD20^+$ B cells, cytotoxic $CD4^+$ and $CD8^+$ T cells, regulatory $FOXP3^+$ T cells, and $CD68^+$ and $CD163^+$ macrophages in both stroma and tumor nests (Table 1). We also performed a hierarchical clustering analysis, shown in Figure 2A. These data further strengthen the close relationship between B cell and T cell infiltration. In particular, tumoral and stromal $CD20^+$ B cell infiltration was more closely related to cytotoxic $CD4^+$ and $CD8^+$ TILs.

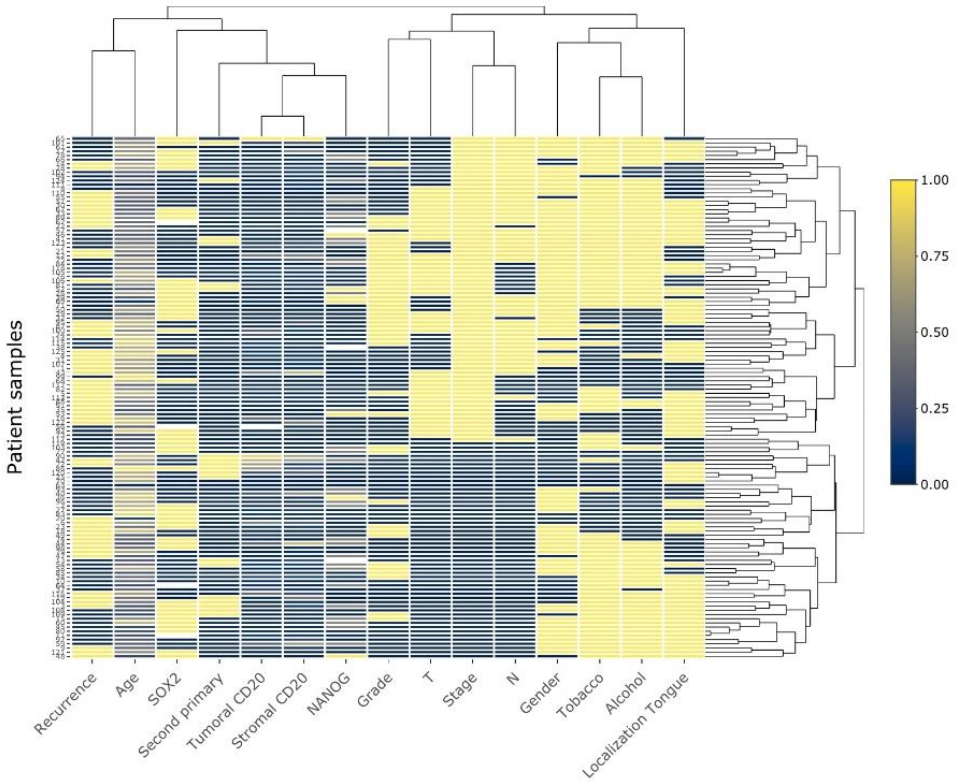
Table 1. Correlations between the mean numbers of $CD20^+$ B cells with $CD4^+$, $CD8^+$, and $FOXP3^+$ TILs, and $CD68^+$ and $CD163^+$ macrophages infiltrating both the tumor nests and surrounding stroma. The Spearman's Rho coefficients and the corresponding *p* values are shown.

Variable	Stromal $CD20^+$ (Mean)	Tumoral $CD20^+$ (Mean)
Stromal $CD8^+$ (mean)	0.772 <0.001	0.624 <0.001
Tumoral $CD8^+$ (mean)	0.404 <0.001	0.358 <0.001
Stromal $CD4^+$ (mean)	0.662 <0.001	0.488 <0.001
Tumoral $CD4^+$ (mean)	0.353 <0.001	0.362 <0.001
Stromal $FOXP3^+$ (mean)	0.326 <0.001	0.328 <0.001
Tumoral $FOXP3^+$ (mean)	0.253 0.005	0.246 0.006
Stromal $CD68^+$ (mean)	0.354 <0.001	0.377 <0.001
Tumoral $CD68^+$ (mean)	0.259 0.004	0.261 0.004
Stromal $CD163^+$ (mean)	0.418 <0.001	0.373 <0.001
Tumoral $CD163^+$ (mean)	0.190 0.03	0.228 0.01



(**A**)

Figure 2. *Cont.*



(**B**)

Figure 2. Heatmap and hierarchical clustering analysis to classify the different variables studied. (**A**) Heatmap displaying the IHC scores for the different immune profiles in both tumoral and stromal compartments. (**B**) Heatmap displaying the clinical characteristics, IHC scores for $CD20^+$ B cell infiltration, Nanog, and SOX2 expression. All data sets were normalized.

2.2. Associations between $CD20^+$ TILs and Clinicopathological Variables

Stromal $CD20^+$ TIL infiltration was significantly associated with T classification and with the occurrence of second primary tumors. In fact, the mean number of stromal $CD20^+$ B cells was significantly lower in T3 and T4 tumors compared with T1 and T2, as well as in those patients who did not develop a second primary oral carcinoma (Table 2). Hierarchical clustering analysis is also shown in Figure 2B, further indicating that $CD20^+$ B cell infiltration was closely related with the development of a second primary tumor, NANOG and SOX2 expression or the presence of tumor recurrence, whereas expectedly T, grade, N, and stage were closely related, or tobacco and alcohol consumption.

Stromal $CD20^+$ B cell infiltration was not associated with any of the remaining clinicopathological parameters studied. Tumoral $CD20^+$ infiltration was not associated with any clinicopathological variables. It was not possible to calculate the ratios of infiltrating $CD20^+$ B cells and the remaining TILs and macrophage markers for all the cases as various markers showed negative expression (scored as 0). In fact, stromal/tumoral $CD20^+/CD8^+$ ratios could thus be respectively determined in 125 and 118 cases, stromal/tumoral $CD20^+/CD4^+$ ratios respectively determined in 125 and 95 cases, stromal/tumoral $CD20^+/FOXP3^+$ ratios in 106 and 77 cases, stromal/tumoral $CD20^+/CD68^+$ ratios in 125 and 123 cases, and finally, stromal/tumoral $CD20^+/CD163^+$ ratios were determined in 125 and 122 cases, respectively. Stromal $CD20^+/CD8^+$ ratio was significantly associated with T classification and second primary tumors, being consistently lower in T3 and T4 tumors and in patients who developed second primary malignancies. The stromal $CD20^+/CD4^+$ ratio was significantly associated with the development of second primary tumors. Stromal and tumoral $CD20^+/FOXP3^+$ ratios were significantly associated with patient age, with both being lower in patients younger than 65 years. In addition, the stromal $CD20^+/FOXP3^+$

ratio was significantly associated with tumor recurrence, being lower in non-recurrent cases (Table 3).

Table 2. Associations between stromal/tumoral $CD20^+$ B infiltration and the clinicopathological parameters in the cohort of 125 OSCC patients.

Variable	Number	Stromal $CD20^+$ Mean (SD)	*p*	Tumoral $CD20^+$ Mean (SD)	*p*
Age (years)					
<65	77	35.06 (74.34)	0.35	1.26 (2.90)	0.14
≥65	48	54.36 (84.50)		2.21 (3.92)	
Gender					
Female	43	46.61 (82.46)	0.81	2.42 (4.31)	0.27
Male	82	40.30 (76.97)		1.22 (2.66)	
Tobacco consumption					
No	41	48.43 (77.72)	0.25	2.40 (3.99)	0.06
Yes	84	39.56 (79.37)		1.26 (2.95)	
Alcohol consumption					
No	56	42.01 (74.87)	0.90	2.14 (3.88)	0.09
Yes	69	42.84 (82.09)		1.11 (2.74)	
pT classification					
T1 + T2	81	48.91 (83.98)	0.02	1.95 (3.76)	0.10
T3 + T4	44	30.62 (66.97)		1.02 (2.29)	
pN classification					
N0	76	41.03 (74.05)	0.63	1.73 (3.42)	0.70
N+	49	44.70 (86.00)		1.47 (3.26)	
Stage					
I + II	52	51.18 (83.82)	0.31	2.22 (3.90)	0.15
III + IV	73	36.26 (74.68)		1.20 (2.84)	0.15
Grade					
Well	80	52.93 (91.88)	0.06	1.99 (3.94)	0.31
Moderate + Poor	45	23.88 (41.41)		1.00 (1.78)	
Site					
Tongue	51	57.11 (103.48)	0.37	2.09 (4.26)	0.74
Other	74	32.38 (53.99)		1.30 (2.51)	
Recurrence					
No	71	39.68 (76.77)	0.36	1.44 (3.32)	0.90
Yes	54	46.14 (81.59)		1.87 (3.39)	
Second primary tumor					
No	106	37.61 (76.86)	0.02	1.30 (2.71)	0.24
Yes	19	69.56 (84.97)		3.45 (5.46)	

All *p*-values were calculated using the Mann–Whitney U test.

Table 3. Associations between stromal and tumoral CD20/CD8, CD20/CD4, and CD20/FOXP3 ratios and clinicopathological parameters in the cohort of 125 OSCC patients.

Variable Mean (SD)	Stromal CD20/CD8 Ratio	*p*	Tumoral CD20/CD8 Ratio	*p*	Stromal CD20/CD4 Ratio	*p*	Tumoral CD20/CD4 Ratio	*p*	Stromal CD20/FOXP3 Ratio	*p*	Tumoral CD20/FOXP3 Ratio	*p*
Age (years)												
<65	0.50 (3.06)	0.50	0.04 (0.10)	0.35	0.71 (1.10)	0.88	1.10 (3.97)	0.92	3.50 (8.34)	0.01	0.40 (0.75)	0.01
≥65	0.20 (0.24)		0.21 (0.60)		0.78 (1.18)		0.73 (2.25)		16.38 (42.95)		1.99 (4.30)	
Gender												
Female	0.16 (0.18)	0.88	0.08 (0.23)	0.46	0.66 (1.07)	0.61	0.69 (2.30)	0.91	14.03 (45.37)	0.76	2.05 (4.57)	0.55
Male	0.50 (2.97)		0.13 (0.47)		0.78 (1.16)		1.08 (3.79)		5.88 (12.30)		0.46 (0.68)	
Tobacco												
No	0.17 (0.16)	0.47	0.07 (0.23)	0.50	0.69 (0.96)	0.68	0.93 (2.51)	0.57	17.25 (45.85)	0.16	1.62 (4.12)	0.20
Yes	0.48 (2.93)		0.13 (0.46)		0.76 (1.20)		0.94 (3.70)		4.34 (11.08)		0.68 (1.72)	
Alcohol												
No	0.15 (0.16)	0.81	0.12 (0.44)	0.32	0.62 (1.00)	0.50	0.65 (2.06)	0.92	12.71 (39.44)	0.46	1.50 (3.64)	0.06
Yes	0.57 (3.23)		0.10 (0.36)		0.83 (1.21)		1.22 (4.19)		5.16 (12.28)		0.60 (1.80)	
pT												
T1 + T2	0.52 (2.98)	0.03	0.15 (0.48)	0.07	0.86 (1.26)	0.13	0.87 (2.27)	0.15	7.60 (15.79)	0.21	1.25 (3.27)	0.75
T3 + T4	0.12 (0.15)		0.03 (0.09)		0.51 (0.79)		1.08 (4.78)		11.02 (46.03)		0.40 (0.65)	
pN												
N0	0.51 (3.08)	0.47	0.07 (0.29)	0.41	0.66 (1.02)	0.53	0.62 (1.88)	0.69	9.75 (34.76)	0.29	1.25 (3.42)	0.82
N+	0.18 (0.23)		0.17 (0.52)		0.85 (1.28)		1.42 (4.70)		7.10 (14.57)		0.56 (0.86)	
Stage												
I + II	0.70 (3.72)	0.35	0.11 (0.35)	0.93	0.76 (1.15)	0.77	0.78 (2.23)	0.14	6.86 (14.90)	0.94	1.61 (3.93)	0.64
III + IV	0.15 (0.20)		0.11 (0.43)		0.72 (1.11)		1.04 (3.86)		10.15 (35.83)		0.46 (0.75)	
Grade												
Well	0.52 (3.00)	0.29	0.08 (0.28)	0.22	0.78 (1.14)	0.19	0.73 (1.97)	0.19	11.36 (34.96)	0.09	1.35 (3.54)	0.69
Moderate + Poor	0.14 (0.17)		0.16 (0.55)		0.67 (1.10)		1.27 (4.72)		4.20 (10.36)		0.47 (0.56)	
Site												
Tongue	0.71 (3.76)	0.23	0.08 (0.28)	0.64	0.73 (1.17)	0.74	1.11 (4.39)	0.33	4.68 (10.99)	0.93	0.94 (2.21)	0.29
Other	0.15 (0.20)		0.13 (0.47)		0.75 (1.10)		0.79 (2.07)		11.38 (35.62)		1.04 (3.15)	
Recurrence												
No	0.16 (0.22)	0.46	0.14 (0.49)	0.91	0.74 (1.15)	0.64	0.53 (1.60)	0.90	6.69 (16.84)	0.01	0.66 (1.86)	0.37
Yes	0.67 (3.65)		0.07 (0.21)		0.73 (1.10)		1.43 (4.58)		11.59 (39.79)		1.56 (3.83)	
Second primary tumor												
No	0.40 (2.61)	0.01	0.12 (0.43)	0.38	0.66 (1.08)	0.02	0.99 (3.58)	0.23	7.88 (29.81)	0.10	0.88 (2.65)	0.57
Yes	0.26 (0.21)		0.07 (0.13)		1.16 (1.28)		0.68 (1.01)		12.87 (22.13)		1.58 (3.42)	

All *p*-values were calculated using the Mann–Whitney U test.

Stromal $CD20^+/CD68^+$ and $CD20^+/CD163^+$ ratios were significantly and consistently associated with T classification, tumor grade, and second primary tumors. Both ratios were lower in T3 and T4 tumors, and in cases that did not develop a second primary tumor. In marked contrast, while the stromal $CD20^+/CD68^+$ ratio was higher in well-differentiated tumors, the stromal $CD20^+/CD163^+$ ratio showed a higher value in moderate and poorly differentiated tumors (Table S2).

2.3. Associations between CD20⁺ TILs, CSC Markers, and PD-L1

Positive staining of SOX2 and NANOG were respectively detected in 49 (40%) and 39 (32%) cases, as previously reported [24,25]. Noteworthy, we found concordant results when assessing the correlation between CD20⁺ B cell infiltration and the expression of these two CSC markers. The mean number of CD20⁺ TILs in both the tumor and the surrounding stroma was consistently higher in tumors harboring negative expression of SOX2 and NANOG, although this inverse relationship only reached statistical significance for the SOX2 marker ($p = 0.008$) (Table 4).

Table 4. Association between CD20⁺ TILs, PD-L1, and CSCs markers.

Factor	Stromal CD20⁺ (Mean, SD)	*p*	Tumoral CD20⁺ (Mean, SD)	*p*
SOX2				
Negative (*N* = 72, 60%)	48.47 (77.74)	0.29	1.86 (3.38)	0.008
Positive (*N* = 49, 40%)	36.23 (82.77)		1.31 (3.39)	
NANOG				
Negative (*N* = 83, 68%)	44.10 (77.05)	0.14	1.86 (3.67)	0.36
Positive (*N* = 39, 32%)	40.23 (85.20)		1.23 (2.65)	
PD-L1				
≤10% (*N* = 104, 83%)	43.96 (79.55)	0.85	1.46 (3.30)	0.21
>10% (*N* = 18, 15%)	39.27 (80.81)		2.70 (3.67)	

Tumoral PD-L1 expression in more than 10% of tumor cells was previously defined as clinically relevant [34] and was detected in 18 (15%) cases in our OSCC cohort. CD20⁺ infiltrating B cells in the tumor nests were higher in positive PD-L1 tumors, whereas stromal CD20⁺ TILs showed a higher density in negative PD-L1 tumors (i.e., expression in less than 10% of tumor cells). However, none of these relationships were statistically significant (Table 4).

2.4. Impact of CD20⁺ TIL Infiltration on the Survival of OSCC Patients

Follow-up data were available for 121 patients (range 6–230 months, mean 74 and median 61 months). Patients with smaller (T1 and T2) tumors as well as patients without neck lymph node metastasis and in I and II clinical stages showed a significantly improved disease-specific survival (DSS) (Table S3) ($p = 0.001$, $p = 0.01$, and $p = 0.02$, respectively). Stromal infiltrating CD20⁺ TILs did not show a significant relationship with survival. However, a significant relationship was detected between the low density of tumoral CD20⁺ B cells and a lower DSS ($p = 0.04$) (Figure 3).

The different ratios between stromal/tumoral CD20⁺ and the other TIL and macrophage markers did not show any significant association with survival (Table S3).

We also performed a stratified univariate Kaplan–Meier analysis according to tumoral CD20 TIL infiltration (Table S4). Low density of tumoral CD20⁺ B cells was significantly associated with a reduced DSS in patients younger than 65 years ($p = 0.03$), male patients ($p = 0.02$), and in cases with tobacco ($p = 0.004$), or alcohol consumption ($p = 0.005$).

Our data showed that a low density of tumoral CD20⁺ TILs were associated with a poorer DSS in patients with positive tumoral PD-L1 expression ($p = 0.008$), negative SOX2 ($p = 0.01$) or negative NANOG expression ($p = 0.03$), in cases with high infiltration of CD8⁺ TILs in the tumor nests ($p = 0.03$), and in cases with low density of stromal CD4⁺ and tumoral CD68⁺ cells ($p = 0.03$) (Table S4). Finally, on multivariate analysis, clinical stage (stages I–II vs. III–IV), and tumoral CD20⁺ infiltrating cells (low vs. high density) were the only parameters independently associated with DSS (HR = 2.42, $p = 0.003$; HR = 0.57, $p = 0.04$, respectively).

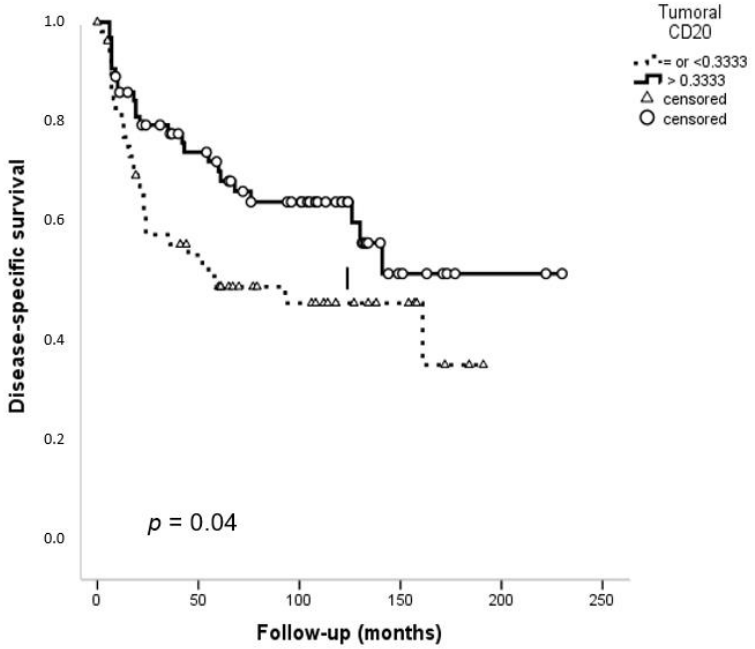


Figure 3. Kaplan–Meier disease-specific survival curves in the cohort of 125 OSCC patients categorized by tumoral CD20$^+$ B cell infiltration. Median values were used as cut-off points. *p* values were estimated using the log-rank test.

3. Discussion

Lymphocyte infiltration into the TME is generally considered to represent host immunity against tumors [35]. To date, most studies have evaluated the relevance of infiltrating T cells in the TME, while less attention has been focused on the significance of B cells in OSCC with conflicting results [7,36,37]. Nonetheless, there is increasing evidence that infiltrating B cells may influence the TME towards tumorigenic or cytotoxic [38]. In tumors such as cutaneous melanoma, tumor-infiltrating B cells have been positively associated with patient prognosis in some studies [39,40], whereas this association was demonstrated in others [5], and even a negative association has also been reported [41]. Here, we found that CD20$^+$ TIL infiltration in the tumor, but not the stroma, is associated with a better outcome. This finding is in agreement with other reports [11,15,33,35,42,43], suggesting that an immune response may be mediated by B lymphocytes. Furthermore, our study revealed that CD20$^+$ TIL density in the tumor nests was an independent prognostic factor in this OSCC patient cohort. A stratified survival analysis showed that tumoral CD20$^+$ TILs were significantly associated with a better prognosis in patients younger than 65 years, male patients, with tobacco or alcohol consumption, positive PD-L1 expression in more than 10% of tumor cells, tumors with negative expression of SOX2 or NANOG, high density of tumoral CD8$^+$ TILs, low density of tumoral infiltration by CD68$^+$ macrophages and low stromal infiltration by CD4$^+$ TILs. Nielsen et al. [44] found that CD20$^+$ B cells co-localized with CD8$^+$ T cells in high-grade serous ovarian cancer, raising the possibility that B lymphocytes may act as antigen-presenting cells to facilitate the antitumoral T cell cytolytic response. Several mechanisms can explain the divergent roles that B cells play in tumor immunology comparable to a double-edged sword. On one hand, tumor-infiltrating B cells can secrete lymphotoxin, which induces angiogenesis, and activates NF-κB signaling and STAT3 in the cancer cells thereby promoting tumor growth [3]. Furthermore, extracellular vesicles derived from tumors are capable of activating B cells to produce antibodies which, in turn, can form immune complexes [45], activating $F_{C\gamma}$ receptors on myeloid cells, and suppressing antitumor CD4$^+$ and CD8$^+$ T cell responses [3]. On the other hand, antibodies against tumor-specific antigens produced by plasma cells have different roles: antibodies mediate complement-dependent tumor cell lysis [44], Fc-mediated phagocytosis by macrophages, as well as antibody-dependent cellular cytotoxicity by natural killer (NK) cells [3,46]. In

addition, antibody-coated tumor cells could also be processed by dendritic cells, which in turn present tumor antigens to $CD4^+$ T cells and cross-present antigens to $CD8^+$ T cells, aiding in the immune response against tumor cells [33,47]. Finally, lymphotoxin produced by B cells has an additional role in promoting the formation of ectopic tertiary lymphoid organs, which correlate directly with a positive outcome in many cancers [3,48,49]. B cells may concentrate on TME or form tumor-associated immune aggregates that include tertiary lymphoid structures (TLS), similar to lymph nodes [50]. TLS can be localized around the tumor or even within the tumor itself, showing different stages of maturation defined by the absence or presence of one or more germinal centers surrounded by T cells, dendritic and plasma cells, along with lymphatic and blood vessels [51]. B cells could be involved in the formation of TLS by producing CXCL13 and lymphotoxin [50,52], and also in the maturation of an antitumoral humoral response in the TME [50]. TLS have been reported in various types of cancers including lung, breast, pancreas, colorectal, and oral cancer [53,54]. These structures have been associated with positive prognostic value in some tumors [55,56], such as high-grade serous ovarian cancer, where tumor infiltration by $CD8^+$ T cells only showed prognostic value when it was combined with the presence of TLS and a high count of plasma cells, $CD4^+$ T cells and $CD20^+$ B cells [56]. In oral cancer, higher grades of TLS have also been associated with improved survival [57]; however, good and poor clinical outcomes have been correlated with different cellular components of TLS, such as dendritic cells, B cells, and different subsets of T cells [58]. This suggests that the cellular components of TLS affect the antitumor immune response in different cancer types, such as gastric cancer, where a high number of $CD20^+$ B cells within lymphoid aggregates was an independent predictor of good prognosis [59]. Moreover, B cells are not only cellular precursors of antibody-producing plasma cells in the TME, but they also act as antigen-presenting cells and are also capable of directly killing cancer cells through the release of granzyme B [60]. These functions support a tumor-suppressive role for B cells; however, immunosuppressive regulatory B cells that produce TGF-β and IL-10, promoting the same effects as Treg cells, have also been found [61]. This heterogeneity in B cell function may explain the contradictory results among studies where pan B cell markers are used. B cells may inhibit the immune response against tumors, but the underlying mechanism is poorly understood. However, it is well-known that B cells expressing PD-L1 interact with follicular T helper (Tfh) cells with high expression of the programmed cell death-1 (PD-1) molecule, thus suppressing CXCR3 upregulation and the follicular recruitment of activated helper T cells [62].

Even though intratumoral $CD20^+$ TIL density was independently associated with the prognosis in this OSCC patient cohort, other important prognostic clinicopathological variables, such as age, tumor stage, neck lymph node metastasis, histological grade, or tumor location within the oral cavity were not associated with $CD20^+$ TIL infiltration. Pretscher et al. [35] found that intratumoral $CD20^+$ B cells were increased in number in metastasis compared to primary tumors, while Taghavi et al. [42] observed a significant inverse association between peritumoral $CD20^+$ B cells and lymph node metastasis. We did not find any association between $CD20^+$ TILs and metastasis or clinical stage. Noteworthy, we found a significant reduction in the number of infiltrating $CD20^+$ B cells in T3 and T4 tumors compared with smaller cancer sizes, which suggests that the reduction in the tumoral B lymphocyte infiltration could be associated with tumor progression. Conversely, it is also plausible that the upfront presence of fewer immune cells and therewith inadequate immunosurveillance of the tumor could ultimately favor tumor growth. In contrast to the above results, other studies have demonstrated an increase in $CD20^+$ B cell infiltration in association with a poorer prognosis [7,8]. The conflicting results may be due to differences in immunohistochemical procedures and/or scoring used.

CSCs can attract macrophages into the tumors [63] and induce the M2 phenotype, secreting IL-6, IL-10, TGF-β, and EGF and driving CSC self-renewal by activating the STAT3/NF-κB signaling pathway [64]. Complementarily exosomes derived from tumors, including OSCC, and released by several types of cells, including B lymphocytes, can

activate M2 TAMs [65]. The cross-talk between CSCs and TAMs is orchestrated by the STAT3 signaling pathway [66], which promotes stemness, survival, and proliferation in CSCs. Conversely, CSCs can induce the immunosuppressive properties of TAMs repressing T lymphocytes [64]. We found a significant and inverse association between a higher tumoral infiltration of $CD20^+$ B cells with negative SOX2 expression. Similarly, $CD163^+$ TAM infiltration in OSCC has also been inversely correlated with the expression of the CSC markers NANOG and SOX2 [67] Together these data suggest an inverse relationship between B-cell infiltration and stemness in OSCC, which could plausibly reflect CSCs role in immune evasion and the contribution to OSCC progression. To the best of our knowledge, this is the first study to provide a potential link between $CD20^+$ B lymphocytes and CSCs.

Regarding the TME, both inflamed and non-inflamed phenotypes have been described [11]. OSCCs have mostly inflamed phenotypes [17], and it has been generally thought that, the more abundant the inflammatory infiltration surrounding the tumor nests, the better the prognosis of the patients. However, inflammation in OSCCs has been generally associated with poor survival [6]. Necrosed tumor cells may contribute to an inflamed TME and also proinflammatory cytokines released by CSCs, such as IL-6, IL-8, IL-10, and IL-13 can contribute to maintaining an inflammatory and suppressive TME representing the "niche" sustaining cellular stemness [68].

4. Materials and Methods

4.1. Patients and Tissue Specimens

A cohort of 125 patients with histologically confirmed OSCC surgically treated at the Hospital Universitario Central de Asturias between 1996 and 2007 was retrospectively selected for the study. This 125 OSCC cohort was previously described [24,25], predominantly male patients (n = 82, 65.6%), ranging in age from 28 to 91 years (mean 58.69, standard deviation 14.34 years). Eighty-four (67%) patients were active smokers and 69 (55%) alcohol drinkers. Primary tumors were located in the mobile tongue (n = 51, 41%), floor of the mouth (n = 37; 30%), gingiva (n = 22; 18%), buccal mucosa (n = 7; 6%), retromolar area (n = 6; 4%), and palate (n = 2; 1%). The patients were staged according to the 8th edition of the TNM classification of malignant tumors [69]. Regarding pT, 27 (22%) cases were classified as T1, 54 (43%) T2, 16 (13%) T3, and 28 (22%) T4. 49 (39%) patients presented lymph node metastasis (pN+), whereas the remaining 76 (61%) patients showed an absence of neck lymph node metastasis (pN0). The distribution according to overall AJCC stages was as follows: 20 (16%) patients were stage I, 32 (26%) patients were stage II, 26 (20%) patients were stage III, and finally, 47 (38%) patients were stage IV. Regarding histopathologic degree of differentiation, 80 (64%) OSCCs were well-differentiated, 41 (33%) moderately, and 4 (3%) poorly-differentiated. None of the patients underwent chemotherapy or radiotherapy before surgical treatment. Complementary radiotherapy (n = 75; 60%), or chemotherapy (n = 14; 11%) were administered when indicated.

53 (44%) patients were alive and free of recurrence during the follow-up period (6 to 230 months), while 19 (15%) suffered from a second primary cancer in the oral cavity, and 51 (42%) died of OSCC or showed a non-treatable recurrence. Disease-specific survival (DSS) was the clinical endpoint, calculated as the time interval from the initial surgical treatment to the date of death by the tumor or the presence of a non-treatable recurrence.

4.2. Immunohistochemistry (IHC)

Tissue microarrays (TMAs) were constructed by collecting three morphologically representative areas (1 mm diameter) from each formalin-fixed, paraffin-embedded (FFPE) tumor block, as previously reported [24,25]. Each TMA contained morphologically normal oral mucosa samples from non-oncological patients undergoing oral surgery, used as internal controls. The TMAs were cut into 3-µm sections and dried on Flex IHC microscope slides (DakoCytomation, Glostrup, Denmark). Tissue sections were deparaffinized in xylene and rehydrated in serial baths of ethanol, and then blocked with 3% hydrogen

peroxide. Antigen retrieval was done using Envision Flex Target Retrieval solution, high pH (Dako), followed by staining on an automatic staining workstation (Dako Autostainer Plus, Dako). Immunohistochemistry was performed incubating the slides with monoclonal antibodies against CD20 (Dako, clone L26, catalogue number M0755; 1:200 dilution), CD4 (Dako, clone 4B12, 1:80 dilution), CD8 (Dako, clone C8/144B, prediluted), FoxP3 (Cell Signaling Technology, clone D6O8R, 1:100 dilution), CD68 (Agilent-Dako, clone KP1, prediluted), CD163 (Biocare Medical, clone 10D6, 1:100 dilution), PD-L1 antibody (clone 22C3; 1:200 dilution; PD-L1 IHC 22C3 pharmDx; Dako SK006), NANOG (D73G4 XP®; 1:200 dilution, Cell Signaling Technology, Inc.), and SOX2 (AB5603; 1:1000 dilution, Merck Millipore). The antibody-antigen complex was visualized with the Dako EnVision Flex + Visualization System (Dako). Tonsil tissue was used as a positive control for CD20.

The IHC results were independently evaluated by four observers (FDI, JSC, JPR, and JMG-P), blinded to clinical information. The number of $CD20^+$, $CD68^+$, $CD163^+$, $CD4^+$, $CD8^+$, and $FOXP3^+$ cells was counted in each 1 mm^2 area from three independent high-power representative microscopic fields (HPFs, 400×; 0.0625 μm^2), both in the tumor nests and tumor stroma. The median was used as a cut-off to separate patient groups based on these lymphocyte markers. Thus, $CD20^+$, $CD4^+$, $CD8^+$, $CD68^+$, $CD163^+$, and $FOXP3^+$ cells immunostaining was classified into two groups, above and below median number of staining for the total patient population. Stromal and tumoral ratios between CD20 and the remaining immune cell markers (CD8, CD4, FOXP3, CD68, and CD163) were calculated. PD-L1 expression in more than 10% of tumor cells was significantly associated with poorer survival in a previous study [34], and accordingly established as a cut-off point for subsequent analyses. SOX2 expression was evaluated as the percentage of tumor cells with positively stained nuclei, as previously described [24,25]. SOX2 staining scores were classified as negative or positive expression as below or above the median cut-off value of 10%, respectively. NANOG staining intensity was scored as negative (score 0) *versus* positive expression (scores 1–2).

4.3. Statistical Analysis

Statistical analyses were carried out using SPSS software version 18 (IBM Co., Armonk, NY, USA). Continuous variables ($CD20^+$, $CD68^+$, $CD163^+$, $CD4^+$, $CD8^+$, and $FOXP3^+$ cells) were expressed as the means ± standard deviations (SD), and absolute and relative frequencies were calculated for categorical variables. The correlations between the numbers of stromal or tumoral $CD20^+$, and $CD68^+$, $CD163^+$, $CD4^+$, $CD8^+$, and $FOXP3^+$ cells were assessed using Spearman's rank correlation coefficient. Mann–Whitney *U* test was used to evaluate the relationship between CD20 expression and the different clinicopathological variables. Analyses of disease-specific survival (DSS) were performed using the Kaplan–Meier method, and a comparison of survival rates was performed by using the log-rank test. Moreover, the univariate and multivariate Cox regression model was applied to calculate hazard ratios (HRs) and 95% confidence intervals (95% CI), as well as to determine independent prognostic factors in the presence of other prognostically relevant covariates. All *p*-values were based on two-sided statistical analysis, and for all analyses a *p*-value, less than 0.05 was considered to be statistically significant.

5. Conclusions

This study thoroughly investigated the clinical relevance of B cell infiltration in the context of oral TME complexity, by jointly evaluating both in the tumor nests and surrounding stroma associations between infiltrating $CD20^+$ B lymphocytes and other immune profiles $CD4^+$, $CD8^+$, and $FOXP3^+$ TILs, $CD68^+$ and $CD163^+$ macrophages in OSCC specimens. Our findings demonstrate that B cell infiltration had an impact on OSCC patient prognosis. Interestingly, high $CD20^+$ TIL density in the tumor nests emerges as an independent good prognostic factor in OSCC, thus underlining a potential role of B lymphocytes in antitumor immunity in oral cancers. Furthermore, this study also uncovered an inverse correlation between infiltration of $CD20^+$ B cells and the expression of CSC markers, in particular SOX2.

Nevertheless, these results need further confirmation using large independent validation cohorts of OSCC and/or other HNSCC patients.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/3/395/s1, Table S1: Summary of immune infiltration levels (mean numbers) in the tumor and surrounding stroma of 125 OSCC patients. Table S2. Associations between stromal and tumoral CD20/CD68 and CD20/CD168 ratios and clinicopathological parameters in the cohort of 125 OSCC patients. Table S3. Univariate Cox regression analysis of disease-specific survival (DSS) in 125 patients with OSCC. Table S4. Stratified univariate Kaplan–Meier analysis to assess significant relationships of tumoral $CD20^{+}$, clinicopathological, and molecular variables on DSS in 125 OSCC patients.

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Institutional Review Board Statement: This study was conducted according to the ethical guidelines of the Declaration of Helsinki, and was approved by the Institutional Ethics Committee of the Hospital Universitario Central de Asturias and by the Regional CEIC from Principado de Asturias (date of approval 14th of May 2019; approval number: 136/19) for the use of histopathological and clinical material for research purposes.

Informed Consent Statement: Informed consent was obtained from all patients involved in this study.

Data Availability Statement: The data presented in this study are available within the article and Supplementary Materials.

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References

1. Ferris, R.L. Immunology and Immunotherapy of Head and Neck Cancer. *J. Clin. Oncol.* **2015**, *3*, 3293–3304. [CrossRef] [PubMed]
2. Chen, D.; Wang, C.Y. Targeting cancer stem cells in squamous cell carcinoma. *Precis Clin. Med.* **2019**, *2*, 152–165. [CrossRef] [PubMed]
3. Yuen, G.J.; Demissie, E.; Pillai, S. B lymphocytes and cancer: A love-hate relationship. *Trends Cancer* **2016**, *2*, 747–757. [CrossRef] [PubMed]
4. Schmidt, M.; Böhm, D.; von Törne, C.; Steiner, E.; Puhl, A.; Pilch, H.; Lehr, H.A.; Hengstler, J.G.; Kölbl, H.; Gehrmann, M. The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer Res.* **2008**, *68*, 5405–5413. [CrossRef]
5. Hussein, M.R.; Elsers, D.A.H.; Fadel, S.A.; Omar, A.E.M. Immunohistological characterisation of tumour infiltrating lymphocytes in melanocytic skin lesions. *J. Clin. Pathol.* **2006**, *59*, 316–324. [CrossRef]
6. Evrard, D.; Szturz, P.; Tijeras-Raballand, A.; Astorgues-Xerri, L.; Abitbol, C.; Paradis, V.; Raymond, E.; Albert, S.; Barry, B.; Faivre, S. Macrophages in the microenvironment of head and neck cancer: Potential targets for cancer therapy. *Oral Oncol.* **2019**, *88*, 29–38. [CrossRef]
7. Hadler-Olsen, E.; Wirsing, A.M. Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma: A systematic review and meta-analysis. *Br. J. Cancer* **2019**, *120*, 714–727. [CrossRef]
8. Wang, T.; Niu, G.; Kortylewski, M.; Burdelya, L.; Shain, K.; Zhang, S.; Bhattacharya, R.; Gabrilovich, D.; Heller, R.; Coppola, D.; et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat. Med.* **2004**, *10*, 48–54. [CrossRef]

9. da Silveira, E.J.; da Costa Miguel, M.C.; Lima, K.C.; de Almeida Freitas, R.; Queiroz, L.M.G. Analysis of local immunity in squamous cell carcinoma of the tongue and lower lip. *Exp. Mol. Pathol.* **2010**, *88*, 171–175. [CrossRef]
10. Stasikowska-Kanicka, O.; Wągrowska-Danilewicz, M.; Danilewicz, M. Immunohistochemical analysis of $Foxp3_+$, $CD4_+$, $CD8_+$ cell infiltrates and PD-L1 in oral squamous cell carcinoma. *Pathol. Oncol. Res.* **2018**, *24*, 497–505. [CrossRef]
11. Wirsing, A.M.; Ervik, I.K.; Seppola, M.; Uhlin-Hansen, L.; Steigen, S.E.; Hadler-Olsen, E. Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma. *Mod. Pathol.* **2018**, *31*, 910–922. [CrossRef] [PubMed]
12. Lund, F.E. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr. Opin. Immunol.* **2008**, *20*, 332–338. [CrossRef] [PubMed]
13. Yarchoan, M.; Ho, W.J.; Mohan, A.; Shah, Y.; Vithayathil, T.; Leatherman, J.; Dennison, L.; Zaidi, N.; Ganguly, S.; Woolman, S.; et al. Effects of B cell-activating factor on tumor immunity. *JCI Insight* **2020**, *5*, e136417. [CrossRef]
14. Disis, M.L. Immune regulation of cancer. *J. Clin. Oncol.* **2010**, *28*, 4531–4538. [CrossRef] [PubMed]
15. Ahn, H.; Yang, J.M.; Kim, H.; Chung, J.H.; Ahn, S.H.; Jeong, W.J.; Paik, J.H. Clinicopathologic implications of the miR-197/PD-L1 axis in oral squamous cell carcinoma. *Oncotarget* **2017**, *8*, 66178–66194. [CrossRef]
16. Mizoguchi, A.M.; Mizoguchi, E.; Takedatsu, H.; Blumberg, R.S.; Bhan, A.K. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* **2002**, *16*, 219–230. [CrossRef]
17. Blair, P.A.; Noreña, L.Y.; Flores-Borja, F.; Rawlings, D.J.; Isenberg, D.A.; Ehrenstein, M.R.; Mauri, C. CD19 (+) CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* **2010**, *32*, 129–140. [CrossRef]
18. Sarvaria, A.; Madrigal, J.A.; Saudemont, A. B cell regulation in cancer and anti-tumor immunity. *Cell Mol. Immunol.* **2017**, *14*, 662–674. [CrossRef]
19. Morrison, B.J.; Steel, J.C.; Morris, J.C. Reduction of MHC-I expression limits T-lymphocyte-mediated killing of cancer initiating cells. *BMC Cancer* **2018**, *18*, 469. [CrossRef]
20. Baniebrahimi, G.; Mir, F.; Khanmohammadi, R. Cancer stem cells and oral cancer: Insights into molecular mechanisms and therapeutic approaches. *Cancer Cell Int.* **2020**, *20*, 113. [CrossRef]
21. Tahmasebi, E.; Alikhani, M.; Yazdanian, A.; Yazdanian, M.; Tebyanian, H.; Seifalian, A. The current markers of cancer stem cell in oral cancers. *Life Sci.* **2020**, *249*, 117483. [CrossRef] [PubMed]
22. Davis, S.J.; Divi, V.; Owen, J.H.; Bradford, C.R.; Carey, T.E.; Papagerakis, S.; Prince, M.E.P. Metastatic potential of cancer stem cells in head and neck squamous cell carcinoma. *Arch. Otolaryngol. Head Neck Surg.* **2010**, *136*, 1260–1266. [CrossRef] [PubMed]
23. Major, A.G.; Pitty, L.P.; Farah, C.S. Cancer stem cell markers in head and neck squamous cell carcinoma. *Stem Cells Int.* **2013**, *2013*, 319489. [CrossRef] [PubMed]
24. de Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Allonca, E.; Vallina, A.; Singhania, A.; Donate-Pérez Del Molino, P.; García-Pedrero, J.M. The emerging role of NANOG as an early cancer risk biomarker in patients with oral potentially malignant disorders. *J. Clin. Med.* **2019**, *8*, 1376. [CrossRef] [PubMed]
25. de Vicente, J.C.; Donate-Pérez Del Molino, P.; Rodrigo, J.P.; Allonca, E.; Hermida-Prado, F.; Granda-Díaz, R.; Rodríguez Santamarta, T.; García-Pedrero, J.M. SOX2 Expression is an independent predictor of oral cancer progression. *J. Clin. Med.* **2019**, *8*, 1744. [CrossRef]
26. Chiou, S.H.; Yu, C.C.; Huang, C.Y.; Lin, S.C.; Liu, C.; Tsai, T.H.; Chou, S.H.; Chien, C.S.; Ku, H.H.; Lo, J.F. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin. Cancer Res.* **2008**, *14*, 4085–4095. [CrossRef]
27. Tsai, L.L.; Yu, C.C.; Chang, Y.C.; Yu, C.H.; Chou, M.Y. Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. *J. Oral Pathol. Med.* **2011**, *40*, 621–628. [CrossRef]
28. Ren, Z.H.; Zhang, C.P.; Ji, T. Expression of SOX2 in oral squamous cell carcinoma and the association with lymph node metastasis. *Oncol. Lett.* **2016**, *11*, 1973–1979. [CrossRef]
29. Miao, Y.; Yang, Y.; Levorse, J.; Yuan, S.; Polak, L.; Sribour, M.; Singh, B.; Rosenblum, M.D.; Fuchs, E. Adaptive immune resistance emerges from tumor-initiating stem cells. *Cell* **2019**, *177*, 1172–1186.e1114. [CrossRef]
30. Chikamatsu, K.; Takahashi, G.; Sakakura, K.; Ferrone, S.; Masuyama, K. Immunoregulatory properties of CD44+ cancer stem-like cells in squamous cell carcinoma of the head and neck. *Head Neck* **2011**, *33*, 208–215. [CrossRef]
31. Piersma, S.J.; Jordanova, E.S.; van Poelgeest, M.I.; Kitty, M.C.; Kwappenberg, J.M.; van der Hulst, M.; Drijfhout, J.W.; Melief, C.J.M.; Kenter, G.G.; Fleuren, G.J.; et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* **2007**, *67*, 354–361. [CrossRef] [PubMed]
32. Han, S.; Zhang, C.; Li, Q.; Dong, J.; Liu, Y.; Huang, Y.; Jiang, T.; Wu, A. Tumour-infiltrating CD4(+) and CD8(+) lymphocytes as predictors of clinical outcome in glioma. *Br. J. Cancer* **2014**, *110*, 2560–2568. [CrossRef]
33. Hladíková, K.; Koucký, V.; Bouček, J.; Laco, J.; Grega, M.; Hodek, M.; Zábrodský, M.; Vošmik, M.; Rozkošová, K.; Vošmiková, H.; et al. Tumor-infiltrating B cells affect the progression of oropharyngeal squamous cell carcinoma via cell-to-cell interactions with $CD8_+$ T cells. *J. Immunother. Cancer* **2019**, *7*, 261. [CrossRef] [PubMed]

34. de Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Blanco-Lorenzo, V.; Allonca, E.; García-Pedrero, J.M. PD-L1 expression in tumor cells is an independent unfavorable prognostic factor in oral squamous cell carcinoma. *Cancer Epidemiol. Biomark. Prev.* **2019**, *28*, 546–554. [CrossRef] [PubMed]
35. Pretscher, D.; Distel, L.V.; Grabenbauer, G.G.; Wittlinger, M.; Buettner, M.; Niedobitek, G. 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. [CrossRef]
36. Lundgren, S.; Berntsson, J.; Nodin, B.; Micke, P.; Jirström, K. Prognostic impact of tumour-associated B cells and plasma cells in epithelial ovarian cancer. *J. Ovarian Res.* **2016**, *9*, 21. [CrossRef]
37. Distel, L.V.; Fickenscher, R.; Dietel, K.; Hung, A.; Iro, H.; Zenk, J.; Nkenke, E.; Büttner, M.; Niedobitek, G.; Grabenbauer, G.G. Tumour infiltrating lymphocytes in squamous cell carcinoma of the oro- and hypopharynx: Prognostic impact may depend on type of treatment and stage of disease. *Oral Oncol.* **2009**, *45*, e167–e174. [CrossRef]
38. Mandal, R.; Şenbabaoğlu, Y.; Desrichard, A.; Havel, J.J.; Dalin, M.G.; Riaz, N.; Lee, K.W.; Ganly, I.; Hakimi, A.A.; Chan, T.A.; et al. The head and neck cancer immune landscape and its immunotherapeutic implications. *JCI Insight* **2016**, *1*, e89829. [CrossRef]
39. Erdag, G.; Schaefer, J.T.; Smolkin, M.E.; Deacon, D.H.; Shea, S.M.; Dengel, L.T.; Patterson, J.W.; Slingluff, C.L., Jr. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res.* **2012**, *72*, 1070–1080. [CrossRef]
40. Garg, K.; Maurer, M.; Griss, J.; Brüggen, M.C.; Wolf, I.H.; Wagner, C.; Willi, N.; Mertz, K.D.; Wagner, S.N. Tumor-associated B cells in cutaneous primary melanoma and improved clinical outcome. *Hum. Pathol.* **2016**, *54*, 157–164. [CrossRef]
41. Martinez-Rodriguez, M.; Thompson, A.K.; Monteagudo, C. A significant percentage of CD20-positive TILs correlates with poor prognosis in patients with primary cutaneous malignant melanoma. *Histopathology* **2014**, *65*, 726–728. [CrossRef] [PubMed]
42. Taghavi, N.; Mohsenifar, Z.; Baghban, A.A.; Arjomandkhah, A. CD20+ Tumor Infiltrating B Lymphocyte in Oral Squamous Cell Carcinoma: Correlation with Clinicopathologic Characteristics and Heat Shock Protein 70 Expression. *Pathol. Res. Int.* **2018**, *2018*, 4810751. [CrossRef] [PubMed]
43. Lao, X.M.; Liang, Y.J.; Su, Y.X.; Zhang, S.E.; Zhou, X.I.; Liao, G.Q. Distribution and significance of interstitial fibrosis and stroma-infiltrating B cells in tongue squamous cell carcinoma. *Oncol. Lett.* **2016**, *11*, 2027–2034. [CrossRef] [PubMed]
44. Nielsen, J.S.; Sahota, R.A.; Milne, K.; Kost, S.E.; Nesslinger, N.J.; Watson, P.H.; Nelson, B.H. CD20+ tumor-infiltrating lymphocytes have an atypical CD27− memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clin. Cancer Res.* **2012**, *18*, 3281–3292. [CrossRef]
45. Raposo, G.; Nijman, H.W.; Stoorvogel, W.; Liejendekker, R.; Harding, C.V.; Melief, C.J.; Geuze, H.J. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **1996**, *183*, 1161–1172. [CrossRef]
46. Li, Q.; Grover, A.C.; Donald, E.J.; Carr, A.; Yu, J.; Whitfield, J.; Nelson, M.; Takeshita, N.; Chang, A.E.J. Simultaneous targeting of CD3 on T cells and CD40 on B or dendritic cells augments the antitumor reactivity of tumor-primed lymph node cells. *J. Immunol.* **2005**, *175*, 1424–1432. [CrossRef]
47. Zhang, Y.; Morgan, R.; Chen, C.; Cai, Y.; Clark, E.; Khan, W.; Shin, S.; Cho, H.; Bayati, A.A.; Pimentel, A.; et al. Mammary-tumor-educated B cells acquire LAP/TGF-β and PD-L1 expression and suppress anti-tumor immune responses. *Int. Immunol.* **2016**, *28*, 423–433. [CrossRef]
48. Schrama, D.; thor Straten, P.; Fischer, W.H.; McLellan, A.D.; Bröcker, E.B.; Reisfeld, R.A.; Becker, J.C. Targeting of lymphotoxin-alpha to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue. *Immunity* **2001**, *14*, 111–121. [CrossRef]
49. Pimenta, E.M.; Barnes, B.J. Role of tertiary lymphoid structures (TLS) in anti-tumor immunity: Potential tumor-induced cytokines/chemokines that regulate TLS formation in epithelial-derived cancers. *Cancers* **2014**, *6*, 969–997. [CrossRef]
50. Sharonov, G.V.; Serebrovskaya, E.O.; Yuzhakova, D.V.; Britanova, O.V.; Chudakov, D.M. B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nat. Rev. Immunol.* **2020**, *20*, 294–307. [CrossRef]
51. Sautès-Fridman, C.; Petitprez, F.; Calderaro, J.; Fridman, W.H. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat. Rev. Cancer* **2019**, *19*, 307–325. [CrossRef] [PubMed]
52. Siliņa, K.; Rulle, U.; Kalniņa, Z.; Linē, A. Manipulation of tumour-infiltrating B cells and tertiary lymphoid structures: A novel anti-cancer treatment avenue? *Cancer Immunol. Immunother.* **2014**, *63*, 643–662. [CrossRef] [PubMed]
53. Yamaguchi, K.; Ito, M.; Ohmura, H.; Hanamura, F.; Nakano, M.; Tsuchihashi, K.; Nagai, S.; Ariyama, H.; Kusaba, H.; Yamamoto, H.; et al. Helper T cell-dominant tertiary lymphoid structures are associated with disease relapse of advanced colorectal cancer. *Oncoimmunology* **2020**, *9*, 1724763. [CrossRef] [PubMed]
54. Wirsing, A.M.; Rikardsen, O.G.; Steigen, S.E.; Uhlin-Hansen, L.; Hadler-Olsen, E. Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma. *BMC Clin. Pathol.* **2014**, *14*, 38. [CrossRef] [PubMed]
55. Colbeck, E.J.; Ager, A.; Gallimore, A.; Jones, G.W. Tertiary lymphoid structures in cancer: Drivers of antitumor immunity, immunosuppression, or bystander sentinels in disease? *Front. Immunol.* **2017**, *9*, 1830. [CrossRef]
56. Kroeger, D.R.; Milne, K.; Nelson, B.H. Tumor-infiltrating plasma cells are associated with tertiary lymphoid structures, cytolytic T-cell responses, and superior prognosis in ovarian cancer. *Clin. Cancer Res.* **2016**, *22*, 3005–3015. [CrossRef]
57. Li, K.; Guo, Q.; Zhang, X.; Dong, X.; Liu, W.; Zhang, A.; Li, Y.; Yan, J.; Jia, G.; Zheng, Z.; et al. Oral cancer-associated tertiary lymphoid structures: Gene expression profile and prognostic value. *Clin. Exp. Immunol.* **2020**, *199*, 172–181. [CrossRef]

58. Dieu-Nosjean, M.C.; Goc, J.; Giraldo, N.A.; Sautès-Fridman, C.; Fridman, W.H. Tertiary lymphoid structures in cancer and beyond. *Trends Immunol.* **2014**, *35*, 571–580. [CrossRef]
59. Sakimura, C.; Tanaka, H.; Okuno, T.; Hiramatsu, S.; Muguruma, K.; Hirakawa, K.; Wanibuchi, H.; Ohira, M. B cells in tertiary lymphoid structures are associated with favorable prognosis in gastric cancer. *J. Surg. Res.* **2017**, *215*, 74–82. [CrossRef]
60. Wang, S.S.; Liu, W.; Ly, D.; Xu, H.; Qu, L.; Zhang, L. Tumor-infiltrating B cells: Their role and application in anti-tumor immunity in lung cancer. *Cell Mol. Immunol.* **2019**, *16*, 6–18. [CrossRef]
61. Tobón, G.J.; Izquierdo, J.H.; Cañas, C.A. B lymphocytes: Development, tolerance, and their role in autoimmunity-focus on systemic lupus erythematosus. *Autoimmune Dis.* **2013**, 827254. [CrossRef] [PubMed]
62. Shi, J.; Hou, S.; Fang, Q.; Liu, X.; Liu, X.; Qi, H. PD-1 controls follicular T helper cell positioning and function. *Immunity* **2018**, *49*, 264–274.e4. [CrossRef] [PubMed]
63. Mehla, K.; Singh, P.K. Metabolic Regulation of Macrophage Polarization in Cancer. *Trends Cancer* **2019**, *5*, 822–834. [CrossRef] [PubMed]
64. Mitchem, J.B.; Brennan, D.J.; Knolhoff, B.L.; Belt, B.A.; Zhu, Y.; Sanford, D.E.; Belaygorod, L.; Carpenter, D.; Collins, L.; Piwnica-Worms, D.; et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Res.* **2013**, *73*, 1128–1141. [CrossRef] [PubMed]
65. Baig, M.S.; Roy, A.; Rajpoot, S.; Liu, D.; Savai, R.; Banerjee, S.; Kawada, M.; Faisal, S.M.; Saluja, R.; Saqib, U.; et al. Tumor-derived exosomes in the regulation of macrophage polarization. *Inflamm. Res.* **2020**, *69*, 435–451. [CrossRef] [PubMed]
66. Grivennikov, S.I.; Greten, F.R.; Karin, M. Immunity, inflammation, and cancer. *Cell* **2010**, *140*, 883–899. [CrossRef]
67. Suárez-Sánchez, F.J.; Lequerica-Fernández, P.; Suárez-Canto, J.; Rodrigo, J.P.; Rodriguez-Santamarta, T.; Domínguez-Iglesias, F.; García-Pedrero, J.M.; de Vicente, J.C. Macrophages in oral carcinomas: Relationship with cancer stem cell markers and PD-L1 expression. *Cancers* **2020**, *12*, 1764. [CrossRef]
68. Ravindran, S.; Rasool, S.; Maccalli, C. The cross talk between cancer stem cells/cancer initiating cells and tumor microenvironment: The missing piece of the puzzle for the efficient targeting of these cells with immunotherapy. *Cancer Microenviron.* **2019**, *12*, 133–148. [CrossRef]
69. Lydiatt, W.M.; Patel, S.G.; Ridge, J.A.; O'Sullivan, B.; Shah, J.P. Staging head and neck cancers. In *AJCC Cancer Staging Manual*, 8th ed.; Springer International Publishing AG: New York, NY, USA, 2017; pp. 55–181.

Tumor-Infiltrating CD20$^+$ B Lymphocytes: Significance and Prognostic Implications in Oral Cancer Microenvironment

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Table S1. Summary of immune infiltration levels (mean numbers) in the tumor and surrounding stroma of 125 OSCC patients.

Tumor-infiltrating Immune Subtypes (cells per mm^2)	Tumor Nests Mean ± SD (range)	Stroma Mean ± SD (range)
CD4$^+$ TILs	6.02 ± 12.29 (0.00 to 86.33)	54.60 ± 68.15 (1.33 to 569.67)
CD8$^+$ TILs	47.88 ± 57.16 (0.00 to 288.33)	178.45 ± 203.21 (0.33 to 1202.67)
FOXP3$^+$ Tregs	3.11 ± 6.47 (0.00 to 50.00)	15.65 ± 24.54 (0.00 to 147.33)
CD68$^+$ macrophages	51.11 ± 45.08 (0.00 to 194.00)	122.72 ± 82.49 (9.67 to 488.67)
CD163$^+$ macrophages	31.11 ± 28.91 (0.00 to 187.33)	168.14 ± 97.59 (14.33 to 476.67)

Table S2. Associations between stromal and tumoral CD20/CD68 and CD20/CD168 ratios and clinicopathological parameters in the cohort of 125 OSCC patients.

Variable Mean (SD)	Number	Stromal CD20/CD68 Ratio	*p*	Tumoral CD20/CD68 Ratio	*p*	Stromal CD20/CD163 Ratio	*p*	Tumoral CD20/CD163 Ratio	*p*
Age (years)									
<65	77	0.29 (0.50)	0.31	0.04 (0.12)	0.24	0.19 (0.39)	0.29	1.10 (3.97)	0.92
≥65	48	0.53 (1.14)		0.10 (0.36)		0.36 (0.79)		0.73 (2.25)	
Gender									
Female	43	0.52 (1.22)	0.71	0.11 (0.38)	0.28	0.33 (0.83)	0.96	0.18 (0.62)	0.25
Male	82	0.30 (0.48)		0.03 (0.11)		0.22 (0.39)		0.07 (0.19)	
Tobacco									
No	41	0.47 (1.14)	0.47	0.05 (0.16)	0.12	0.34 (0.83)	0.36	0.09 (1.73)	0.11
Yes	84	0.34 (0.59)		0.06 (0.27)		0.21 (0.40)		0.11 (0.47)	
Alcohol									
No	56	0.41 (1.05)	0.52	0.08 (0.33)	0.16	0.28 (0.74)	0.77	0.08 (0.16)	0.25
Yes	69	0.36 (0.55)		0.03 (1.28)		0.23 (0.42)		0.13 (0.53)	
pT									
T1 + T2	81	0.47 (0.95)	0.03	0.07 (0.28)	0.05	0.32 (0.69)	0.03	0.13 (0.46)	0.14
T3 + T4	44	0.21 (0.41)		0.03 (0.15)		0.14 (0.28)		0.07 (0.24)	
pN									
N0	76	0.43 (0.99)	0.51	0.07 (0.29)	0.87	0.30 (0.71)	0.76	0.12 (0.48)	0.94
N+	49	0.30 (0.41)		0.04 (0.14)		0.18 (0.25)		0.08 (0.23)	
Stage									
I + II	52	0.54 (1.14)	0.48	0.10 (0.35)	0.12	0.39 (0.84)	0.30	0.16 (0.57)	0.23
III + IV	73	0.26 (0.42)		0.03 (0.12)		0.16 (0.26)		0.07 (0.20)	
Grade									
Well	80	0.48 (0.98)	0.02	0.07 (0.28)	0.52	0.34 (0.70)	0.03	0.12 (0.46)	0.24
Moderate + Poor	45	0.21 (0.31)		0.04 (0.15)		1.19 (1.17)		0.08 (0.24)	
Site									
Tongue	51	0.36 (0.59)	0.61	0.07 (0.32)	0.67	0.26 (0.46)	0.74	0.05 (0.10)	0.75
Other	74	0.39 (0.94)		0.05 (0.17)		0.25 (0.65)		0.14 (0.51)	
Recurrence									
No	71	0.31 (0.53)	0.30	0.03 (0.12)	0.95	0.18 (0.31)	0.29	0.12 (0.51)	0.84
Yes	54	0.47 (1.08)		0.09 (0.34)		0.35 (0.80)		0.09 (0.17)	
Second primary tumor									
No	106	0.32 (0.80)	0.01	0.04 (0.14)	0.25	0.24 (0.61)	0.01	0.10 (0.43)	0.17
Yes	19	0.71 (0.84)		0.15 (0.52)		0.36 (0.37)		0.11 (0.16)	

All *p*-values were calculated using the U Mann-Whitney test.

Table S3. Univariate Cox regression analysis of disease-specific survival (DSS) in 125 patients with OSCC.

Variable	Number	Censored Patients (%)	DSS (95% CI)	*p*	Hazard Ratio	95% CI
Age						
<65 years	77	46 (60)	142.32 (118.63–166.01)	0.23	1.39	0.80–2.42
≥65 years	48	26 (54)	91.11 (70.43–111.79)			
Gender						
Female	43	25 (58)	130.56 (99.15–161.97)	0.77	1.08	0.61–1.92
Male	82	47 (57)	131.61 (107.28–155.94)			
Tobacco						
No	41	24 (59)	106.57 (83.24–129.91)	0.97	0.98	0.55–1.76
Yes	84	48 (57)	132.42 (108.70–156.14)			
Alcohol						
No	56	31 (55)	125.45 (98.13–152.76)	0.53	0.84	0.49–1.44
Yes	69	41 (59)	136.11 (109.92–162.29)			
pT						
T1–T2	81	53 (65)	151.82 (129.03–174.99)	0.001	2.49	1.44 – 4.30
T3–T4	44	19 (43)	77.62 (54.19–101.04)			
pN						
N0	76	49 (65)	127.96 (109.43–146.49)	0.01	1.92	1.12–3.31
N+	49	23 (4)	108.58 (77.88–139.28)			
Stage						
I + II	52	36 (69)	140.09 (120.15–160.04)	0.002	2.40	1.33–4.32
III + IV	73	36 (49)	113.33 (87.73–138.93)			
Grade						
Well	80	44 (55)	127.85 (103.73–151.98)	0.59	0.85	0.48–1.52
Moderate + Poor	45	28 (62)	121.63 (96.25–147.01)			
Site						
Tongue	51	28 (55)	124.43 (94.00–154.86)	0.31	0.75	0.43–1.30
Other	74	44 (60)	101.48 (123.52–139.47)			
Radiotherapy						
No	50	44 (88)	167.58 (150.15–185.00)	<0.0001	6.58	2.81–15.41
Yes	75	28 (37)	95.89 (73.23–118.55)			
Stromal $CD20^+$ TILs						
≤12.33	61	36 (59)	131.31 (104.39–158.23)	0.95	0.98	0.57–1.68
>12.33	64	36 (56)	132.74 (106.95–159.00)			
Tumoral $CD20^+$ TILs						
≤0.33	57	28 (49)	99.07 (76.37–121.78)	0.04	0.58	0.34–1.00
>0.33	68	44 (65)	148.94 (123.52–174.35)			
Stromal $CD20^+/CD8^+$ ratio						
≤0.1003	63	37 (59)	115.86 (94.45–137.28)	0.94	1.02	0.59–1.74
>0.1003	62	35 (57)	134.60 (108.12–161.07)			
Tumoral $CD20^+/CD8^+$ ratio						
≤0.0096	59	31 (53)	105.31 (83.00–127.63)	0.23	0.71	0.40–1.24
>0.0096	59	37 (63)	142.67 (114.81–170.54)			
Stromal $CD20^+/CD4^+$ ratio						
≤0.3136	65	36 (55)	126.46 (99.20–153.73)	0.33	0.76	0.44–1.32
>0.3136	60	36 (60)	118.54 (98.73–138.35)			
Tumoral $CD20^+/CD4^+$ ratio						
≤0.0909	47	26 (55)	126.83 (94.91–158.76)	0.36	0.75	0.40–1.40
>0.0909	48	29 (60)	134.30 (105.21–163.39)			
Stromal $CD20^+/FOXP3^+$ ratio						
≤0.7975	49	33 (67)	156.99 (127.91–186.07)	0.14	1.54	0.85–2.78
>0.7975	76	39 (51)	103.26 (85.13–121.39)			

Tumoral $CD20^+/FOXP3^+$ ratio						
≤0.1250	39	24 (62)	119.64 (92.81–146.48)	0.47	1.24	0.68–2.61
>0. 1250	86	48 (56)	128.83 (105.59–152.07)			
Stromal $CD20^+/CD68^+$ ratio						
≤0.0997	63	37 (59)	128.03 (100.77–155.29)	0.80	0.93	0.54–1.60
>0.0997	62	35 (57)	134.15 (107.83–160.46)			
Tumoral $CD20^+/CD68^+$ ratio						
≤0.04	62	32 (52)	103.62 (81.83–125.42)	0.14	0.66	0.38–1.15
>0.004	63	40 (64)	145.68 (118.96–172.37)			
Stromal $CD20^+/CD163^+$ ratio						
≤0.0781	63	37 (59)	128.43 (101.31–155.54)	0.86	0.95	0.55–1.63
>0.0781	62	35 (57)	133.70 (107.22–160.18)			
Tumoral $CD20^+/CD163^+$ ratio						
≤0.0061	61	33 (54)	107.44 (85.25–129.63)	0.31	0.76	0.44–1.30
> 0. 0061	64	39 (61)	140.47 (114.06–166.88)			

Table S4. Stratified univariate Kaplan-Meier analysis to assess significant relationships of tumoral CD20⁺, clinicopathological and molecular variables on DSS in 125 OSCC patients.

Parameter	CD20⁺ Median	Number	Censored patients (%)	DSS (95% CI)	*P*
Age					
<65 years	≤0.33	38	18 (47)	102.02 (75.38–128.67)	0.03
	>0.33	39	28 (72)	168.57 (138.01–199.13)	
≥65 years	≤0.33	19	10 (53)	71.17 (42.86–99.49)	0.24
	>0.33	29	16 (55)	99.21 (74.51–123.92)	
Gender					
Female	≤0.33	17	10 (59)	116.40 (75.59–157.20)	0.87
	>0.33	26	15 (58)	127.37 (87.06–167.77)	
Male	≤ 0.33	40	18 (45)	91.40 (65.78–117.01)	0.02
	> 0.33	42	29 (69)	158.66 (127.24–190.07)	
Tobacco					
No	≤0.33	14	10 (71)	119.58 (78.32–160.84)	0.47
	>0.33	27	14 (52)	100.17 (72.24–128.10)	
Yes	≤0.33	43	18 (42)	90.44 (65.28–115.60)	0.004
	>0.33	41	30 (73)	164.76 (132.83–196.70)	
Alcohol					
No	≤0.33	20	12 (60)	102.94 (67.33–138.55)	0.97
	>0.33	36	19 (53)	123.92 (90.80–157.05)	
Yes	≤0.33	37	16 (43)	93.48 (66.44–120.52)	0.005
	>0.33	32	25 (78)	174.84 (140.22–209.47)	
pT					
T 1 + T2	≤0.33	35	21 (60)	118.94 (90.87–147.00)	0.20
	>0.33	46	32 (70)	163.15 (134.90–191.39)	
T 3 + T4	≤0.33	22	7 (32)	62.85 (32.71–92.99)	0.20
	>0.33	22	12 (55)	85.20 (55.70–114.70)	
pN					
N0	≤0.33	33	19 (58)	116.72 (88.11–145.33)	0.30
	>0.33	43	30 (70)	126.67 (106.30–147.03)	
N+	≤0.33	24	9 (38)	65.81 (35.35–96.27)	0.09
	>0.33	25	14 (56)	132.89 (90.81–174.98)	
Stage					
I + II	≤0.33	22	14 (64)	132.13 (100.24–164.02)	0.20
	>0.33	30	22 (73)	135.78 (114.71–156.85)	

III + IV	≤0.33	35	14 (40)	71.27 (45.58–96.96)	0.07
	>0.33	38	22 (58)	134.86 (99.96–169.76)	
Grade					
Well	≤0.33	33	15 (45)	84.41 (61.60–117.23)	0.08
	>0.33	47	29 (62)	143.87 (113.17–174.58)	
Moderate-poorly	≤0.33	24	13 (54)	109.75 (74.87–144.63)	0.22
	>0.33	21	15 (71)	125.89 (95.76–156.02)	
Site					
Tongue	≤0.33	23	11 (48)	90.59 (55.98–125.20)	0.33
	>0.33	28	17 (61)	135.99 (94.53–177.44)	
Other	≤0.33	34	17 (50)	104.08 (75.33–132.83)	0.09
	>0.33	40	27 (68)	126.23 (105.59–146.86)	
Radiotherapy					
No	≤0.33	21	17 (81)	155.63 (124.71–186.56)	0.22
	>0.33	29	27 (93)	159.74 (142.75–176.73)	
Yes	≤0.33	36	11 (31)	66.48 (43.57–89.39)	0.12
	>0.33	39	17 (44)	112.69 (80.85–144.53)	
PD-L1					
≤10%	≤0.33	49	27 (55)	110.66 (86.33–134.99)	0.24
	>0.33	55	36 (66)	150.75 (122.85–178.64)	
>10%	≤0.33	7	1 (14)	16.17 (6.59–25.74)	0.008
	>0.33	11	6 (55)	94.81 (54.41–135.22)	
SOX2					
Negative	≤0.33	25	9 (36)	70.02 (38.21–101.83)	0.01
	>0.33	47	28 (60)	134.65 (103.10–166.20)	
Positive	≤0.33	31	19 (61)	121.59 (92.03–151.15)	0.32
	>0.33	18	13 (72)	167.93 (127.86–208.01)	
NANOG					
Negative	≤0.33	35	15 (43)	85.89 (57.28–114.50)	0.03
	>0.33	48	30 (63)	143.79 (113.07–174.52)	
Positive	≤0.33	20	11 (55)	97.68 (68.72–126.65)	0.39
	>0.33	19	13 (68)	126.95 (94.90–159.00)	
Tumoral $CD8^+$ TIL					
≤24.65	≤0.33	36	19 (53)	104.44 (76.43–132.46)	0.33
	>0.33	25	16 (64)	149.32 (108.38–190.26)	
>24.65	≤0.33	21	9 (43)	83.16 (46.99–119.33)	0.03
	>0.33	43	28 (65)	114.07 (94.33–133.80)	

Stromal CD8⁺ TIL					
≤118	≤0.33	44	21 (48)	93.62 (67.85–119.40)	0.46
	>0.33	19	11 (58)	129.19 (81.85–176.53)	
>118	≤0.33	13	7 (54)	109.14 (64.89–153.40)	0.32
	>0.33	49	33 (67)	156.70 (127.92–185.48)	
Tumoral CD4⁺ TIL					
≤2.666	≤0.33	35	16 (46)	94.18 (67.62–120.75)	0.14
	>0.33	24	15 (63)	143.41 (104.49–182.34)	
>2.666	≤0.33	22	12 (55)	103.19 (65.05–141.33)	0.23
	>0.33	44	29 (66)	150.28 (118.18–182.38)	
Stromal CD4⁺ TIL					
≤32.66	≤0.33	39	18 (46)	96.37 (69.14–123.60)	0.03
	>0.33	23	16 (70)	159.58 (121.89–197.27)	
>32.66	≤0.33	18	10 (56)	111.68 (74.31–149.04)	0.64
	>0.33	45	28 (62)	141.54 (109.47–173.62)	
Tumoral FOXP3⁺ Tregs					
≤0.8333	≤0.33	31	13 (42)	83.25 (54.17–112.33)	0.16
	>0.33	31	17 (55)	123.72 (87.69–159.76)	
>0.8333	≤0.33	26	15 (58)	118.77 (86.42–151.13)	0.23
	>0.33	37	27 (73)	168.34 (136.25–200.44)	
Stromal FOXP3⁺ Tregs					
≤5.6667	≤0.33	35	14 (40)	80.82 (53.99–107.65)	0.06
	>0.33	28	17 (61)	116.20 (88.52–143.89)	
>5.6667	≤0.33	22	14 (64)	128.84 (94.45–163.22)	0.70
	>0.33	40	27 (68)	153.58 (120.65–186.51)	
Tumoral CD68⁺ macrophages					
≤36.1667	≤0.33	34	13 (38)	83.23 (56.17–110.30)	0.03
	>0.33	29	18 (62)	147.28 (110.31–184.26)	
>36.1667	≤0.33	23	15 (65)	121.95 (87.65–156.24)	0.74
	>0.33	39	26 (67)	114.70 (93.39–136.00)	
Stromal CD68⁺ macrophages					
≤108.000	≤0.33	40	19 (48)	92.74 (65.72–119.77)	0.24
	>0.33	16	10 (63)	140.87 (91.37–190.37)	
>108.000	≤ 0.33	17	9 (53)	108.18 (69.83–146.54)	0.36
	>0.33	52	34 (65)	151.42 (122.78–180.07)	
Tumoral CD163⁺ macrophages					
≤25.5833	≤0.33	37	19 (51)	105.70 (78.31–133.08)	0.25

	>0.33	27	18 (67)	152.69 (112.65–192.74)	
>25.5833	≤0.33	20	9 (45)	79.15 (43.94–114.36)	0.06
	>0.33	41	26 (63)	111.93 (91.72–132.15)	
Stromal $CD163^+$ macrophages					
≤147.3333	≤0.33	37	18 (49)	101.02 (73.08–128.95)	0.25
	>0.33	24	15 (63)	111.00 (83.98–138.02)	
>147.3333	≤0.33	20	10 (50)	99.84 (63.29–136.38)	0.15
	>0.33	44	29 (66)	149.72 (117.98–181.46)	

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Prognostic Relevance of CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs in Oral Squamous Cell Carcinoma and Correlations with PD-L1 and Cancer Stem Cell Markers

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Abstract: This study investigates the relevance of tumor-infiltrating lymphocytes (TILs) in oral squamous cell carcinoma (OSCC). Immunohistochemical analysis of stromal/tumoral CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs is performed in 125 OSCC patients. Potential relationships with the expression of tumoral PD-L1 and cancer stem cell (CSC) markers (NANOG, SOX2, OCT4, Nestin and Podoplanin (PDPN)) are assessed. CD4$^+$ and CD8$^+$ TILs are significantly associated with smoking and alcohol habits. CD4$^+$ and CD8$^+$ TILs show an inverse relationship with NANOG and SOX2 expression, and FOXP3$^+$ TILs is significantly correlated with Nestin and PDPN expression. High infiltration of CD4$^+$ and CD8$^+$ TILs and a high tumoral CD8$^+$/FOXP3$^+$ ratio are significantly associated with tumors harboring positive PD-L1 expression. Infiltration of stromal/tumoral FOXP3$^+$ TILs and a low stromal CD8$^+$/FOXP3$^+$ ratio are significantly associated with better disease-specific survival. Multivariate analysis reveals that the stromal CD8$^+$/FOXP3$^+$ TILs ratio is a significant independent prognostic factor. Regarding OSCC patient survival, the CD8$^+$/FOXP3$^+$ TILs ratio is an independent prognostic factor. TILs may act as biomarkers and potential therapeutic targets for OSCC.

Keywords: CD4; CD8; FOXP3; OCT4; Nestin; Podoplanin; TILs; oral cancer

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1. Introduction

Cancer is not only a genetic disease, it also involves an immunological basis. Oral squamous cell carcinoma (OSCC), one of the most common head and neck squamous cell carcinomas (HNSCC), is an immunosuppressive disease able to evade immune surveillance avoiding an effective immune response [1]. The tumor microenvironment (TME) refers to the cellular environment in which tumor cells or cancer stem cells (CSCs) exist [2,3]. This is the scenario where tumor-infiltrating lymphocytes (TILs) may play a role in the

development of OSCC [3]. Studies to date generally agree that cytotoxic T lymphocytes (CTLs) and $CD4^+$ Th1 cells are involved in effective antitumor immunity while $FOXP3^+$ regulatory T cells are associated with suppression of antitumor immunity [4]. Interestingly, $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes can be easily evaluated by immunohistochemistry.

Cancer cells overexpress the programmed death-ligand 1 (PD-L1), which binds to the programmed death-1 receptor (PD-1 or CD274) on activated T cells delivering an inhibitory signal to cytotoxic T lymphocytes that prevents tumor elimination from the immune system [5]. PD-L1 expression is affected by the surrounding microenvironment, and found to be generally associated with poor prognosis [6]. CSCs constitute a subpopulation of tumor cells endowed with properties such as self-renewal and long-term clonal persistence [7], maintained in specific niches within the tumor in which the interaction with the TME is crucial [8]. In turn, CSCs bear stemness properties supported by gene master regulators, such as NANOG, SOX2 and OCT4 [9], which have been implicated in oral carcinogenesis, poor differentiation and poor prognosis [10–17]. Accumulating evidence indicates that these transcription factors correlate with Nestin expression [18]. Nestin is a class VI intermediate filament protein identified as a stem cell marker during the development of the central nervous system [19]. Nestin is expressed in several tumors including OSCC, and found to regulate invasion, metastasis, cell cycle and apoptosis through regulation of cytoskeletal proteins and stemness [20]. Atsumi et al. [21] first identified Podoplanin (PDPN) as a CSC marker in squamous carcinoma cells. It has been shown that PDPN regulates stem cells in normal and tumor tissues [22], and also reported as a valuable biomarker for cancer risk assessment of malignant transformation in oral leukoplakia [23]. NANOG is also a pluripotency/CSC regulatory factor, found to be widely detected in multiple cancers, enriched in tumor cells that exhibit stem cell-like properties and with important prognostic implications in several cancer types, including OSCC [11,13]. The association between TILs and patient survival has been described in various types of cancer [1,24–33]. Specifically, in HNSCC, $CD8^+$ T cells have been related to a favorable prognosis, although few studies have examined the prognostic relevance of $CD8^+$ T cells' density in OSCC [3,34,35]. The types and functional statuses of different TILs as well as their tissue localizations within the TME can determine the balance between control and promotion of cancer [35]. In a recent meta-analysis, de Ruiter et al. [36] confirmed the favorable prognostic role of $CD8^+$ T cells in HNSCC, while the potential impact on OSCC prognosis remains a matter of controversy [26]. Additionally, in another recent meta-analysis, Borsetto et al. [37] investigated the prognostic role of $CD4^+$ and $CD8^+$ T cells in HNSCC, and found a significant reduction in the risk of death for both high $CD4^+$ and $CD8^+$ lymphocytes in oropharyngeal and for $CD8^+$ TILs in hypopharyngeal cancers. Nevertheless, neither high $CD4^+$ nor $CD8^+$ lymphocytes were significantly associated with improved survival for patients with oral or laryngeal cancer, indicating that tumor location could be a more discriminating factor than TILs in terms of clinical outcome in HNSCC. In addition, the prognostic value of $CD4^+$ T and $FOXP3^+$ T cells requires further investigation [38,39] due to protumoral and antitumoral functions that have been postulated for FOXP3 TILs' (Tregs') expression in different cancers. Thus, Tregs recruitment has been associated with a worse prognosis in some tumors [28–30,40], while it has been unexpectedly correlated with good prognosis in head and neck, esophageal and colorectal cancers [24,39]. Therefore, the roles of $CD4^+$, $CD8^+$ and $FOXP3^+$ T cell subpopulations in OSCC remain still inconclusive [25–27]. Further clarification is needed on whether lymphocyte infiltration represents a beneficial antitumor immune response or a poor prognostic factor involved in OSCC progression. The purpose of this study is to thoroughly evaluate the infiltration of $CD4^+$, $CD8^+$ and $FOXP3^+$ TILs in a large and homogeneous cohort of OSCC specimens, their relationships with clinicopathological features, and impact on patient prognosis. Additionally, correlations between Tregs and CTLs infiltration and the expression of tumor PD-L1 and various well-established CSCs markers (i.e. NANOG, SOX2, OCT4, Nestin and PDPN) are also assessed. A better understanding of TILs as clinically relevant biomarkers in OSCC is a prerequisite to adopt new immunotherapy treatments.

2. Materials and methods

2.1. Patients and Tissue Specimens

Following approval by the Regional Ethics Committee from Principado de Asturias (date of approval 14 May 2019; approval number 136/19, for the project PI19/01255), this retrospective study was performed using archived formalin-fixed paraffin-embedded (FFPE) tissue samples derived from 125 OSCC patients treated by surgery between January 1996 and November 2007 at the Hospital Universitario Central de Asturias, Spain. FFPE tissue specimens were sourced from the Principado de Asturias BioBank (PT17/0015/0023) and processed following standard operating procedures. Clinicopathologic data were collected from clinical records. Written informed consent was obtained from all patients. The inclusion criteria for all the participants enrolled were: (i) primary OSCC (International Classification of Disease-10 diagnosis codes: C02.0, C02.1, C02.2, C02.3, C03.0, C03.1, C04.0, C04.1, C05.0, C06.0, C06.1 and C06.2), (ii) treatment-naïve patients from whom we had formalin-fixed paraffin-embedded (FFPE) primary biopsy tissue samples and (iii) with a minimum follow-up of at least three years for alive patients. In addition, the exclusion criteria were: (i) OSCC with immediate postoperative death, (ii) recurrent disease, (iii) neoadjuvant chemo- or radiotherapy and (iv) missing survival data. The tumor histological grade was determined according to the World Health Organization classification [41] and the clinical staging was assessed according to the eighth edition of the AJCC [42] classification.

All patients underwent surgery of the primary tumor with curative intention as well as neck dissection without receiving any preoperative treatment with radiotherapy and/or chemotherapy. All research procedures have been performed in accordance with the World Medical Association Declaration of Helsinki.

2.2. Immunohistochemistry (IHC)

OSCC samples were processed for tissue microarray (TMA) as previously described [10,11,43]. Infiltration of TIL subtypes as well as PD-L1 expression were evaluated in the total selected cohort of 125 OSCC patients. However, immunohistochemical analysis of some CSC markers could not be evaluated in the whole sample due to the lack of representative tumor samples available in the OSCC TMAs. The following primary antibodies were used: mouse monoclonal anti-CD4 (Dako, clone 4B12, 1:80 dilution), mouse monoclonal anti-CD8 (Dako, clone C8/144B, prediluted), rabbit monoclonal anti-FoxP3 (Cell Signalling Technology, clone D6O8R, 1:100 dilution), mouse monoclonal PD-L1 antibody (clone 22C3, PD-L1 IHC 22C3 pharmDx, Dako SK006; 1:200 dilution), NANOG (D73G4) XP® rabbit monoclonal antibody (Cell Signaling technology, Inc.; 1:200 dilution), anti-SOX2 rabbit polyclonal antibody (AB5603, Merck Millipore; 1:1000 dilution), OCT4 (clone MRQ-10, Roche; prediluted), Nestin (clone 10C2, Invitrogen; 1:200 dilution) and IgG anti-podoplanin monoclonal antibody (clone D2-40, Covance Inc. formerly Signet Catalog No. 730-01; 1:100 dilution) using the Dako EnVision Flex+ Visualization System (Dako Autostainer) and diaminobenzidine chromogen as the substrate. Negative controls were prepared by omitting the primary antibody. Positive controls were prepared using appropriate positive control slides. Tonsil tissue was used as a positive control for CD4, CD8 and FOXP3; PD-1 or placenta for PD-L1; seminoma for NANOG and OCT4; and squamous carcinoma for SOX2, Nestin and PDPN.

The IHC results were independently evaluated by five observers (three pathologists (F.J.S.S., F.D.I. and V.B.L.), J.P.R. and J.M.G.-P.), blinded to clinical data. CD4, CD8 and FOXP3 immunostainings in both the tumor nests and the surrounding stroma were scored using the average of positively stained cells in each 1 mm^2 area from three independent high-power representative microscopic fields (HPFs, 400×; 0.0625 μm^2). The CD8/FOXP3 ratio was calculated using the CD8 and FOXP3 results. Since the cut-off value for TILs has not been standardized, the median of the number of $CD4^+$, $CD8^+$ and $FOXP3^+$ cells/mm^2 was chosen and used for survival analysis with patients categorized into high (above the median) and low (below the median) subgroups. Semiquantitative scoring was applied

for PD-L1 staining, which was considered positive if more than 10% of tumor cells were positively stained, as described previously [43]. NANOG immunostaining was scored as negative or positive, following previously described methods [10,11]. SOX2 staining scores were classified as negative or positive expression on the basis of values below or above the median cut-off value of 10%, respectively. Nestin staining was classified as low or high expression on the basis of values below or above the median cut-off value of 60% of cells with positive membranes and/or cytoplasmic staining. For PDPN assessment, immunostaining intensity was graded on a scale from zero to three (zero = negative, one = weak, two = moderate, three = strong), and in addition, the proportion of PDPN immunoreactive tumor cells was scored as zero (0%), one (1 to 33%), two (34 to 66%) or three (more than 66 % of tumor cells positive) [44]. To grade Podoplanin expression, each tumor was assigned an immunoreactive score (IRS) [44] based on the percentage of Podoplanin-positive tumor cells and the intensity of the staining. The IRS was calculated by multiplying the staining intensity by the percentage of positive cells resulting in a range from zero to nine. IRS equal or less than four was considered low, and the remaining values were seen as high [45].

The density and distribution of TILs in OSCC, CD4, CD8 and FOXP3 expression was analyzed by immunohistochemistry in 125 OSCC samples using tissue microarrays; these figures are shown elsewhere [46] (Figure 1A,B). The mean numbers of $CD4^+$ TILs (Figure 1C,D) in the tumor nests and the surrounding stroma were 6.02 $\pm$ 12.29 cells per mm^2 and 54.60 $\pm$ 68.15 cells per mm^2, respectively. The mean $CD8^+$ TILs (Figure 1E,F) in the tumor nests and the tumor stroma were 47.88 $\pm$ 57.16 and 178.45 $\pm$ 203.21 cells per mm^2, respectively; the mean numbers of $FOXP3^+$ lymphocytes (Figure 1G,H) in the tumor nests and the surrounding stroma were 3.11 $\pm$ 6.47 per mm^2 and 15.65 $\pm$ 24.54 per mm^2, respectively (Supplementary Figure S1).

2.3. Statistical Analysis

Data were analyzed using SPSS software version 18 (IBM Co., Armonk, NY, USA). Continuous variables ($CD4^+$, $CD8^+$ and $FOXP3^+$ cells) were expressed as the means $\pm$ standard deviation (SD), and absolute and relative frequencies were calculated for categorical variables. Correlations between the number of stromal or tumoral $CD4^+$, $CD8^+$ and $FOXP3^+$ cells were calculated using Spearman's correlation coefficient. TILs means per mm^2 for $CD4^+$, $CD8^+$ and $FOXP3^+$ cells were correlated with clinicopathological variables using Student's *t* test or the Mann-Whitney U test when variables had non-normal distributions. The endpoint of this study was disease-specific survival (DSS), calculated as the time from the date of surgery to death for the tumor. Censoring was defined as loss of follow-up or alive and free of recurrence at the end of the follow-up. Receiver operating characteristic (ROC) and area under the curve (AUC) analysis were used to estimate the predictive value of infiltrating TILs in OSCC patient survival. The survival rates were estimated by the Kaplan-Meier method and compared using the log-rank test. Moreover, the Cox regression model was applied to calculate hazard ratios (HRs) with 95% confidence intervals (95% CI). The factors that were significant in univariate analysis were then analyzed using the multivariate Cox regression model to determine the independent prognostic factors in the presence of other prognostically relevant covariates. All *p*-values were based on the two-sided statistical analysis, and a *p*-value less than 0.05 was considered statistically significant.

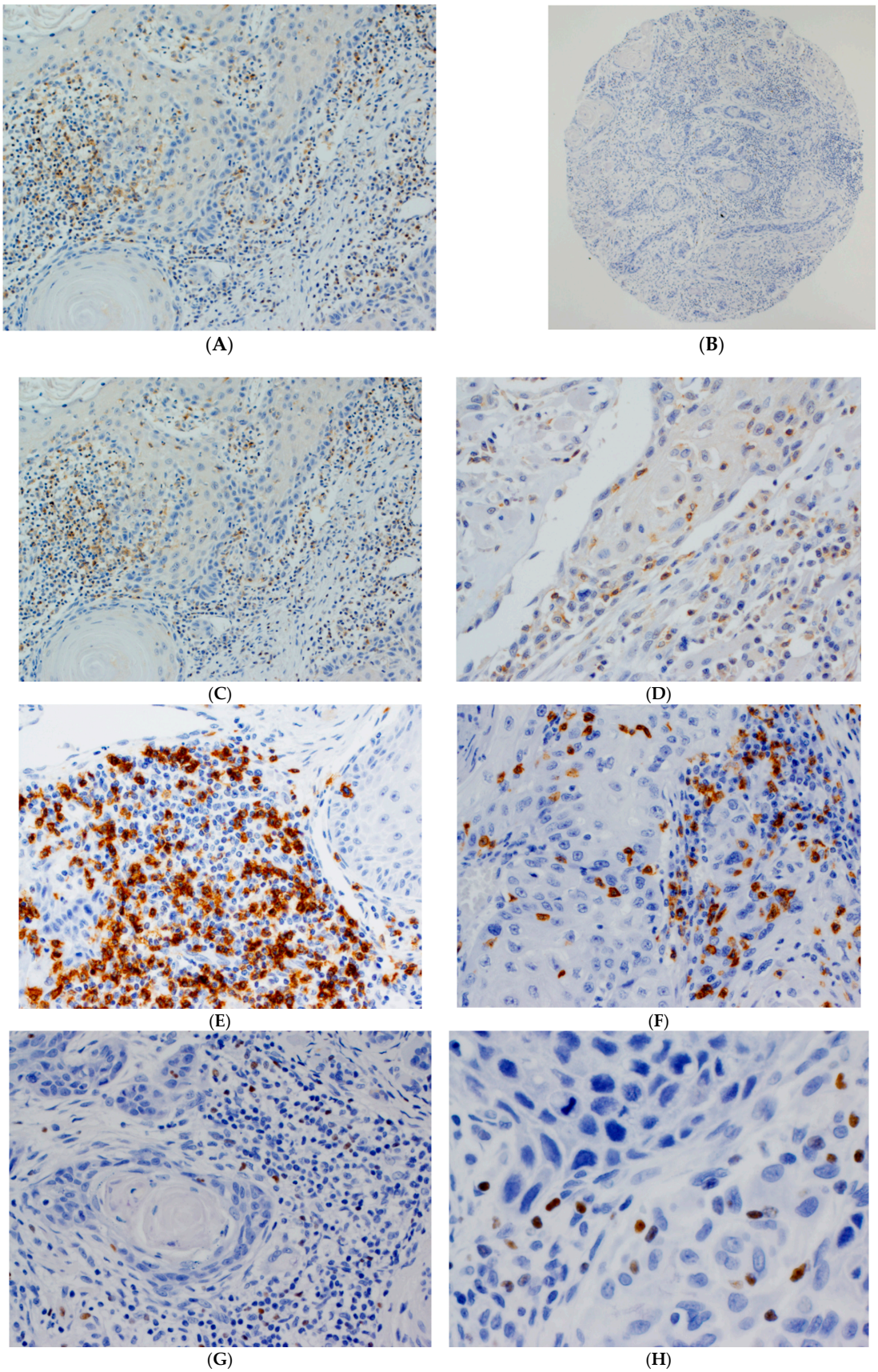


Figure 1. Representative examples of oral squamous cell carcinomas (OSCC) showing high (**A**) and low (**B**) densities of TILs. Stromal and tumoral staining of $CD4^+$ (**C**,**D**), $CD8^+$ (**E**,**F**) and $FOXP3^+$ (**G**,**H**) TILs. Magnification: 50× (**B**); 100× (**A**,**C**,**E**); 200× (**D**,**F**,**G**); 400× (**H**).

3. Results

3.1. Patient Characteristics

The patients were predominantly men (82 vs 43) and ranged in age from 28 to 91 years (mean 58.69, standard deviation 14.34 years). The tumor subsite was classified as the tongue (n = 51, 41%), floor of the mouth (n = 37, 30%), gingiva (n = 22, 18%), buccal mucosa (n = 7, 6%), retromolar area (n = 6, 4%) or palate (n = 2, 1%). Smoking habit and alcohol consumption were respectively reported in 84 (67%) and 69 (55%) patients. AJCC classification [42] T1 was detected in 27 (22%) patients, T2 in 54 (43%), T3 in 16 (13%) and T4 in 28 (22%) patients. pN0 classification was observed in 76 (61%), pN1 in 25 (20%) and pN2 in 24 (19%) patients. According to overall AJCC disease stages, 20 (16%) had stage I, 32 (26%) had stage II, 26 (20%) had stage III and finally, 47 (38%) had stage IV. Regarding histopathologic degree of differentiation, 80 (64%) OSCCs were well-differentiated, 41 (33%) moderately and 4 (3%) poorly-differentiated.

3.2. Immunohistochemical Evaluation of CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs in OSCC Tissue Specimens

There was a strong positive correlation between stromal and tumoral infiltration of the different TILs subtypes (Figure 1, Supplementary Figure S1), with the only exceptions being tumoral CD8$^+$ and stromal FOXP3$^+$, which were not significantly correlated (Supplementary Table S1).

3.3. Associations between CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs Density and Clinicopathological Variables

All TILs markers (CD4, CD8, FOXP3) were evaluated for the whole series (125 cases). Stromal CD4$^+$ TILs infiltration was significantly associated with a non-smoking habit, well-differentiated tumors and tongue tumoral location. On the other hand, tumoral CD4$^+$ TILs infiltration was significantly associated with older age, female gender and tobacco or alcohol consumption, as well as tongue location. Stromal CD8$^+$ TILs were significantly associated with well-differentiated tumors, and tumoral CD8$^+$ TILs were associated with the absence of tobacco or alcohol consumption. Finally, stromal FOXP3$^+$ TILs showed a significant association with T classification and with tumors located in the tongue (Table 1).

3.4. Associations between CD4$^+$, CD8$^+$, FOXP3$^+$ TILs Density and Expression of CSC Markers

The expression of NANOG and OCT4 was evaluated by immunohistochemistry in 122 cases, SOX2 in 121 cases, Nestin in 93 cases and PDPN in 85 cases in which representative tumor tissue was available. Positive SOX2 and NANOG expression was respectively detected in 49 (40%) and 39 (32%) of the OSCC samples in this series, as we previously reported [10,11]. Overall, infiltration of CD4$^+$ and CD8$^+$ TILs but not FOXP3$^+$ TILs was inversely correlated with the expression of the CSC markers NANOG and SOX2. Concordantly, stromal/tumoral CD4$^+$ and CD8$^+$ TILs were significantly higher in tumors harboring negative expression of NANOG, except for tumoral CD8$^+$ TILs that did not attain significance (Table 2). Noteworthy, the CD8$^+$/FOXP3$^+$ TILs ratios in both tumor nests and stroma were consistently higher in tumors harboring negative expressions of NANOG and SOX2; however, significant associations were only reached between the tumoral CD8$^+$/FOXP3$^+$ ratio and both SOX2 and NANOG. None of the tumor samples showed OCT4 expression. Positive Nestin and PDPN expressions were respectively detected in 86 of 93 (92%) and 28 of 85 (33%) OSCC samples in this series. Similar to our observations for NANOG and SOX2, higher infiltrations of CD4$^+$ and CD8$^+$ TILs, but not FOXP3$^+$ TILs, were observed in those tumors harboring low expressions of Nestin and PDPN (Table 2). In addition, stromal/tumoral FOXP3$^+$ TILs were significantly higher in tumors with a high PDPN expression. In turn, higher stromal/tumoral FOXP3$^+$ TILs densities were associated with tumors harboring low Nestin expression, reaching significance in the case of stromal FOXP3$^+$ TILs (Table 2).

Table 1. Associations between stromal and tumoral CD4, CD8 and FOXP3 TILs and the clinicopathological parameters in the cohort of 125 OSCC patients.

Variable	Number	Stromal CD4 Mean (SD)	*p*	Tumoral CD4 Mean (SD)	*p*	Stromal CD8 Mean (SD)	*p*	Tumoral CD8 Mean (SD)	*p*	Stromal FOXP3 Mean (SD)	*p*	Tumoral FOXP3 Mean (SD)	*p*
Age (years)													
<65	77	43.87 (42.35)	0.05	3.51 (5.43)	0.01	158.24 (160.38)	0.16	43.47 (58.60)	0.28	18.32 (27.33)	0.13	3.79 (7.70)	0.09
≥65	48	71.82 (94.14)		10.01 (17.94)		210.88 (256.15)		54.85 (54.68)		11.46 (18.92)		2.05 (3.67)	
Gender													
Female	43	66.37 (96.18)	0.25	7.92 (13.85)	0.03 *	205.31 (276.68)	0.37	51.88 (67.82)	0.57	16.19 (22.44)	0.85	3.40 (6.00)	0.71
Male	82	48.43 (46.92)		5.02 (11.34)		164.37 (151.46)		45.75 (50.94)		15.35 (25.73)		2.95 (6.74)	
Tobacco													
No	41	76.18 (96.80)	0.03 *	10.26 (18.58)	0.01 *	215.76 (226.61)	0.18	67.25 (69.83)	0.02	14.65 (22.50)	0.75	2.76 (4.95)	0.67
Yes	84	44.07 (45.62)		3.93 (6.70)		160.25 (189.54)		47.34 (5.19)		16.15 (25.62)		3.28 (7.13)	
Alcohol consumption													
No	56	67.10 (86.31)	0.08	8.54 (16.18)	0.004 *	208.14 (222.55)	0.14	58.69 (64.46)	0.04 *	13.84 (20.70)	0.45	2.38 (4.40)	0.25
Yes	69	44.46 (47.02)		3.95 (7.27)		154.36 (184.20)		38.98 (49.08)		17.15 (27.40)		3.72 (7.78)	
T classification													
T1 + 2	81	55.93 (54.83)	0.76	6.28 (12.52)	0.75	192.29 (206.21)	0.3	45.69 (58.84)	0.56	17.96 (26.50)	0.02 *	3.55 (7.14)	0.3
T3 + 4	44	52.15 (88.27)		5.56 (11.99)		152.99 (197.33)		51.85 (54.40)		11.34 (19.97)		2.30 (4.96)	
N classification													
N0	76	52.74 (50.56)	0.7	5.22 (8.31)	0.43	176.77 (196.70)	0.9	50.73 (59.57)	0.49	14.24 (21.01)	0.42	2.78 (4.83)	0.48
N+	49	57.48 (89.39)		7.25 (16.68)		181.07 (214.97)		43.52 (53.56)		17.84 (29.32)		3.62 (8.46)	
Stage													
I + II	52	57.77 (55.79)	0.66	5.93 (9.88)	0.94	197.14 (224.40)	0.38	47.67 (63.87)	0.97	16.93 (24.08)	0.62	3.43 (5.61)	0.64
III + IV	73	52.34 (76.04)		6.09 (13.80)		165.14 (187.14)		48.02 (52.42)		14.74 (24.98)		2.88 (7.05)	
Grade													
Well	80	63.23 (80.43)	0.02	6.09 (11.39)	0.94	204.61 (235.63)	0.02	48.23 (59.11)	0.92	14.70 (23.95)	0.57	3.15 (6.98)	0.92
Moderate + Poor	45	39.26 (33.17)		5.91 (13.86)		131.96 (115.05)		47.25 (54.20)		17.28 (25.72)		3.04 (5.56)	
Site													
Tongue	51	73.87 (90.52)	0.01 *	9.52 (17.06)	0.02	200.96 (229.44)	0.3	43.11 (50.32)	0.44	21.67 (32.63)	0.04	4.26 (8.85)	0.15
Others	74	41.32 (42.89)		3.58 (6.43)		162.95 (183.01)		51.21 (61.61)		11.52 (15.92)		2.33 (4.01)	
Recurrence													
No	71	47.50 (49.79)	0.25	4.78 (8.86)	0.23	171.85 (173.57)	0.67	45.65 (55.24)	0.62	18.85 (27.78)	0.06	3.61 (7.26)	0.05
Yes	54	62.63 (86.47)		7.64 (15.61)		187.13 (238.10)		50.76 (59.96)		11.55 (19.12)		2.47 (5.30)	
Second primary tumor													
No	106	53.13 (70.30)	0.57	5.72 (12.24)	0.52	170.75 (205.11)	0.31	44.95 (51.89)	0.18	15.99 (25.91)	0.71	3.09 (6.64)	0.92
Yes	19	62.79 (55.56)		7.70 (12.80)		221.45 (191.76)		64.07 (80.24)		13.76 (15.36)		3.24 (5.65)	

Tumoral but not stromal $CD8^+$ infiltration was significantly associated with non-smokers ($p = 0.02$) and non-alcohol drinkers ($p = 0.04$), and stromal $CD8^+$ infiltration was significantly higher in well-differentiated tumors ($p = 0.02$). Higher stromal, but not tumoral $FOXP3^+$ TILs infiltration was significantly associated with smaller tumors (T1 and T2) ($p = 0.02$), as well as tumors arisen in the tongue ($p = 0.04$) (Table 1). It was not possible to calculate the $CD8^+$ /$FOXP3^+$ ratio in all the cases since for several of them, the TIL density was zero. In fact, the stromal $CD8^+/FOXP3^+$ ratio was determined in 106 cases, and the tumoral $CD8^+/FOXP3^+$ ratio in 80 cases. Higher stromal and tumoral $CD8^+/FOXP3^+$ ratios were significantly found in older patients ($p = 0.001$), and a higher tumoral $CD8^+/FOXP3^+$ ratio was significantly associated with non-smokers ($p = 0.01$) and non-alcohol drinkers ($p = 0.005$). No other significant associations were observed with any of the remaining clinicopathological variables (Supplementary Table S2). * U Mann-Whitney test.

Table 2. Associations between tumoral and stromal $CD4^+$, $CD8^+$ and $FOXP3^+$ TILs and expression of CSCs markers.

Factor	SOX2		*p*	NANOG		*p*	Nestin		*p*	PDPN (IRS)		*p*
	Negative	Positive		Negative	Positive		Low	High		Low	High	
Stromal CD4 (mean, SD)	63.66 (81.02)	43.61 (43.92)	0.11	63.00 78.30)	36.71 (35.64)	0.01	58.28 (53.67)	49.61 (52.18)	0.30	61.45 (88.09)	47.78 (36.98)	0.61
Tumoral CD4 (mean, SD)	6.89 (14.65)	5.06 (8.13)	0.43	7.51 (14.32)	3.29 (6.12)	0.03	6.26 (6.83)	5.15 (11.36)	0.21	8.02 (16.48)	4.63 (7.09)	0.96
Stromal CD8 (mean, SD)	215.23 (234.37)	128.21 (142.87)	0.01	200.83 (229.44)	134.07 (131.46)	0.04	229.90 (172.05)	163.05 (187.08)	0.13	189.45 (239.22)	186.60 (161.68)	0.45
Tumoral CD8 (mean, SD)	53.44 (58.72)	41.95 (55.66)	0.04	53.95 (61.19)	36.11 (48.35)	0.11	39.28 (67.17)	45.52 (58.46)	0.55	51.36 (63.00)	45.38 (42.81)	0.90
Stromal FOXP3 (mean, SD)	15.57 (22.17)	16.48 (28.32)	0.84	14.81 (24.46)	17.05 (24.92)	0.64	32.85 (37.47)	13.48 (20.90)	0.01	9.75 (17.19)	20.02 (21.76)	0.003
Tumoral FOXP3 (mean, SD)	2.54 (4.60)	4.12 (8.57)	0.24	3.22 (7.37)	2.90 (4.36)	0.80	7.54 (9.36)	2.25 (4.39)	0.05	2.10 (4.20)	3.29 (5.53)	0.03
Stromal CD8/FOXP3 ratio (mean, SD)	56.48 (124.57)	38.03 (78.71)	0.15	62.08 (129.78)	25.05 (36.24)	0.64	14.15 (10.93)	52.28 (127.31)	0.54	71.66 (137.10)	48.51 (113.58)	0.08
Tumoral CD8/FOXP3 ratio (mean, SD)	35.32 (56.90)	22.39 (57.74)	0.008	39.29 (67.86)	12.58 (15.63)	0.02	7.42 (6.04)	35.28 (68.76)	0.35	46.52 (84.19)	29.58 (35.36)	0.96

3.5. Associations between CD4$^+$, CD8$^+$, FOXP3$^+$ TILs Density and PD-L1 Expression

Positive PD-L1 expression in more than 10% of tumor cells was detected in 18 (15%) of our series of OSCC samples, as previously reported [43]. High tumoral CD4$^+$ and CD8$^+$ -infiltrating TILs were significantly associated with PD-L1-positive tumors (Table 3). The tumoral CD8$^+$/FOXP3$^+$ TILs ratio was significantly higher in PD-L1-positive tumors, while the stromal CD8$^+$/FOXP3$^+$ ratio was higher in PD-L1-negative tumors (Table 3).

Table 3. Association between CD4$^+$, CD8$^+$ and FOXP3$^+$TILs and PD-L1 expression (clone 22C3) in the cohort of 125 OSCC patients.

Factor	Tumor PD-L1		*p*
	≤10%	>10%	
Stromal CD4 (mean, SD)	50.09 (48.50)	86.40 (134.59)	0.27
Tumoral CD4 (mean, SD)	4.57 (7.89)	15.16 (24.77)	0.01
Stromal CD8 (mean, SD)	161.87 (158.97)	285.01 (365.01)	0.17
Tumoral CD8 (mean, SD)	40.10 (49.76)	98.49 (73.77)	0.004
Stromal FOXP3 (mean, SD)	16.18 (25.41)	14.57 (21.23)	0.80
Tumoral FOXP3 (mean, SD)	3.22 (6.86)	2.98 (4.43)	0.88
Stromal CD8/FOXP3 ratio (mean, SD)	50.03 (116.61)	43.89 (49.56)	0.08
Tumoral CD8/FOXP3 ratio (mean, SD)	26.63 (48.44)	49.02 (92.22)	0.01

3.6. Impact of CD4$^+$, CD8$^+$ and FOXP3$^+$ Infiltrating TILs on the Survival of OSCC Patients

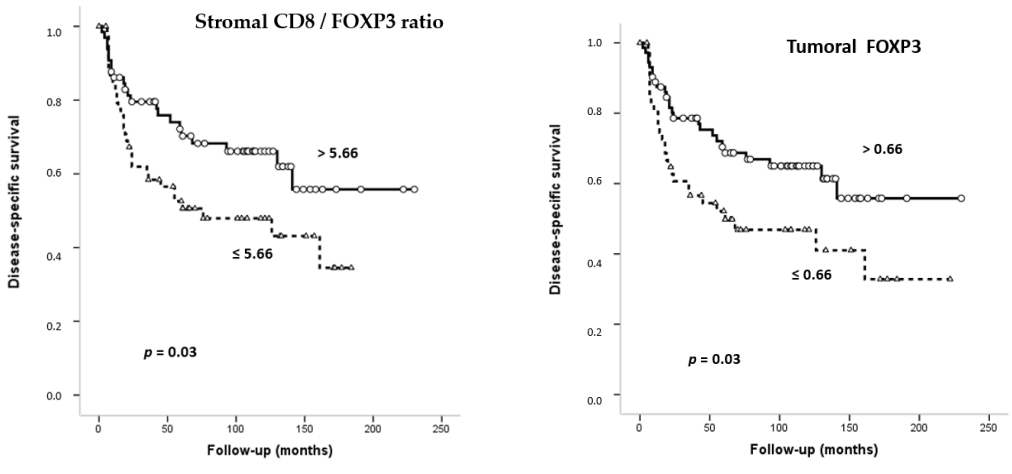
Follow-up information was available for 125 OSCC patients, ranging from 6 to 230 months with a mean of 74.10 (SD: 57.08) and a median of 61 months. At the end of this study, 17 (14%) patients were lost during the follow-up period, 53 (44%) patients were alive and free of recurrence and finally, 51 (42%) of them died of cancer or showed a non-treatable recurrence. Stromal/tumoral CD4$^+$, CD8$^+$ and FOXP3$^+$-infiltrating TILs were divided into two categories (high/low density) according to their respective median values. As shown in Supplementary Figure S2 and Table S3, stromal and tumoral FOXP3$^+$ TILs were superior to other TIL markers in terms of determining patient prognosis (AUC = 0.384, 95% CI = 0.285–0.483, p = 0.028; and AUC = 0.368, 95% CI = 0.270–0.466, p = 0.012; respectively). Contrasting this, tumoral and stromal CD4$^+$ and CD8$^+$ TILs did not show any significant relationship with patient survival (Table 4, Supplementary Figure S2 and Table S3). However, high densities of stromal and tumoral FOXP3$^+$ TILs were significantly associated with more favorable prognosis (p = 0.03 and p = 0.03, respectively; Table 4 and Figure 2A,B). The median value of the tumoral CD8$^+$/FOXP3$^+$ ratio was 11.22 and the median value of the stromal CD8$^+$/FOXP3$^+$ ratio was 10.98. A low tumoral CD8$^+$/FOXP3$^+$ ratio was found to significantly associate with a better prognosis (p = 0.04; Figure 2C).

We also performed a stratified univariate Kaplan-Meier analysis according to CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs densities (Supplementary Table S4). A high stromal CD8$^+$ TILs density (above the median) was significantly associated with a better survival in patients with ages lower than 65 years (p = 0.01), male gender (p = 0.02), tobacco (p = 0.02) and alcohol consumption (p = 0.02), neck lymph node metastasis (p = 0.03), well-differentiated tumors (p = 0.03), complementary radiotherapy (p = 0.01), negative SOX2 expression (p = 0.03) and negative NANOG expression (p = 0.04). On the other hand, high stromal FOXP3$^+$ TILs density (above the median) was significantly associated with better prognosis in female patients (p = 0.03), smokers (p = 0.02), drinkers (p = 0.02), patients with tumors arisen in the tongue (p = 0.03) and negative PD-L1 expression (p = 0.02). Similarly, a high tumoral FOXP3$^+$ density was associated with increased survival in patients younger than 65 years (p = 0.02), female patients (p = 0.01), smokers (p = 0.01), alcohol drinkers (p = 0.01), patients treated with complementary radiotherapy (p = 0.04) and tumors with negative PD-L1 expression (p = 0.01). A low stromal CD8$^+$/FOXP3$^+$ ratio was significantly associated with a better prognosis in patients with female gender (p = 0.009), history of alcohol consumption (p = 0.03), tongue tumor location (p = 0.01) and well-differentiated tumors (p = 0.03) (Supplementary Table S4).

Table 4. Kaplan-Meier and univariate Cox cancer-free survival analysis in the cohort of 125 patients with OSCC.

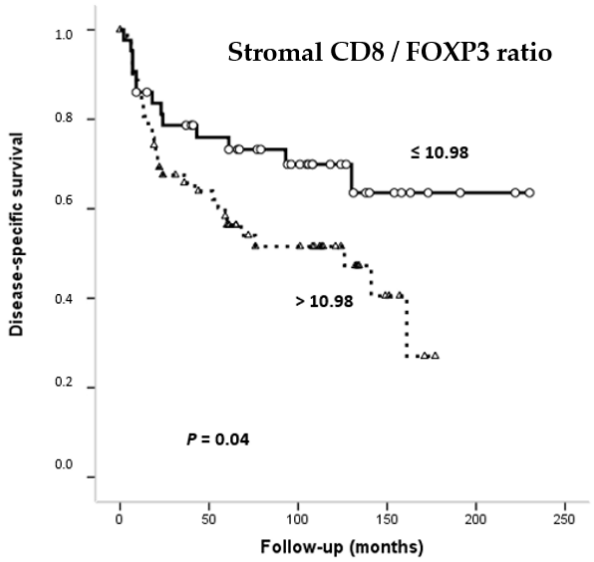
Parameter	Number	Censored Patients (%)	Cancer-Free Survival Time (95% CI)	*p*	Hazard Ratio	95% CI
pT classification						
• T1–T2	81	53 (65)	151.82 (129.03–174.99)	0.001	2.49	1.44–4.30
• T3–T4	44	19 (43)	77.62 (54.19–101.04)			
pN classification						
• N0	76	49 (65)	127.96 (109.43–146.49)	0.01	1.92	1.12–3.31
• N+	49	23 (4)	108.58 (77.88–139.28)			
Stage						
• I + II	52	36 (69)	140.09 (120.15–160.04)	0.002	2.4	1.33–4.32
• III + IV	73	36 (49)	113.33 (87.73–138.93)			
Grade						
• Well	80	44 (55)	127.85 (103.73–151.98)	0.59	0.85	0.48–1.52
• Moderate + Poor	45	28 (62)	121.63 (96.25–147.01)			
Stromal $CD4^+$						
• ≤32.66	62	34 (55)	127.86 (102.28–153.45)	0.6	0.86	0.50–1.49
• >32.66	63	38 (60)	137.49 (110.54–164.44)			
Tumoral $CD4^+$						
• ≤2.66	59	31 (53)	120.39 (93.85–146.93)	0.42	0.8	0.47–1.38
• >2.66	66	41 (62)	142.01 (115.42–168.60)			
Stromal $CD8^+$						
• ≤118	63	32 (51)	109.62 (81.85–137.39)	0.05	0.58	0.33–1.01
• >118	62	40 (65)	152.28 (126.67–177.90)			
Tumoral $CD8^+$						
• ≤24.65	61	35 (57)	133.16 (105.84–160.47)	0.95	1.01	0.59–1.74
• >24.65	64	37 (58)	111.28 (90.91–131.66)			
Stromal $FOXP3^+$						
• ≤5.66	60	29 (48)	96.14 (75.24–117.05)	0.03	0.56	0.32–0.98
• >5.66	65	43 (66)	152.79 (126.93–178.66)			
Tumoral $FOXP3^+$						
• ≤0.66	53	25 (47)	105.47 (77.32–133.62)	0.03	0.54	0.31–0.93
• >0.66	72	47 (65)	151.97 (127.55–176.38)			
Stromal $CD8/FOXP3^+$ ratio						
• ≤10.9853	43	30 (70)	162.89 (132.67–193.11)	0.04	1.9	0.99–3.64
• >10.9853	63	32 (51)	97.82 (78.78–116.87)			
Tumoral $CD8/FOXP3^+$ ratio						
• ≤11.2254	39	28 (72)	163.64 (131.68–195.59)	0.06	2.01	0.95–4.27
• >11.2254	41	22 (54)	100.67 (77.71–123.63)			

p values were estimated using the log-rank test.



(**A**) (**B**)

Figure 2. *Cont.*



(**C**)

Figure 2. Kaplan-Meier disease-specific survival curves in the cohort of 125 OSCC patients categorized by stromal FOXP3$^+$ (**A**), tumoral FOXP3$^+$ (**B**) or stromal CD8$^+$/FOXP3$^+$ ratio (**C**). Median values were used as cut-off points. *p* values were estimated using the log-rank test.

Multivariate analysis further revealed that clinical stage, which combines pT and pN classification, and a high stromal CD8$^+$/FOXP3$^+$ ratio were the only parameters independently associated with DSS (HR = 2.195, $p = 0.01$ and HR = 2.039, $p = 0.03$, respectively) (Supplementary Table S5).

4. Discussion

In this study, we assessed the relationship between CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs, as well as with PD-L1 and CSC markers in OSCC, and their association with clinicopathologic features and disease prognosis. We found a strong positive correlation among stromal and tumoral CD4$^+$, CD8$^+$ and FOXP3$^+$ cell numbers, with the only exceptions being tumoral CD8 and stromal FOXP3. These findings support the presence of different TILs subtypes in OSCC TME interacting with one another to exert their effects, which highlights the idea that the numbers and functions of TILs are dynamic in nature. The relationship between cancer cells and host immune cells is very complex. The immune system promotes the elimination of tumor cells, but this system plays a dual role in tumor evolution, and cancer cells can display an escape from the immune system, maintaining the tumor progression [47]. In this study, the numbers of CD4$^+$ and CD8$^+$ lymphocytes were not significant predictors of the patient's survival in the multivariate analysis, results that are consistent with other reports [3,37,48–51]. Of interest, here, CD4$^+$ and CD8$^+$ cells showed higher numbers in patients who did not have tobacco or alcohol habits; these associations suggest that tobacco and alcohol consumption not only play a carcinogenic role in OSCC but may also influence the host's immune system response. Studies showed that CD8$^+$ cells are a critical barrier to the initial development of tumors [52]. CD8$^+$ TIL infiltration of tumors has been largely related to favorable outcomes in different cancers and to a favorable response to chemoradiotherapy [53,54], while immunosuppressive Tregs can confer good or poor prognosis depending on the context. Some evidence has been provided that higher numbers of Tregs are linked to worse prognosis in HNSCCs [55], but others have noticed conflicting results [56]. We herein identified stromal and tumoral FOXP3$^+$ TILs as independent prognostic factors in the whole OSCC series, and all the studied lymphocyte subtypes also showed prognostic relevance in different strata of other variables. Our results indicate a major impact of FOXP3$^+$ Tregs, more so than CD8$^+$ T cells and CD4$^+$ T helpers, on the patient's outcome. Furthermore, in a multivariate analysis, the stromal CD8$^+$/FOXP3$^+$

ratio was revealed as an independent prognostic indicator together with the clinical stage of the disease. Immunohistochemistry allows for the location of TILs in the tumor nests or tumor stroma separately, and this could be of a paramount importance because in previous reports, stromal TILs seem more relevant than intra-epithelial ones [57].

OSCC is a cancer exposed to a microbial environment within the oral cavity, and it is often ulcerated and heavily infiltrated by TILs. Troiano et al. [58] classified oral tongue squamous cell carcinomas into three groups: immune-inflamed, when TILs were found inside the tumor mass in proximity to tumor cells; immune-excluded, when TILs were located into the stroma, outside the tumor; and finally, immune-desert, when tumors lacked TILs. Tumors of the latter group showed the worse prognosis among tongue carcinomas. It is not entirely understood how higher Treg infiltrates could be beneficial for patient survival [59]; however, we can speculate that this phenomenon may be due to the relationship between Tregs and an inflamed TME [60]. In this regard, various immunosuppressive markers, such as PD-L1 and FOXP3, have been associated with T-cell-inflamed tumors and better prognosis [61]. On the other hand, FOXP3 is essential to functional maintenance of Tregs, whose infiltration in the TME negatively regulates the immune response against tumors, favoring tumor growth and consequently related to a poor prognosis in HNC [62]. However, we found here a paradoxical relationship between high $FOXP3^+$-infiltrating TILs and good prognosis. A meta-analysis comprising 15,512 patients demonstrated that the prognostic role of FOXP3 was highly influenced by the tumor site and tumor stage [38]. Thus, when analyzing the impact of immunological parameters on OSCC patient survival, we identified that a low density of both stromal and tumoral $FOXP3^+$ lymphocytes showed a poorer outcome compared with their counterparts with high $FOXP3^+$ lymphocyte numbers. Thus, high $FOXP3^+$ cell infiltration was associated with poor prognosis in the majority of solid tumors and no prognostic effect was observed in ovarian and pancreatic cancers, whereas in others, such as head and neck cancers, tumor infiltrating $FOXP3^+$ T cells were associated with favorable prognosis [33,38]. Our results are in line with some authors [3,24,36,59,63], but contrasted to a few others [24,50,64]. Hence, the prognostic relevance of $FOXP3^+$ Tregs is not completely understood. Furthermore, it is necessary to take in account that $FOXP3^+$ T cells are highly heterogeneous with respect to their genotype and phenotype, having been subdivided into three functional subpopulations: effector, resting and non-suppressive cytokine-secreting Tregs [65]. Here a high density of $FOXP3^+$ T cells was associated with extended survival in patients in the whole sample, but especially in patients treated not only by surgery but also by radiotherapy. Interestingly, in patients who underwent surgery alone, only a modest and non-significant improvement in DSS was noticed. Radiotherapy affects the TME, and it has been observed that is able to enhance the antitumor immune response in rectal and pancreatic cancer [66,67]. It has been suggested that the composition of the TME before treatment is of less importance than the antitumor immune response induced by the radiotherapy [68]. In this sense, we studied the composition of TME in OSCC tissue specimens obtained from primary surgical treatment, i.e., pre-radiotherapy TME, and still a relationship with a better prognosis was observed. The conflicting results from different studies suggest that $FOXP3^+$ T cells act differently in different anatomical subsites and tumor stages [69], changing to pro-tumorigenic cells as the tumor progresses, or that it might depend on their origin, with only $FOXP3^+$ T cells recruited from the peripheral blood involved in tumor elimination [57]. Together with the aforementioned functional heterogeneity of $FOXP3^+$ subpopulations, a possible explanation for the striking association of Tregs with a favorable prognosis could be related to a role in suppressing or at least down-regulating inflammatory reactions that promote tumor progression by growth factor and cytokine production by immune cells, killing macrophages and monocytes [33,38,70]. Importantly, we have identified various subsets of patients based on clinicopathological factors and TILs subtypes that aid in establishing different outcomes. Nevertheless, some caution is warranted because of possible over fitting of the data as well as power loss in the data analysis due to the large number of variables analyzed and the small size in

some clinical groups. The anatomical location of the tumor within the oral cavity may influence the prognostic role of T cells. Oral cancer arises from diverse subsites, and tongue carcinomas should be studied separately as they have unique epidemiological characteristics different from those of other oral cavity cancers [69]. The present study reported that stromal and tumoral $CD4^+$ T cell densities and also stromal $FOXP3^+$ T cell density were higher in tumors located in the tongue in comparison to tumors arisen in other sites within the oral cavity. These results seem to be partially consistent with findings provided by Kashima et al., [71] who found a predominance of $CD8^+$ and $FOXP3^+$ T cells in tongue squamous cell carcinomas. In this study, a high stromal $FOXP3^+$ TILs density and a low stromal $CD8^+/FOXP3^+$ ratio were associated with a better outcome in tongue carcinomas, while $CD4^+$ T cell density did not show any prognostic relevance, possibly due to the diversity of T-helper subtypes and hence their functional complexity. Here, TILs' density was separately evaluated in two different compartments within OSCC TME. Using a similar procedure, Naito et al. [72] found that $CD8^+$ T cells infiltrating the tumor islands affected prognosis positively in colorectal cancer, while $CD8^+$ T cells located in the tumor stroma had no effect on prognosis. Conversely, Menon et al. [73] showed the prognostic relevance of stromal $CD8^+$ TILs in colorectal cancer.

In addition, we also found a positive association between PD-L1 expression and a higher $CD4^+$ and $CD8^+$ TILs densities within the tumor islands, which is in accordance with previous studies [68]. Even though $CD8^+$ TILs' density did not globally show a significant impact on the prognosis in our cohort of OSCC patients, it is worth mentioning that a higher TILs density was associated with a better prognosis in specific subsets of OSCC patients with neck node metastasis, well-differentiated tumors and patients treated with surgery and radiotherapy. It has been shown that PD-L1 correlates with the presence of $CD8^+$ TILs in the TME, [74] and PD-L1 can induce apoptosis in activated antigen-specific $CD8^+$ cells [75]. A negative correlation between $PD\text{-}L1^+$ and $CD8^+$ cells in OSCC has been observed, which implies that the blockade of PD-L1 induces local immune activation [76]. However, we found a significant positive association between $CD8^+$ TILs in the tumor compartment and positive PD-L1 expression, which could reflect the presence of an adaptive immune resistance mechanism triggered by $CD8^+$ T cells that secretes IFNγ, suggesting that $CD8^+$ TILs may have a function other than a cytotoxic one [77]. Thus, if T cells are exposed to a persisting antigen for two to four weeks, T-cell exhaustion may be established [48]. OSCC comprises a heterogeneous cell population, including CSCs, with tumor-associated antigens present, which can be potentially targeted by immune cells [59]. CSCs may escape from the host's immunosurveillance by producing cytokines in the TME and paralyzing the immune system responses, by converting a subset of immature myeloid DCs into TGF-β-secreting cells, thus attracting Tregs to tumors, facilitating spreading and metastasis [74,75,77–79] and inhibiting $CD8^+$ T cells [14]. CSCs may express different stem-like markers such as SOX2, NANOG and OCT4 that allow them to or preclude them from adapting to challenging environmental situations. Thus, various CSs subpopulations can co-exist within a tumor and expand according to their own hierarchy, driving tumor evolution [8]. Notably, in this study, $CD4^+$ and $CD8^+$ TILs were inversely and significantly associated with the expression of two important CSC-related regulators NANOG and SOX2, and analogous associations were observed for two additional CSC markers, Nestin and PDPN, with the only exceptions being tumoral $CD8^+$ and Nestin. Moreover, the tumoral $CD8^+/FOXP3^+$ ratio was inversely and significantly associated with the expression of NANOG and SOX2, thereby suggesting an inverse association between cytotoxic and helper TILs infiltration and stemness in OSCC. A similar trend was found for PDPN but not Nestin. NANOG not only contributes to the regulation of pluripotency in stratified epithelia [80]; it also mediates tumor cell proliferation, epithelial-mesenchymal transition and escape from immune system [78]. NANOG expression in OSCC is regulated by SOX2, OCT4, KLF4, AGR2, NOTCH1 and miR-34a [13]. However, OCT4 expression was not detected in any tumor in our OSCC cohort. Concordantly, analogous observation was obtained from the analysis of a series of 348 tumors located in oropharynx, hypopharynx

and larynx [81], while quite remarkably, different OCT4 antibodies were employed in both studies. Ghazi et al. [82] found that SOX2 expression did not correlate with OCT4 expression in OSCC. Additionally, Vijayakumar et al. [83] studied 20 cases of OSCC and 20 cases of oral epithelial dysplasia (OED), and found that SOX2 expression was higher in OSCC than in OED; most cases predominantly showed high SOX2 expression accompanied by negative OCT4 expression. Together these data highlight that SOX2 expression in HNSCC and OSCC seems to primarily be independent of OCT4 transcriptional control. OCT4 and SOX2 have been reported to play different roles in CSC biology. In the absence of OCT4 expression, neoplasms could not be initiated from normal tissues, but without SOX2 expression, the neoplastic cells could not be self-renewed to maintain tumor growth [82]. Nestin is considered a CSC marker in epithelial neoplasms and has been shown to play key roles in differentiation, proliferation, migration, invasion, metastasis and survival of malignant neoplastic cells through regulation of the cytoskeleton and progenitor cells [83]. In this study, Nestin expression was present mainly in the cytoplasm of tumor cells and showed an inverse relationship with the stromal/tumoral $CD8^+$, $CD4^+$ and $FOXP3^+$ cell densities and $CD8^+/FOXP3^+$ ratios similar to other CSC markers studied (i.e., NANOG and SOX2), although the only significant association reached was with stromal $FOXP3^+$ TILs. In contrast, PDPN expression, a demonstrated CSC marker in squamous carcinoma cells [21], while showing similar relationships with different TILs subtypes such as SOX2 and NANOG, was only significantly correlated with stromal and tumoral $FOXP3^+$ cells. As far as we know, our study is the first to consistently show striking inverse correlations between $CD8^+$ TIL, $CD4^+$ TIL and $CD8^+/FOXP3^+$ ratios in OSCC TME and the expression of various well-established CSC markers such as NANOG, SOX2 and Nestin, and also between $FOXP3^+$ lymphocytes and PDPN expression. According to these findings, we hypothesize that cytotoxic and helper TILs' infiltration does not seem to be related to CSC niche maintenance, but opposed to stemness maintenance and CSCs' escape from the immune system. These findings are in line with our previous study demonstrating an inverse association between both stromal and tumoral M2 macrophages and NANOG expression [84]. Our results also pose a possible link between PDPN expression and immunosuppression in OSCC. The literature on the prognostic significance of TILs is heterogeneous in terms of sample sizes, patient cohorts and methodological differences among studies mainly due to the diverse techniques, scoring methods and cut-off values used, which may altogether contribute to contradictory results [45]. Moreover, immunohistochemically phenotyping T lymphocyte subsets is limited by the fact that this technique does not provide any information on their functionality [54].

5. Conclusions

These findings demonstrate that stromal/tumoral $FOXP3^+$ TILs infiltration and a low stromal $CD8^+/FOXP3^+$ ratio are significantly associated with improved survival, and the stromal $CD8^+/FOXP3^+$ ratio emerges as a significant independent prognostic factor in OSCC. Moreover, high $CD4^+$ and $CD8^+$ T infiltration and a high tumoral $CD8^+/FOXP3^+$ ratio are significantly associated with high tumoral PD-L1. Strikingly, this study uncovers an inverse relationship between high infiltration of $CD8^+$ and $CD4^+$TILs with the absence/low expression of various CSC markers (i.e. NANOG, SOX2 and Nestin), while $FOXP3^+$ Treg infiltration is significantly correlated with PDPN expression. Further research is warranted to validate these results.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biomedicines9060653/s1. Figure S1. Stromal and tumoral distribution of CD8+, CD4+ and FOXP3+ T cell subsets in OSCC patients. Whiskers indicate variability outside the 75 and 25 percentiles. The Y axis represents the number of infiltrating T cells. Circles represent outliers and asterisks extreme values; Figure S2. ROC curves of stromal/tumoral CD4, CD8, and FOXP3 (A) and stromal/tumoral CD8/FOXP3 ratio (B). Table S1. Correlations between the mean numbers of CD4, CD8 and FOXP3 TIL infiltration in the tumor nests and surrounding stroma. The Spearman's Rho coefficients and the corresponding p values are shown; Table S2. Associations between stromal

and tumoral CD8/FOXP3 ratios and clinicopathological parameters in the cohort of 125 OSCC patients. Table S3. DSS predictive ac-curacy of CD4, CD8 and FOXP3 infiltrating TILs; Table S4. Stratified univariate Kaplan-Meier analysis of stromal and tumoral CD8+ and FOXP3+ TILs with clinicopathological variables in 125 OSCC patients. Median values were used as cut-off points. No correlations were found with stromal and tumoral CD4; Table S5. Multivariate Cox regression of disease-specific survival for clinical variables and TILs infiltration.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available within the article and Supplementary Materials.

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References

1. Ferris, R.L. Immunology and immunotherapy of head and neck cancer. *J. Clin. Oncol.* **2015**, *33*, 3293–3304. [CrossRef]
2. Arneth, B. Tumor microenvironment. *Medicina* **2019**, *56*, 15. [CrossRef]
3. Nguyen, N.; Bellile, E.; Thomas, D.; McHugh, J.; Rozek, L.; Virani, S.; Peterson, L.; Carey, T.E.; Walline, H.; Moyer, J.; et al. Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. *Head Neck* **2016**, *38*, 1074–1084. [CrossRef] [PubMed]
4. Luen, S.; Virassamy, B.; Savas, P.; Salgado, R.; Loi, S. The genomic landscape of breast cancer and its interaction with host immunity. *Breast* **2016**, *29*, 241–250. [CrossRef] [PubMed]
5. Zou, W.; Chen, L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat. Rev. Immunol.* **2008**, *8*, 467–477. [CrossRef]
6. Kythreotou, A.; Siddique, A.; Mauri, F.A.; Bower, M.; Pinato, D.J. PD-L1. *J. Clin. Pathol.* **2018**, *71*, 189–194. [CrossRef]
7. Valle, S.; Martin-Hijano, L.; Alcalá, S.; Alonso-Nocelo, M.; Sainz, B., Jr. The ever-evolving concept of the cancer stem cell in pancreatic cancer. *Cancers* **2018**, *10*, 33. [CrossRef]
8. Martin-Hijano, L.; Sainz, B., Jr. The interactions between cancer stem cells and the innate interferon signaling pathway. *Front. Immunol.* **2020**, *11*, 526. [CrossRef]
9. Davis, S.J.; Divi, V.; Owen, J.H.; Bradford, C.R.; Carey, T.E.; Papagerakis, S.; Prince, M.E.P. Metastatic potential of cancer stem cells in head and neck squamous cell carcinoma. *Arch. Otolaryngol. Head Neck Surg.* **2010**, *136*, 1260–1266. [CrossRef] [PubMed]
10. de Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Allonca, E.; Vallina, A.; Singhania, A.; Donate-Pérez Del Molino, P.; García-Pedrero, J.M. The emerging role of NANOG as an early cancer risk biomarker in patients with oral potentially malignant disorders. *J. Clin. Med.* **2019**, *8*, 1376. [CrossRef]
11. de Vicente, J.C.; Donate-Pérez Del Molino, P.; Rodrigo, J.P.; Allonca, E.; Hermida-Prado, F.; Granda-Díaz, R.; Rodríguez Santamarta, T.; García-Pedrero, J.M. SOX2 expression is an independent predictor of oral cancer progression. *J. Clin. Med.* **2019**, *8*, 1744. [CrossRef] [PubMed]

12. Major, A.G.; Pitty, L.P.; Farah, C.S. Cancer stem cell markers in head and neck squamous cell carcinoma. *Stem Cells Int.* **2013**, *2013*, 319489. [CrossRef] [PubMed]
13. Grubelnik, G.; Boštjančič, E.; Grošelj, A.; Zidar, N. Expression of NANOG and its regulation in oral squamous cell carcinoma. *Biomed. Res. Int.* **2020**, *2020*, 8573793. [CrossRef] [PubMed]
14. Tsai, L.L.; Yu, C.C.; Chang, Y.C.; Yu, C.H.; Chou, M.Y. Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. *J. Oral. Pathol. Med.* **2011**, *40*, 621–628. [CrossRef]
15. Chiou, S.H.; Yu, C.C.; Huang, C.Y.; Lin, S.C.; Liu, C.J.; Tsai, T.H.; Chou, S.H.; Chien, C.S.; Ku, H.H.; Lo, J.F. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin. Cancer Res.* **2008**, *14*, 4085–4095. [CrossRef] [PubMed]
16. Curtarelli, R.B.; Gonçalves, J.M.; Dos Santos, L.G.P.; Savi, M.G.; Nör, J.E.; Mezzomo, L.A.M.; Rodríguez Cordeiro, M.M. Expression of cancer stem cell biomarkers in human head and neck carcinomas: A systematic review. *Stem. Cell Rev. Rep.* **2018**, *14*, 769–784. [CrossRef] [PubMed]
17. Lee, S.H.; Oh, S.Y.; Do, S.I.; Lee, H.J.; Kang, H.J.; Rho, Y.S.; Bae, W.J.; Lim, Y.C. SOX2 regulates self-renewal and tumorigenicity of stem-like cells of head and neck squamous cell carcinoma. *Br. J. Cancer* **2014**, *111*, 2122–2130. [CrossRef] [PubMed]
18. Luo, W.; Li, S.; Peng, B.; Ye, Y.; Deng, X.; Yao, K. Embryonic stem cells markers SOX2, OCT4 and Nanog expression and their correlations with epithelial-mesenchymal transition in nasopharyngeal carcinoma. *PLoS ONE* **2013**, *8*, e56324.
19. Lothian, C.; Lendahl, U. An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. *Eur. J. Neurosci.* **1997**, *9*, 452–462. [CrossRef]
20. Ravindran, G.; Devaraj, H. Prognostic significance of neural stem cell markers, Nestin and Musashi-1, in oral squamous cell carcinoma: Expression pattern of Nestin in the precancerous stages of oral squamous epithelium. *Clin. Oral Investig.* **2015**, *19*, 1251–1260. [CrossRef] [PubMed]
21. Atsumi, N.; Ishii, G.; Kojima, M.; Sanada, M.; Fujii, S.; Ochiai, A. Podoplanin, a novel marker of tumor-initiating cells in human squamous cell carcinoma A431. *Biochem. Biophys. Res. Commun.* **2008**, *373*, 36–41. [CrossRef]
22. Hu, L.; Zhang, P.; Mei, Q.; Sun, W.; Zhou, L.; Yin, T. Podoplanin is a useful prognostic marker and indicates better differentiation in lung squamous cell cancer patients? A systematic review and meta-analysis. *BMC Cancer* **2020**, *20*, 424. [CrossRef] [PubMed]
23. de Vicente, J.C.; Rodrigo, J.P.; Rodriguez-Santamarta, T.; Lequerica-Fernández, P.; Allonca, E.; García-Pedrero, J.M. Podoplanin expression in oral leukoplakia: Tumorigenic role. *Oral Oncol.* **2013**, *49*, 598–603. [CrossRef]
24. Kindt, N.; Descamps, G.; Seminerio, I.; Bellier, J.; Lechien, J.R.; Mat, Q.; Pottier, C.; Delvenne, P.; Journé, F.; Saussez, S. High stromal Foxp3-positive T cell number combined to tumor stage improved prognosis in head and neck squamous cell carcinoma. *Oral Oncol.* **2017**, *67*, 183–191. [CrossRef] [PubMed]
25. Zeng, C.; Kuang, H.; Fan, W.; Chen, X.; Yu, T.; Tang, Q.; Zhou, Z.; Liang, F. Downregulation of *FOXP3* in neutrophils by IL-8 promotes the progression of oral squamous cell carcinoma. *Oncol. Lett.* **2019**, *18*, 4771–4777. [CrossRef] [PubMed]
26. Zhang, B.; Wu, C.; Zhang, Z.; Yan, K.; Li, C.; Li, Y.; Li, L. CXCL12 is associated with FoxP3^{+} tumor-infiltrating lymphocytes and affects the survival of patients with oral squamous cell carcinoma. *Oncol. Lett.* **2019**, *18*, 1099–1106. [CrossRef] [PubMed]
27. Song, J.J.; Zhao, S.J.; Fang, J.; Ma, D.; Liu, X.Q.; Chen, X.B.; Wang, Y.; Cheng, B.; Wang, Z. Foxp3 overexpression in tumor cells predicts poor survival in oral squamous cell carcinoma. *BMC Cancer* **2016**, *16*, 530. [CrossRef]
28. Tao, H.; Mimura, Y.; Aoe, K.; Kobayashi, S.; Yamamoto, H.; Matsuda, E.; Okabe, K.; Matsumoto, T.; Sugi, K.; Ueoka, H. Prognostic potential of FOXP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells. *Lung Cancer* **2012**, *75*, 95–101. [CrossRef]
29. Tang, Y.; Xu, X.; Guo, S.; Zhang, C.; Tang, Y.; Tian, Y.; Ni, B.; Lu, B.; Wang, H. An increased abundance of tumor-infiltrating regulatory T cells is correlated with the progression and prognosis of pancreatic ductal adenocarcinoma. *PLoS ONE* **2014**, *9*, e91551. [CrossRef]
30. Sayour, E.J.; McLendon, P.; McLendon, R.; De Leon, G.; Reynolds, R.; Kresak, J.; Sampson, J.H.; Mitchell, D.A. Increased proportion of FoxP3+ regulatory T cells in tumor infiltrating lymphocytes is associated with tumor recurrence and reduced survival in patients with glioblastoma. *Cancer Immunol. Immunother.* **2015**, *64*, 419–427. [CrossRef]
31. Vlad, C.; Kubelac, P.; Fetica, B.; Vlad, D.; Irimie, A.; Achimas-Cadariu, P. The prognostic value of FOXP3+ T regulatory cells in colorectal cancer. *J. Buon.* **2015**, *20*, 114–119.
32. Haas, M.; Dimmler, A.; Hohenberger, W.; Grabenbauer, G.G.; Niedobitek, G.; Distel, L.V. Stromal regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia. *BMC Gastroenterol.* **2009**, *9*, 65. [CrossRef]
33. Badoual, C.; Hans, S.; Rodriguez, J.; Peyrard, S.; Klein, C.; Agueznay, N.E.H.; Mosseri, V.; Laccourreye, O.; Bruneval, P.; Fridman, W.H.; et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin. Cancer Res.* **2006**, *12*, 465–472. [CrossRef]
34. Shimizu, S.; Hiratsuka, H.; Koike, K.; Tsuchihashi, K.; Sonoda, T.; Ogi, K.; Miyakawa, A.; Kobayashi, J.; Kaneko, T.; Igarashi, T.; et al. Tumor-infiltrating CD8^{+} T-cell density is an independent prognostic marker for oral squamous cell carcinoma. *Cancer Med.* **2019**, *8*, 80–93. [CrossRef] [PubMed]
35. Huang, Z.; Xie, N.; Liu, H.; Wan, Y.; Zhu, Y.; Zhang, M.; Tao, Y.; Zhou, H.; Liu, X.; Hou, J.; et al. The prognostic role of tumour-infiltrating lymphocytes in oral squamous cell carcinoma: A meta-analysis. *J. Oral Pathol. Med.* **2019**, *48*, 788–798. [CrossRef] [PubMed]

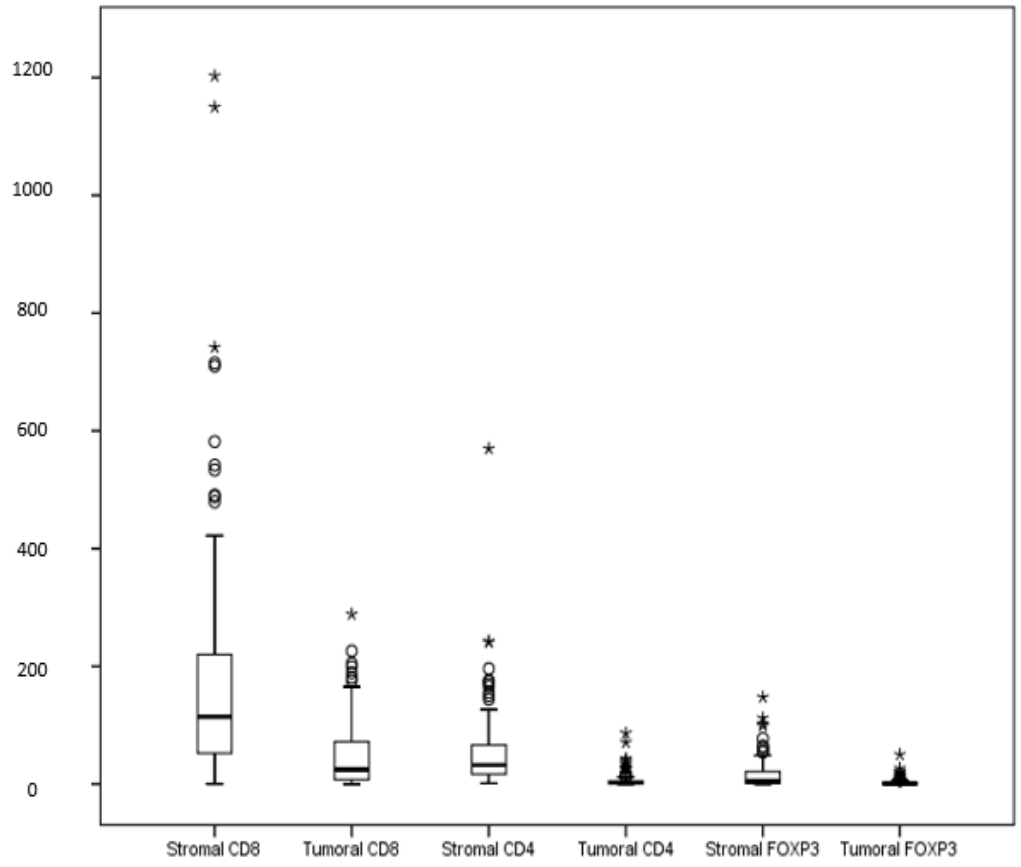
36. de Ruiter, E.J.; Ooft, M.L.; Devriese, L.A.; Willems, S.M. The prognostic role of tumor infiltrating T-lymphocytes in squamous cell carcinoma of the head and neck: A systematic review and meta-analysis. *Oncoimmunology* **2017**, *6*, e1356148. [CrossRef]
37. Borsetto, D.; Tomasoni, M.; Payne, K.; Polesel, J.; Deganello, A.; Bossi, P.; Tysome, J.R.; Masterson, L.; Tirelli, G.; Tofanelli, M.; et al. Prognostic Significance of CD4+ and CD8+ tumor-infiltrating lymphocytes in head and neck squamous cell carcinoma: A meta-analysis. *Cancers* **2021**, *13*, 781. [CrossRef] [PubMed]
38. Shang, B.; Liu, Y.; Jiang, S.J.; Liu, Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: A systematic review and meta-analysis. *Sci. Rep.* **2015**, *5*, 15179. [CrossRef]
39. Park, K.; Cho, K.J.; Lee, M.; Yoon, D.H.; Kim, S.B. Importance of FOXP3 in prognosis and its relationship with p16 in tonsillar squamous cell carcinoma. *Anticancer Res.* **2013**, *33*, 5667–5673.
40. de Leeuw, R.J.; Kost, S.E.; Kakal, J.A.; Nelson, B.H. The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: A critical review of the literature. *Clin. Cancer Res.* **2012**, *18*, 3022–3029. [CrossRef]
41. Müller, S. Update from the 4th Edition of the World Health Organization of Head and Neck Tumours: Tumours of the Oral Cavity and Mobile Tongue. *Head Neck Pathol.* **2017**, *11*, 33–40. [CrossRef]
42. Amin, M.B. *AJCC Cancer Staging Manual*, 8th ed.; Springer: Chicago, IL, USA, 2017; pp. 79–94.
43. de Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Blanco-Lorenzo, V.; Allonca, E.; García-Pedrero, J.M. PD-L1 expression in tumor cells is an independent unfavorable prognostic factor in oral squamous cell carcinoma. *Cancer Epidemiol. Biomark. Prev.* **2019**, *28*, 546–554. [CrossRef]
44. de Vicente, J.C.; Santamarta, T.R.; Rodrigo, J.P.; García-Pedrero, J.M.; Allonca, E.; Blanco-Lorenzo, V. Expression of podoplanin in the invasion front of oral squamous cell carcinoma is not prognostic for survival. *Virchows Arch.* **2015**, *466*, 549–558. [CrossRef] [PubMed]
45. Remmele, W.; Schicketanz, K.H. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs. Subjective grading (IRS). *Pathol. Res. Pract.* **1993**, *189*, 862–866. [CrossRef]
46. Suárez-Sánchez, F.J.; Lequerica-Fernández, P.; Rodrigo, J.P.; Hermida-Prado, F.; Suárez-Canto, J.; Rodríguez-Santamarta, T.; Domínguez-Iglesias, F.; García-Pedrero, J.M.; de Vicente, J.C. Tumor-infiltrating $CD20^+$ B lymphocytes: Significance and prognostic implications in oral cancer microenvironment. *Cancers* **2021**, *13*, 395. [CrossRef] [PubMed]
47. DiPaolo, R.J.; Glass, D.D.; Bijwaard, K.E.; Shevach, E.M. CD4+CD25+ T cells prevent the development of organ-specific autoimmune disease by inhibiting the differentiation of autoreactive effector T cells. *J. Immunol.* **2005**, *175*, 7135–7142. [CrossRef]
48. Swann, J.B.; Smyth, M.J. Immune surveillance of tumors. *J. Clin. Investig.* **2007**, *117*, 1137–1146. [CrossRef] [PubMed]
49. Quan, H.; Shan, Z.; Liu, Z.; Liu, S.; Yang, L.; Fang, X.; Li, K.; Wang, B.; Deng, Z.; Hu, Y.; et al. The repertoire of tumor-infiltrating lymphocytes within the microenvironment of oral squamous cell carcinoma reveals immune dysfunction. *Cancer Immunol. Immunother.* **2020**, *69*, 465–476. [CrossRef]
50. Wolf, G.T.; Chepeha, D.B.; Bellile, E.; Nguyen, A.; Thomas, D.; McHugh, J. University of Michigan Head and Neck SPORE Program. Tumor infiltrating lymphocytes (TIL) and prognosis in oral cavity squamous carcinoma: A preliminary study. *Oral Oncol.* **2015**, *51*, 90–95. [CrossRef]
51. Ono, T.; Azuma, K.; Kawahara, A.; Sasada, T.; Hattori, S.; Sato, F.; Shin, B.; Chitose, S.I.; Akiba, J.; Hirohito, U. Association between PD-L1 expression combined with tumor-infiltrating lymphocytes and the prognosis of patients with advanced hypopharyngeal squamous cell carcinoma. *Oncotarget* **2017**, *8*, 92699–92714. [CrossRef]
52. Hu, C.; Tian, S.; Lin, L.; Zhang, J.; Ding, H. Prognostic and clinicopathological significance of PD-L1 and tumor infiltrating lymphocytes in hypopharyngeal squamous cell carcinoma. *Oral Oncol.* **2020**, *102*, 104560. [CrossRef] [PubMed]
53. Peske, J.D.; Woods, A.B.; Engelhard, V.H. Control of CD8 T-cell infiltration into tumors by vasculature and microenvironment. *Adv. Cancer Res.* **2015**, *128*, 263–307.
54. Balermpas, P.; Rödel, F.; Weiss, C.; Rödel, C.; Fokas, E. Tumor-infiltrating lymphocytes favor the response to chemoradiotherapy of head and neck cancer. *Oncoimmunology* **2014**, *3*, e27403. [CrossRef]
55. Balermpas, P.; Michel, Y.; Wagenblast, J.; Seitz, O.; Weiss, C.; Rödel, F.; Rödel, C.; Fokas, E. Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer. *Br. J. Cancer* **2014**, *110*, 501–509. [CrossRef]
56. Drennan, S.; Stafford, N.D.; Greenman, J.; Green, V.L. Increased frequency and suppressive activity of CD127(low/-) regulatory T cells in the peripheral circulation of patients with head and neck squamous cell carcinoma are associated with advanced stage and nodal involvement. *Immunology* **2013**, *140*, 335–343.
57. Schott, A.K.; Pries, R.; Wollenberg, B. Permanent up-regulation of regulatory T-lymphocytes in patients with head and neck cancer. *Int. J. Mol. Med.* **2010**, *26*, 67–75. [PubMed]
58. Troiano, G.; Rubini, C.; Togni, L.; Caponio, V.C.A.; Zhurakivska, K.; Santarelli, A.; Cirillo, N.; Lo Muzio, L.; Mascitti, M. The immune phenotype of tongue squamous cell carcinoma predicts early relapse and poor prognosis. *Cancer Med.* **2020**, *9*, 8333–8344. [CrossRef]
59. De Meulenaere, A.; Vermassen, T.; Aspeslagh, S.; Zwaenepoel, K.; Deron, P.; Duprez, F.; Rottey, S.; Ferdinande, L. Prognostic markers in oropharyngeal squamous cell carcinoma: Focus on CD70 and tumour infiltrating lymphocytes. *Pathology* **2017**, *49*, 397–404. [CrossRef]
60. Lei, Y.; Xie, Y.; Tan, Y.S.; Prince, M.E.; Moyer, J.S.; Nör, J.; Wolf, G.T. Telltale tumor infiltrating lymphocytes (TIL) in oral, head & neck cancer. *Oral Oncol.* **2016**, *61*, 159–165.

61. Gentles, A.J.; Newman, A.M.; Liu, C.L.; Bratman, S.V.; Feng, W.; Kim, D.; Nair, V.S.; Xu, Y.; Khuong, A.; Hoang, C.D.; et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat. Med.* **2015**, *21*, 938–945. [CrossRef]
62. Spranger, S.; Gajewski, T.F. Tumor-intrinsic oncogene pathways mediating immune avoidance. *Oncoimmunology* **2015**, *5*, e1086862. [CrossRef]
63. Schipmann, S.; Wermker, K.; Schulze, H.J.; Kleinheinz, J.; Brunner, G. Cutaneous and oral squamous cell carcinoma-dual immunosuppression via recruitment of FOXP3+ regulatory T cells and endogenous tumour FOXP3 expression? *J. Craniomaxillofac. Surg.* **2014**, *42*, 1827–1833. [CrossRef]
64. Salama, P.; Phillips, M.; Grieu, 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*, 186–192. [CrossRef]
65. Boxberg, M.; Leising, L.; Steiger, K.; Jesinghaus, M.; Alkhamas, A.; Mielke, M.; Pfarr, N.; Götz, C.; Wolff, C.D.; Weichert, W.; et al. Composition and clinical impact of the immunologic tumor microenvironment in oral squamous cell carcinoma. *J. Immunol.* **2019**, *202*, 278–291. [CrossRef]
66. Miyara, M.; Sakaguchi, S. Human FoxP3(+)CD4(+) regulatory T cells: Their knowns and unknowns. *Immunol. Cell Biol.* **2011**, *89*, 346–351. [CrossRef]
67. Teng, F.; Mu, D.; Meng, X.; Kong, L.; Zhu, H.; Liu, S.; Zhang, J.; Yu, J. Tumor infiltrating lymphocytes (TILs) before and after neoadjuvant chemoradiotherapy and its clinical utility for rectal cancer. *Am. J. Cancer Res.* **2015**, *5*, 2064–2074.
68. Homma, Y.; Taniguchi, K.; Murakami, T.; Nakagawa, K.; Nakazawa, M.; Matsuyama, R.; Mori, R.; Takeda, K.; Ueda, M.; Ichikawa, Y.; et al. Immunological impact of neoadjuvant chemoradiotherapy in patients with borderline resectable pancreatic ductal adenocarcinoma. *Ann. Surg. Oncol.* **2014**, *21*, 670–676. [CrossRef] [PubMed]
69. de Ruiter, E.J.; de Roest, R.H.; Brakenhoff, R.H.; Leemans, C.R.; de Bree, R.; Terhaard, C.H.J.; Willems, S.M. Digital pathology-aided assessment of tumor-infiltrating T lymphocytes in advanced stage, HPV-negative head and neck tumors. *Cancer Immunol. Immunother.* **2020**, *69*, 581–591. [CrossRef] [PubMed]
70. Ni, Y.H.; Zhang, X.X.; Lu, Z.Y.; Huang, X.F.; Wang, Z.Y.; Yang, Y.; Dong, Y.C.; Jing, Y.; Song, Y.; Hou, Y.Y.; et al. Tumor-infiltrating $CD1a_+$ DCs and $CD8_+/FoxP3_+$ ratios served as predictors for clinical outcomes in tongue squamous cell carcinoma patients. *Pathol. Oncol. Res.* **2019**, *26*, 1687–1695. [CrossRef] [PubMed]
71. Kashima, Y.; Nishii, N.; Tachinami, H.; Furusawa, E.; Nagai, S.; Harada, H.; Azuma, M. Orthotopic tongue squamous cell carcinoma (SCC) model exhibiting a different tumor-infiltrating T-cell status with margin-restricted $CD8^+$ T cells and regulatory T cell-dominance, compared to skin SCC. *Biochem. Biophys. Res. Commun.* **2020**, *526*, 218–224. [CrossRef]
72. 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*, 3491–3494. [PubMed]
73. Menon, A.G.; Janssen-van Rhijn, C.M.; Morreau, H.; Putter, H.; Tollenaar, R.A.; van de Velde, C.J.; Fleuren, G.J.; Kuppen, P.J. Immune system and prognosis in colorectal cancer: A detailed immunohistochemical analysis. *Lab. Investig.* **2004**, *84*, 493–501. [CrossRef] [PubMed]
74. Kim, T.K.; Herbst, R.S.; Chen, L. Defining and understanding adaptive resistance in cancer immunotherapy. *Trends Immunol.* **2018**, *39*, 624–631. [CrossRef]
75. Zhu, Q.; Cai, M.Y.; Weng, D.S.; Zhao, J.J.; Pan, Q.Z.; Wang, Q.J.; Tang, Y.; He, J.; Li, M.; Xia, J.C. PD-L1 expression patterns in tumour cells and their association with CD8(+) tumour infiltrating lymphocytes in clear cell renal cell carcinoma. *J. Cancer* **2019**, *10*, 154–1161. [CrossRef]
76. Piazzolla, D.; Palla, A.R.; Pantoja, C.; Cañamero, M.; Perez de Castro, I.; Ortega, S.; Gómez-López, G.; Dominguez, O.; Megías, D.; Roncador, G.; et al. Lineage-restricted function of the pluripotency factor NANOG in stratified epithelia. *Nat. Comm.* **2015**, *5*, 4226. [CrossRef]
77. Stasikowska-Kanicka, O.; Wągrowska-Danilewicz, M.; Danilewicz, M. CD8+ and CD163+ infiltrating cells and PD-L1 immunoexpression in oral leukoplakia and oral carcinoma. *APMIS* **2018**, *126*, 732–738. [CrossRef]
78. Thompson, E.D.; Zahurak, M.; Murphy, A.; Cornish, T.; Cuka, N.; Abdelfatah, E.; Yang, S.; Duncan, M.; Ahuja, N.; Taube, J.M.; et al. Patterns of PD-L1 expression and CD8 T cell infiltration in gastric adenocarcinomas and associated immune stroma. *Gut* **2017**, *66*, 794–801. [CrossRef]
79. Sharma, A.; Rudra, D. Emerging functions of regulatory T cells in tissue homeostasis. *Front. Immunol.* **2018**, *9*, 883. [CrossRef]
80. Ghiringhelli, F.; Puig, P.E.; Roux, S.; Parcellier, A.; Schmitt, E.; Solary, E.; Kroemer, G.; Martin, F.; Chauffert, B.; Zitvogel, L. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J. Exp. Med.* **2005**, *202*, 919–929. [CrossRef]
81. Pedregal-Mallo, D.; Hermida-Prado, F.; Granda-Díaz, R.; Montoro-Jiménez, I.; Allonca, E.; Pozo-Agundo, E.; Álvarez-Fernández, M.; Álvarez-Marcos, C.; García-Pedrero, J.M.; Rodrigo, J.P. Prognostic significance of the pluripotency factors NANOG, SOX2, and OCT4 in head and neck squamous cell carcinomas. *Cancers* **2020**, *12*, 1794. [CrossRef]
82. Ghazi, N.; Aali, N.; Shahrokhi, V.R.; Mohajertehran, F.; Saghravanian, N. Relative expression of SOX2 and OCT4 in oral squamous cell carcinoma and oral epithelial dysplasia. *Rep. Biochem. Mol. Biol.* **2020**, *9*, 171–179. [CrossRef] [PubMed]
83. Vijayakumar, G.; Narwal, A.; Kamboj, M.; Sen, R. Association of SOX2, OCT4 and WNT5A expression in oral epithelial dysplasia and oral squamous cell carcinoma: An immunohistochemical study. *Head Neck Pathol.* **2020**, *14*, 749–757. [CrossRef] [PubMed]

84. Suárez-Sánchez, F.J.; Lequerica-Fernández, P.; Suárez-Canto, J.; Rodrigo, J.P.; Rodriguez-Santamarta, T.; Domínguez-Iglesias, F.; García-Pedrero, J.M.; de Vicente, J.C. Macrophages in oral carcinomas: Relationship with cancer stem cell markers and PD-L1 expression. *Cancers* **2020**, *12*, 1764. [CrossRef]

Supplementary material

Supplementary Figure S1. Stromal and tumoral distribution of $CD8^+$, $CD4^+$ and $FOXP3^+$ T cell subsets in OSCC patients. Whiskers indicate variability outside the 75 and 25 percentiles. The Y axis represents the number of infiltrating T cells. Circles represent outliers and asterisks extreme values.



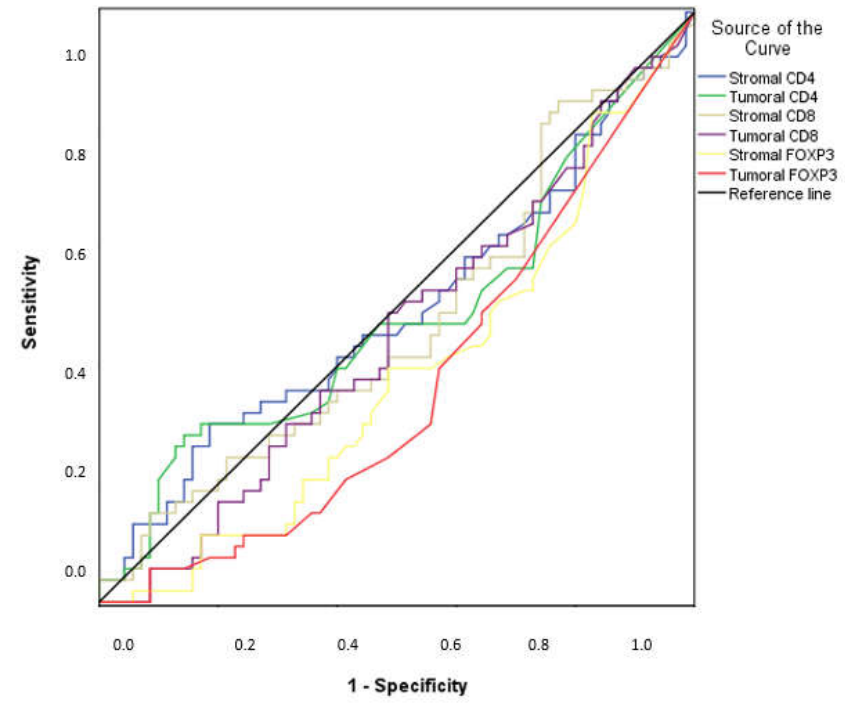
Supplementary Table S1. Correlations between the mean numbers of CD4, CD8 and FOXP3 TIL infiltration in the tumor nests and surrounding stroma. The Spearman's Rho coefficients and the corresponding *p* values are shown.

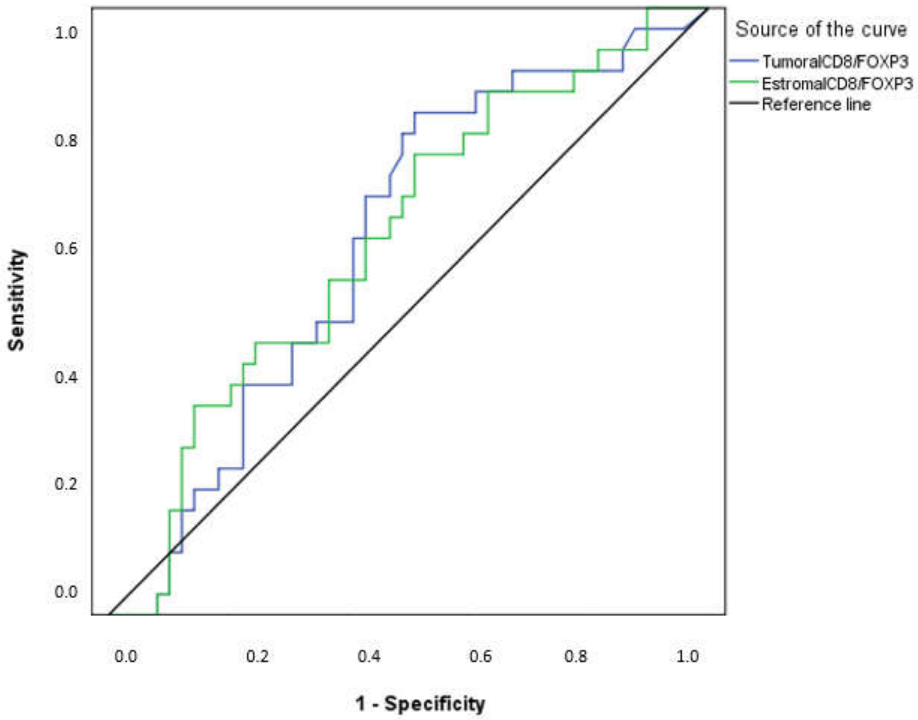
Factor	*Tumoral CD4 (mean)*	*Stromal CD8 (mean)*	*Tumoral CD8 (mean)*	*Stromal FOXP3 (mean)*	*Tumoral FOXP3 (mean)*
Stromal CD4 (mean)	0.693 < 0.001	0.693 < 0.001	0.401 < 0.001	0.365 < 0.001	0.262 0.003
Tumoral CD4 (mean)		0.467 < 0.001	0.418 < 0.001	0.252 0.005	0.224 0.013
Stromal CD8 (mean)			0.515 < 0.001	0.320 < 0.001	0.265 0.003
Tumoral CD8 (mean)				0.094 0.299	0.259 0.004
Stromal FOXP3 (mean)					0.817 < 0.001

Supplementary Table S2. Associations between stromal and tumoral CD8/FOXP3 ratios and clinicopathological parameters in the cohort of 125 OSCC patients.

Variable	Stromal CD8/FOXP3 ratio Mean (SD)	*p*	Tumoral CD8/FOXP3 ratio Mean (SD)	*p*
Age (years)				
< 65	26.74 (41.18)	0.001	14.80 (20.47)	0.001
≥ 65	80.96 (157.80)		55.79 (84.12)	
Gender				
Female	54.87 (133.00)	0.52	33.07 (70.27)	0.67
Male	45.45 (92.93)		28.43 (49.00)	
Tobacco				
No	74.12 (150.44)	0.10	51.66 (87.01)	0.01
Yes	35.68 (76.03)		18.59 (25.57)	
Alcohol consumption	60.98 (130.48)	0.11	47.90 (78.43)	0.005
No	37.81 (82.87)		15.50 (21.53)	
Yes				
T classification				
T1 + 2	36.02 (56.97)	0.76	27.05 (53.78)	0.11
T3 + 4	76.87 (172.71)		36.75 (63.93)	
N classification				
N0	58.92 (134.09)	0.73	34.79 (68.05)	0.90
N+	32.60 (38.09)		21.56 (26.38)	
Stage				
I + II	35.16 (63.75)	0.40	29.67 (63.06)	0.12
III + IV	59.15 (132.01)		30.35 (51.72)	
Grade				
Well	49.62 (102.66)	0.12	29.21 (55.88)	0.87
Moderate + Poor	47.21 (118.14)		31.29 (59.19)	
Site				
Tongue	22.80 (32.87)	0.16	21.40 (34.04)	0.35
Rest	65.76 (134.23)		36.05 (68.13)	
Recurrence				
No	48.00 (106.23)	0.08	32.54 (68.62)	0.11
Yes	49.77 (111.83)		25.81 (28.03)	
Second primary tumor	45.64 (110.95)	0.37	29.61 (58.50)	0.76
No	63.87 (93.98)		32.20 (49.80)	
Yes				

All *p*-values were calculated using the U Mann-Whitney test.





Supplementary Figure S2. ROC curves of stromal/tumoral CD4, CD8, and FOXP3 (A) and stromal/tumoral CD8/FOXP3 ratio (B).

Supplementary Table S3. DSS predictive accuracy of CD4, CD8 and FOXP3 infiltrating TILs.

Predictive factor	AUC	SE	*p*	95% CI
Stromal CD8	0.472	0.053	0.594	0.368 – 0.576
Tumoral CD8	0.455	0.052	0.391	0.352 – 0.557
Stromal CD4	0.494	0.054	0.902	0.388 – 0.599
Tumoral CD4	0.484	0.054	0.755	0.377 – 0.590
Stromal CD8/CD4 ratio	0.460	0.053	0.450	0.356 – 0.564
Tumoral CD8/CD4 ratio	0.462	0.053	0.476	0.359 – 0.566
Stromal FOXP3	0.384	0.051	0.028	0.285 – 0.483
Tumoral FOXP3	0.368	0.050	0.012	0.270 – 0.466
Stromal CD8/FOXP3 ratio	0.621	0.065	0.076	0.494 – 0.748
Tumoral CD8/FOXP3 ratio	0.616	0.065	0.089	0.489 – 0.742

Supplementary Table S4. Stratified univariate Kaplan-Meier analysis of stromal and tumoral $CD8^+$ and $FOXP3^+$ TILs with clinicopathological variables in 125 OSCC patients. Median values were used as cut-off points. No correlations were found with stromal and tumoral CD4.

Parameters	Number	Censored patients (%)	Cancer-free survival time (95% CI)	*p*
Age, stromal $CD8^+$				
< 65 years				
• ≤ 118	38	17 (45)	106.58 (74.56 – 138.60)	0.01
• > 118	39	29 (74)	175.97 (147.19 – 204.76)	
≥ 65 years				
• ≤ 118	25	15 (60)	82.36 (58.26 – 106.47)	0.89
• > 118	23	11 (48)	93.20 (66.04 – 120.36)	
Age, tumoral $FOXP3^+$				
< 65 years				
• ≤ 0.666	30	13 (43)	107.69 (71.99 – 143.39)	0.02
• > 0.666	47	33 (70)	166.16 (138.40 – 193.91)	
≥ 65 years				
• ≤ 0.666	23	12 (52)	78.05 (50.08 – 106.02)	0.47
• > 0.666	25	14 (56)	93.20 (69.40 – 124.63)	
Gender, stromal $CD8^+$				
Female				
• ≤ 118	23	14 (61)	135.66 (92.84 – 178.49)	0.81
• > 118	20	11 (55)	113.00 (84.50 – 141.49)	
Male				
• ≤ 118	40	18 (52)	84.07 (61.26 – 106.89)	0.02
• > 118	42	29 (56)	161.57 (131.04 – 192.10)	
Gender, stromal $FOXP3^+$				
Female				
• ≤ 5.666	19	9 (47)	71.52 (44.59 – 98.45)	0.03
• > 5.666	24	16 (67)	155.47 (118.21 – 192.73)	

Male • ≤ 5.666 • > 5.666	 41 41	 20 (49) 27 (66)	 100.56 (75.60 – 125.52) 149.26 (115.52 – 183.00)	 0.28
Gender, tumoral FOXP3+ Female • ≤ 0.666 • > 0.666 Male • ≤ 0.666 • > 0.666	 20 23 33 49	 9 (45) 16 (70) 16 (48) 31 (63)	 93.61 (48.10 – 139.13) 141.86 (112.04 – 171.8) 99.79 (71.88 – 127.71) 145.57 (115.14 – 176.00)	 0.01 0.28
Gender, stromal CD8/FOXP3+ Female • ≤ 10.9853 • > 10.9853 Male • ≤ 10.9853 • > 10.9853	 18 19 25 44	 14 (78) 7 (37) 16 (64) 25 (57)	 178.76 (141.20 – 216.32) 76.45 (48.82 – 104.09) 145.09 (101.67 – 188.50) 107.18 (83.93 – 130.43)	 0.009 0.63
Tobacco, stromal CD8+ No • ≤ 118 • > 118 Yes • ≤ 118 • > 118	 17 24 46 38	 11 (65) 13 (54) 21 (46) 27 (71)	 89.20 (57.66 – 120.74) 106.56 (78.23 – 134.89) 103.66 (73.71 – 133.60) 165.05 (133.02 – 197.09)	 0.92 0.02
Tobacco, stromal FOXP3+ No • ≤ 5.666 • > 5.666 Yes • ≤ 5.666 • > 5.666	 21 20 39 45	 12 (57) 12 (60) 17 (44) 31 (69)	 103.91 (71.07 – 136.74) 155.47 (75.17 – 137.00) 90.67 (75.60 – 125.52) 159.52 (129.59 – 189.45)	 0.78 0.02
Tobacco, tumoral FOXP3+				

No				
• ≤ 0.666	17	10 (59)	103.25 (66.07 – 140.44)	0.74
• > 0.666	24	14 (58)	109.26 (79.72 – 138.79)	
Yes				
• ≤ 0.666	36	15 (42)	100.20 (67.33 – 133.07)	0.01
• > 0.666	48	33 (69)	159.12 (130.00 – 188.24)	
Alcohol, stromal $CD8^+$				
No				
• ≤ 118	25	14 (56)	122.59 (80.18 – 165.00)	0.65
• > 118	31	17 (55)	105.97 (80.51 – 131.43)	
Yes				
• ≤ 118	38	18 (47)	96.37 (69.78 – 122.96)	0.02
• > 118	31	23 (74)	171.48 (137.03 – 205.94)	
Alcohol, stromal $FOXP3^+$				
No				
• ≤ 5.666	27	14 (52)	95.55 (66.73 – 124.37)	0.55
• > 5.666	29	17 (59)	135.95 (98.90 – 173.00)	
Yes				
• ≤ 5.666	33	15 (45)	93.79 (65.88 – 121.70)	0.02
• > 5.666	36	26 (72)	165.31 (132.39 – 198.23)	
Alcohol, tumoral $FOXP3^+$				
No				
• ≤ 0.666	25	13 (52)	113.43 (72.02 – 154.85)	0.45
• > 0.666	31	18 (58)	108.18 (81.99 – 134.36)	
Yes				
• ≤ 0.666	28	12 (43)	89.92 (59.68 – 120.15)	0.01
• > 0.666	41	29 (71)	163.06 (132.05 – 194.08)	
Alcohol, stromal $CD8/FOXP3^+$				
No	16	9 (56)	135.15 (86.44 – 183.87)	0.68
• ≤ 10.9853	34	18 (53)	97.16 (71.57 – 122.76)	
• > 10.9853				

Yes	27	21 (78)	177.29 (140.49 – 214.09)	0.03
• ≤ 10.9853	29	14 (48)	98.76 (71.74 – 125.78)	
• > 10.9853				
Tumor site, stromal FOXP3+				
Tongue				
• ≤ 5.666	20	8 (40)	73.61 (37.53 – 109.68)	0.03
• > 5.666	31	20 (65)	147.75 (110.08 – 185.42)	
Other				
• ≤ 5.666	40	21 (53)	101.60 (78.64 – 124.56)	0.17
• > 5.666	34	23 (68)	135.44 (108.80 – 162.08)	
Tumor site, stromal CD8/FOXP3+				
Tongue				
• ≤ 10.9853	17	13 (77)	180.37 (138.19 – 222.53)	0.01
• > 10.9853	25	10 (40)	75.44 (45.34 – 105.54)	
Other				
• ≤ 10.9853	26	17 (65)	129.64 (97.54 – 161.74)	0.58
• > 10.9853	38	22 (58)	109.58 (87.11 – 132.04)	
N classification, stromal CD8+				
N0				
• ≤ 118	39	24 (62)	118.84 (91.70 – 145.99)	0.37
• > 118	37	25 (68)	133.75 (110.74 – 156.76)	
N+				
• ≤ 118	24	8 (33)	71.93 (33.70 – 110.17)	0.03
• > 118	25	15 (60)	141.96 (100.24 – 183.68)	
Grade, stromal CD8+				
Well differentiated				
• ≤ 118	38	17 (45)	97.72 (63.86 – 131.58)	0.03
• > 118	42	27 (64)	151.03 (119.76 – 182.30)	
Moderate-poorly differentiated	25	15 (60)	112.06 (75.92 – 148.21)	0.57
• ≤ 118	20	13 (65)	117.90 (85.90 – 149.90)	
• > 118				
Grade, stromal CD8/FOXP3+				

Well differentiated				
• ≤ 10.9853	24	17 (71)	180.37 (138.19 – 222.53)	0.01
• > 10.9853	43	19 (44)	75.44 (45.34 – 105.54)	
Moderate-poorly differentiated	19	13 (68)	131.76 (93.63 – 169.90)	0.85
• ≤ 10.9853	20	13 (65)	111.49 (77.60 – 145.38)	
• > 10.9853				
Radiotherapy, stromal $CD8^+$				
No				
• ≤ 118	27	23 (85)	163.76 (139.24 – 188.28)	0.50
• > 118	23	21 (91)	133.75 (145.45 – 188.54)	
Yes				
• ≤ 118	36	9 (25)	64.86 (38.00 – 91.73)	0.01
• > 118	39	19 (49)	123.34 (91.03 – 155.64)	
Radiotherapy, tumoral $FOXP3^+$				
No				
• ≤ 0.666	21	17 (81)	142.44 (107.90 – 176.98)	0.15
• > 0.666	29	27 (93)	178.24 (161.19 – 195.29)	
Yes				
• ≤ 0.666	32	8 (25)	70.28 (39.90 – 100.66)	0.04
• > 0.666	43	20 (47)	115.05 (84.23 – 145.87)	
PD-L1, stromal $FOXP3^+$				
Negative (≤ 10% tumor cells)				
• ≤ 5.666	50	25 (50)	99.36 (76.40 – 122.32)	0.02
• > 5.666	54	38 (70)	163.75 (137.04 – 190.45)	
Positive (> 10% tumor cells)				
• ≤ 5.666	8	3 (38)	52.28 (13.99 – 90.57)	0.66
• > 5.666	10	4 (40)	75.60 (31.13 – 120.06)	
PD-L1, tumoral $FOXP3^+$				
Negative (≤ 10% tumor cells)				
• ≤ 0.666	44	21 (48)	107.44 (76.61 – 138.27)	0.01
• > 0.666	60	42 (70)	163.68 (138.49 – 188.87)	

Positive (> 10% tumor cells) • ≤ 0.666 • > 0.666	 6 12	 2 (33) 5 (42)	 41.80 (1.00 – 82.60) 76.75 (37.37 – 116.12)	 0.37
SOX2, stromal $CD8^+$ Negative • ≤ 118 • > 118 Positive • ≤ 118 • > 118	 34 38 28 21	 14 (41) 23 (61) 18 (64) 14 (67)	 67.04 (46.25 – 87.82) 138.78 (104.52 – 173.05) 140.19 (101.09 – 179.29) 127.15 (99.33 – 154.97)	 0.03 0.64
NANOG, stromal $CD8^+$ Negative • ≤ 118 • > 118 Positive • ≤ 118 • > 118	 38 45 24 15	 17 (45) 28 (62) 14 (58) 10 (67)	 97.72 (64.16 – 131.29) 146.36 (115.80 – 176.91) 102.06 (76.12 – 127.99) 124.56 (87.43 – 161.70)	 0.04 0.67

p values were estimated using the log-rank test.

Supplementary Table S5. Multivariate Cox regression of disease-specific survival for clinical variables and TILs infiltration.

Variable	*p*	Hazard Ratio	95% CI
Stage (I + II vs. III + IV)	0.01	2.195	1.170 – 4.119
Stromal CD8/FOXP3 ratio (≤10.9853 vs. > 10.9853)	0.03	2.039	1.059 – 3.925

4. DISCUSIÓN

4.1 CD68^{+}, CD163^{+} en OSCC

En este estudio la infiltración por macrófagos CD68^{+}, tanto de los nidos tumorales como del estroma, no mostró asociaciones significativas con las variables clínico-patológicas y la supervivencia. Lin y cols. [56] estudiaron la infiltración de TAMs evaluando la expresión de CD68^{+} en 84 carcinomas laríngeos. Encontraron que 60 de ellos se asociaban significativamente, con recurrencia del tumor y menor supervivencia. En disparidad con este estudio, Troiano y cols. [12] realizaron un metaanálisis en HNSCC y no encontraron ninguna asociación entre la expresión tumoral o estromal de macrófagos CD68^{+}y supervivencia. En nuestro estudio se realizó un análisis adicional de la expresión de CD163 –un marcador altamente específico de macrófagos M2– de forma individualizada, tanto en los nidos tumorales como en el estroma. No encontramos asociaciones significativas con ningún parámetro clínico-patológico o pronóstico, siendo los resultados similares a los de otros estudios de OSCC [4, 11, 57]. Sin embargo, en algunos estudios se evidenció que en los tumores con un alto porcentaje de macrófagos CD163^{+} este se correlacionó significativamente con una peor supervivencia [5, 12, 15, 58]. En otros estudios, no obstante, la expresión estromal de CD163^{+} resultó estar significativamente asociada con la supervivencia, mientras que no se detectó ninguna relación significativa con los valores de expresión intratumoral [12]. La explicación para estas inconsistencias puede estar relacionada con posibles diferencias en la biología del tumor, la metodología utilizada para evaluar la infiltración de macrófagos, la evaluación IHC, diferencias en el número de casos clínicos entre estudios, la extensión del tumor, el estadio del mismo y también el papel, aún no bien entendido, de los macrófagos M2 en la biología del tumor.

Las células troncales del cáncer (CSCs) representan una pequeña subpoblación de células en los tumores. Mediante la secreción de diferentes factores promueven la autorreplicación y plasticidad favoreciendo la tomorigénesis y la progresión tumoral. NANOG es un factor de transcripción nuclear que se expresa en gran medida en las CSCs para mantener la autorreplicación y la pluripotencialidad de las

células embrionarias. Desempeña un papel importante en la tumorigénesis y metástasis dentro del OSCC [40]. Se ha descrito un sistema de retroalimentación entre las CSCs y TAMs basado en la migración y el reclutamiento de los TAMs a través de los vasos sanguíneos y la liberación de citoquinas por parte de los TAMs, los cuales mantienen la quiescencia de las CSCs [2]. Nuestro estudio demostró una fuerte asociación inversa entre la expresión estromal/intratumoral de $CD163^{+}$ y la expresión NANOG. De acuerdo con nuestros hallazgos, sostenemos la hipótesis de que al menos la infiltración de TAMs tipo M2 no parece estar relacionada con el mantenimiento del nicho de CSC.

La expresión de PDL1 en el cáncer es un mecanismo de resistencia inmunitaria adaptativa para evitar la respuesta anticancerígena mediada por TILs. Actúa favoreciendo la proliferación, supervivencia y evasión de la celularidad tumoral. En diversos estudios recientes se describe cómo las células tumorales pueden inducir el fenotipo de macrófagos M2 aumentando la expresión de PD-L1 [59]. La expresión de PD-L1 se estimula después de la exposición al Interferón-γ (IFN- γ) liberado por las células T efectoras, sin obviar otras señales TNF-α, VEGF y CXCL8, promoviendo todo ello la progresión del cáncer de pulmón [60, 61, 62, 63]. En sintonía con estos estudios, encontramos que tanto en los nidos tumorales como en el estroma los macrófagos M2 se asociaron con la expresión PD-L1, la cual se asociaba previamente con un mal pronóstico en el OSCC [52]. Los receptores TAM (Tyro3, Axl y Mertk) son una familia de receptores tirosin-quinasa que promueven la polarización de los macrófagos hacia un fenotipo protumoral similar a M2 y pueden activar la expresión de PD-L1 en las células tumorales [64]. Adicionalmente, el IFN-γ liberado por células inflamatorias en el TME induce la expresión de PD-L1 en células tumorales y también la isoforma 2 de la proteína quinasa D (PKD2), un importante regulador de PD-L1 en el OSCC [65]. Podemos especular con que esta relación entre la expresión de CD163 y PD-L1 podría ser el vínculo entre inflamación y escape inmunológico en la carcinogénesis oral [66, 67], y que en la génesis del OSCC pueden estar presentes inflamaciones crónicas como la periodontitis [68]. Una posible limitación de este estudio es que utilizamos $CD163^{+}$ como marcador de macrófagos M2, el más utilizado actualmente en la bibliografía. Los fenotipos M1 y M2 son los extremos de un espectro continuo de la polarización de los macrófagos, que a su vez muestran una alta plasticidad en

respuesta a diferentes estímulos. Por consiguiente, se considera que la tinción única con CD163 no es suficiente para asignar los macrófagos hacia la polarización de M2. Otra posible limitación sería que se trata de un estudio retrospectivo, por lo que no se puede excluir un posible sesgo de selección.

4.2 Expresión de $CD20^+$ en muestras de tejido de OSCC relacionada con otros subtipos inmunes

Los linfocitos B $CD20^+$ representan del 25% al 40% de todas las células que componen el TME en los diferentes tumores sólidos [69].

Son varios los mecanismos pueden explicar las funciones divergentes desempeñadas por los linfocitos B en la inmunología tumoral. Por un lado, los linfocitos B infiltrantes de tumor secretan linfotoxinas que inducen angiogénesis y activan las vías de señalización NF-kB y STAT3 en las células cancerosas, promoviendo el crecimiento del tumor. Además, los exomas (vesículas extracelulares) derivados de las células tumorales son capaces de activar los linfocitos B, que, como precursores celulares de las células plasmáticas productoras de anticuerpos en el TME, forman complejos inmunes que activan los receptores FCγ en las células mieloides y suprimen la respuesta antitumoral de las células T $CD4^+$ y $CD8^+$ [69]. En consonancia con ello, se han encontrado células B reguladoras inmunosupresoras que producen TGF-β e IL-10, promoviendo los mismos efectos que las células *Treg* [70].

En contraposición a este papel inmunosupresor, los anticuerpos liberados por las células plasmáticas contra los antígenos específicos del tumor realizan diferentes funciones. Median en la lisis de las células tumorales por activación del complemento [71], facilitan la fagocitosis de las células neoplásicas mediada por macrófagos o por estar las células tumorales revestidas de Ac (citotoxicidad celular dependiente de anticuerpos), facilitando la acción de la células NK [72] y la liberación de granzima B por los propios linfocitos B, con capacidad de destrucción de las células tumorales [73].

Los linfocitos B pueden concentrarse en el TME o formar agregados inmunitarios asociados a tumores que incluyen estructuras linfoides terciarias (TLS), similares a los ganglios linfáticos [74]. Dichas estructuras TLS pueden

localizarse dentro del tumor o adyacentes al mismo, mostrando diferentes etapas de maduración definidas por la ausencia o presencia de centros germinales. Los linfocitos B podrían estar implicados en la formación de TLS al producir CXCL13 y linfotoxina, y también en la maduración de una respuesta antitumoral en el TME. Estas estructuras linfoides terciarias se han asociado con un valor pronóstico positivo en algunos tumores. En el cáncer oral, los grados más altos de TLS se han asociado con una mejor supervivencia; sin embargo, los resultados clínicos favorables/desfavorables se han correlacionado con los diferentes componentes celulares de TLS. Esto sugiere que los componentes celulares de TLS afectan a la respuesta inmune antitumoral en diferentes tipos de cáncer. Todas estas funciones que acabamos de describir apoyan el papel supresor de tumores mostrado por los linfocitos B. En resumen, esta heterogeneidad en las funciones de los linfocitos B puede explicar los resultados contradictorios entre los estudios donde se utilizan marcadores de linfocitos pan-B.

4.2.1 Asociación entre CD20⁺ y las variables clínico-patológicas

Pretscher y cols. [75] determinaron que una alta densidad intratumoral de las células B CD20⁺ aumentaba el número de las metástasis ganglionares en comparación con los tumores primarios. Por el contrario, Taghavi y cols. [76] observaron una asociación inversa significativa entre la infiltración estromal por células B CD20⁺ y las metástasis en ganglios linfáticos.

En nuestro estudio no encontramos asociación entre TILs CD20⁺ y metástasis o estadiaje clínico. Sí cabe destacar que encontramos una asociación significativa entre la baja densidad de linfocitos B CD20⁺ y el estadio T –específicamente en los tumores T3 y T4–, lo que sugiere que la reducción en el número de linfocitos B tumorales podría estar asociada con la progresión tumoral. También es plausible que la presencia inicial de menos células inmunitarias –y por tanto una inmunovigilancia deficiente del tumor– pueda favorecer la progresión de este.

En contraste con nuestros resultados, otros estudios han demostrado un aumento de la infiltración de células B CD20⁺ en asociación con un peor pronóstico [15, 16]. Estos resultados contradictorios pueden deberse a diferencias en las técnicas de procesado inmunohistoquímico, así como a la técnica de cuantificación empleada.

4.2.2 Asociación entre CD20⁺ y la expresión de CSCs y PD-L1

En nuestro estudio encontramos una asociación significativa e inversa entre una infiltración tumoral de linfocitos B CD20⁺ más abundante y con expresión negativa de SOX2, lo que podría reflejar el papel de las CSCs en la evasión inmune y la contribución a la progresión del OSCC. Que conozcamos, este es el primer estudio que proporciona un vínculo potencial entre los linfocitos B CD20⁺ y las CSCs.

En cuanto a la asociación del linfocito B CD20⁺ y la expresión de PD-L1 en más del 10% de las células tumorales, no hemos obtenido ninguna relación estadísticamente significativa en nuestro estudio.

4.2.3 Asociación entre CD20⁺ y la supervivencia de los pacientes con OSCC.

Hemos observado que la densidad de linfocitos CD20⁺ en los nidos tumorales fue un factor pronóstico independiente en nuestra muestra de pacientes.

Un análisis estratificado de supervivencia mostró que los linfocitos CD20⁺ tumorales se asociaron significativamente con un mejor pronóstico en pacientes menores de 65 años varones, con hábito de consumo de tabaco o alcohol, expresión de PD-L1 (en más del 10% de células tumorales), tumores con expresión negativa de SOX2 o NANOG, con alta densidad de TILs CD8⁺ en nidos tumorales, baja infiltración estromal de TILs CD4⁺ y baja densidad en nidos tumorales para macrófagos CD68⁺ [77, 78, 79].

4.3 Evaluación inmunohistoquímica de CD4⁺, CD8⁺ y FOXP3⁺ en OSCC

El TME está constituido por multitud de células linfoides y mieloides. La composición exacta del infiltrado inmune puede variar ampliamente entre tumores y en un mismo tumor, modulando la efectividad de la respuesta antitumoral. En este estudio, evaluamos la relación entre los TILs CD4⁺, CD8⁺ y FOXP3⁺, así como los marcadores PD-L1 y CSC en el OSCC.

Los estudios realizados hasta la fecha coinciden en que los linfocitos T $CD8^+$ (citotóxicos, CTL) y los linfocitos Th1 están implicados en una inmunidad antitumoral eficaz, mientras que las células *Treg* $FOXP3^+$ están asociadas con la supresión de la inmunidad antitumoral.

Las células cancerosas sobreexpresan PD-L1, lo que se ve afectado por el microambiente circundante y se asocia, en general, con un mal pronóstico [53].

Las CSCs constituyen una subpoblación de células tumorales dotadas de propiedades de autorreplicación y clonalidad a largo plazo [37], así como propiedades de multidiferenciación sustentadas por factores de transcripción nuclear como NANOG, SOX2 y OCT4 [39].

Las subpoblaciones linfocitarias con fenotipo citotóxico (CD8) –colaborador (CD4) y regulador (FOXP3) en el microambiente de OSCC– pueden evaluarse fácilmente mediante IHC, empleando anticuerpos específicos contra marcadores de superficie de las diferentes poblaciones linfocitarias.

4.3.1. Asociación entre $CD4^+$, $CD8^+$, $FOXP3^+$ y las variables clínico-patológicas.

En este estudio hemos podido identificar varios subconjuntos de pacientes basados en factores clínico-patológicos y subtipos de TILs que ayudan a establecer diferentes resultados.

La ubicación anatómica del tumor dentro de la cavidad oral puede influir en el papel pronóstico de los linfocitos T. El cáncer oral surge de diversas localizaciones, y los carcinomas de lengua deben estudiarse por separado al tener características epidemiológicas únicas, diferentes de las de otros cánceres de la cavidad oral [80]. Hemos observado que las densidades de linfocitos T $CD4^+$ estromales e intratumorales, además de las de linfocitos T $FOXP3^+$ estromales, fueron mayores en los tumores localizados en la lengua al compararse con los tumores surgidos en otros sitios de la cavidad oral. Estos resultados parecen ser parcialmente consistentes con los hallazgos proporcionados por Kashima y cols. [81], quienes encontraron un predominio de linfocitos T $CD8^+$ y $FOXP3^+$ en los carcinomas de células escamosas de la lengua. Si bien en este estudio la densidad de linfocitos T $CD4^+$ no mostró ninguna relevancia pronostica –posiblemente debido a la diversidad de subtipos *T-helper*, de ahí su complejidad funcional–.

En nuestro estudio, la densidad de TILs se evaluó por separado en dos compartimentos diferentes dentro del TME del OSCC. Utilizando un procedimiento similar, Naito y cols. [82] encontraron que los linfocitos T $CD8^+$ infiltrados en los nidos tumorales afectaban al pronóstico de manera positiva en el cáncer colorrectal, mientras que los linfocitos T $CD8^+$ ubicados en el estroma no tenían ningún efecto sobre el pronóstico de la enfermedad. Por el contrario, Menon y cols. [83] mostraron relevancia pronóstica de los TILs $CD8^+$ estromales en el cáncer colorrectal.

En nuestro estudio se observó que los TILs $CD4^+$ y $CD8^+$ presentaban una densidad más alta –tanto a nivel intratumoral como en el estroma– en los pacientes que no tenían hábitos de consumo de tabaco o alcohol. Estas asociaciones sugieren que el consumo de tabaco y alcohol no solo juega un papel carcinogénico en el OSCC, sino que también puede influir en la respuesta del sistema inmunológico del huésped. Se ha observado que los linfocitos $CD8^+$ son una barrera relevante para el desarrollo inicial de tumores [84]. La infiltración de TILs $CD8^+$ intratumoral se ha relacionado en gran medida con resultados favorables en diferentes cánceres y con una respuesta positiva a la quimioterapia y a la radioterapia [85, 86], mientras que los *Treg* pueden conferir un pronóstico favorable o desfavorable según el contexto.
Aunque la densidad de TILs $CD8^+$ no mostró globalmente un impacto significativo en el pronóstico, en nuestro estudio vale la pena mencionar que una densidad más alta de TILs se asoció con un mejor pronóstico en subconjuntos específicos de pacientes con OSCC bien diferenciados y a su vez tratados con cirugía y radioterapia.

4.3.2. Asociación entre $CD4^+$, $CD8^+$, $FOXP3^+$ y la expresión de marcadores de CSCs

Las CSCs pueden evadirse de la inmunovigilancia del huésped al producir citoquinas en el TME e inhibir la respuesta inmunológica mediante la polarización de TILs *Treg* intratumorales, facilitando la propagación y las metástasis [87, 88, 89, 90, 91] e inhibiendo los linfocitos T $CD8^+$. Las CSCs tienen propiedades de clonalidad respaldadas por factores de transcripción nuclear como NANOG, SOX2 y OCT4 [44], que se han implicado en la carcinogénesis oral, diferenciación deficiente y peor pronóstico [92].

La expresión de NANOG en OSCC está regulada por SOX2, OCT4, KLF4, AGR2, NOTCH1 y miR-34a [43]. Sin embargo, la expresión de OCT4 no se detectó en ningún tumor de nuestro estudio. También se obtuvo una observación análoga a partir de una serie de 348 tumores localizados en orofaringe, hipofaringe y laringe [93]. Ghazi y cols. [94] encontraron que la expresión de SOX2 no se correlacionó con la expresión de OCT4 en OSCC. Vijayakumar y cols. [95] estudiaron 20 casos de OSCC y 20 casos de displasia epitelial oral (DEO), encontrando que la expresión de SOX2 era más abundante en OSCC que en DEO. La mayoría de los casos mostraron una alta expresión de SOX2 acompañada de una ausencia de expresión de OCT4. La valoración de estos resultados destaca que la expresión de SOX2 en HNSCC y OSCC parece ser independiente del control transcripcional de OCT4. SOX2 y OCT4 juegan diferentes roles en la biología de las CSCs. En ausencia de expresión de OCT4, la neoplasia no podría iniciarse a partir de tejidos normales, pero sin la expresión de SOX2 las células neoplásicas no podrían replicarse para mantener el crecimiento tumoral [94]. En nuestro estudio, los TILs $CD4^+$ y $CD8^+$ se asociaron inversa y significativamente con la expresión de dos importantes reguladores relacionados con CSCs: NANOG y SOX2 –excepto para TILs $CD8^+$ de localización intratumoral, para los cuales no se alcanzó significación–. La relación $CD8^+/FOXP3^+$ tumoral se asoció inversa y significativamente con la expresión de NANOG y SOX2, lo que sugiere una asociación inversa entre la infiltración de TILs citotóxicos y auxiliares y la pluripotencialidad de las CSCs en el OSCC.

Se observaron asociaciones análogas para otros dos marcadores adicionales de CSC, como son Nestina y PDPN.

Nestina es un biomarcador de CSCs en neoplasias epiteliales, desempeña un papel clave en la diferenciación, proliferación, invasión, metástasis y supervivencia de las células neoplásicas malignas. En nuestro estudio, la expresión de Nestina en el citoplasma de las células tumorales mostró una relación inversa con las densidades de TILs $CD4^+$, $CD8^+$ y $FOXP3^+$, tanto estromales como intratumorales, y, además, las proporciones de $CD8^+/FOXP3^+$ fueron similares a otros marcadores de CSCs estudiados (NANOG, SOX2), aunque la única asociación significativa observada fue con los TILs $FOXP3^+$ estromales.

PDPN es otro biomarcador que regula las células troncales en los tejidos normales y tumorales. Actúa como un biomarcador importante en la evaluación del riesgo de malignidad en las leucoplasias orales [51]. En nuestro estudio solo se

correlacionó significativamente con las células estromales y tumorales que expresaban FOXP3^{+}. De acuerdo con estos hallazgos, planteamos la hipótesis de que la infiltración de TILs citotóxicos y auxiliares no parece estar relacionada con el mantenimiento de los nidos de CSCs, sino que se opone al mantenimiento de la pluripotencialidad y el escape de las CSCs del sistema inmunológico. Estos hallazgos están en consonancia con la asociación inversa entre los macrófagos M2 estromales/tumorales y la expresión de NANOG que hemos observado [93]. Nuestros resultados también plantean un posible vínculo entre la expresión de PDPN y la inmunosupresión en OSCC.

4.3.3 Asociación entre CD4^{+}, CD8^{+}, FOXP3^{+} y la expresión de PD-L1

Se ha demostrado que PD-L1 se correlaciona con la presencia de TILs CD8^{+} en el TME [87]. Además, PD-L1 puede inducir la apoptosis en linfocitos CD8^{+} activados específicos de antígeno [88]. Se ha observado una correlación negativa entre las células PD-L1 y CD8^{+} en OSCC, lo que implica que el bloqueo de PD-L1 induce la activación inmunitaria local [96]. En contraposición, encontramos una asociación positiva significativa entre los TILs CD8^{+}intratumorales y la expresión de PD-L1, lo que podría reflejar la presencia de un mecanismo de inmunorresistencia adaptativa desencadenado por los linfocitos T CD8^{+} que secretan IFN-γ. Esto sugiere que los TILs CD8^{+} pueden tener otras funciones diferentes a la citotoxicidad [89].

4.3.4 Asociación entre CD4^{+}, CD8^{+}, FOXP3^{+} y la supervivencia de los pacientes con OSCC

En nuestro estudio, el número de linfocitos CD4^{+} y CD8^{+} no fue un predictor significativo de la supervivencia de los pacientes en el análisis multivariante, lo que es consistente con los hallazgos de otros autores [18, 22, 97, 98, 99, 100].

En el análisis multivariante, la relación entre TILs CD8^{+}/FOXP3^{+} estromales se reveló como un indicador pronóstico independiente junto con el estadio clínico de la enfermedad.

Al estudiar el impacto de los parámetros inmunológicos en la supervivencia de los pacientes con OSCC, identificamos que una alta densidad de TILs $FOXP3^+$ tumorales se asoció con un pronóstico favorable. Nuestros hallazgos están en concordancia con algunos autores [18, 21, 29, 101, 102], pero contrastan con los de otros [29, 99, 103]. Una alta densidad de linfocitos $FOXP3^+$ se asoció con una supervivencia prolongada en todos los pacientes de la muestra, pero especialmente en aquellos que tras la cirugía recibieron radioterapia adyuvante. En este sentido, se ha sugerido que la composición del TME antes del tratamiento tiene menos importancia que la respuesta inmunitaria antitumoral inducida por la radioterapia [104].

5. CONCLUSIONES

1. Existe una asociación inversa entre la presencia estromal y tumoral de macrófagos $CD163^+$ y la expresión NANOG, lo que sugiere que la presencia de macrófagos M2 no parece estar relacionada con el mantenimiento de nichos de células troncales cancerosas (*stemcells*).

2. Una elevada densidad de macrófagos $CD68^+$ y $CD163^+$, tanto intratumorales como estromales, se asoció significativamente con una alta expresión de PD-L1. Esto sugiere un vínculo entre la infiltración de macrófagos y el escape inmunológico en carcinomas orales de células escamosas.

3. Ni la infiltración tumoral o estromal de macrófagos ni su densidad se asociaron con la progresión o el pronóstico de la enfermedad.

4. La alta densidad de linfocitos $CD20^+$ en los nidos tumorales es un factor independiente de buen pronóstico en pacientes con carcinoma de células escamosas de la cavidad oral.

5. Existe una asociación inversa entre la infiltración de linfocitos B $CD20^+$ y la expresión de marcadores de células troncales pluripotenciales, especialmente SOX2.

6. Tanto una elevada densidad de linfocitos $FOXP3^+$ infiltrantes estromales y tumorales como una baja proporción de linfocitos $CD8^+/FOXP3^{+,}$ estromales se asociaron significativamente con una mejor supervivencia. La proporción de linfocitos $CD8^+/FOXP3^+$ estromales se configura así como un potencial factor pronóstico independiente en pacientes con carcinoma de células escamosas de la cavidad oral.

7. La alta densidad de linfocitos infiltrantes $CD4^+$ y $CD8^+$, sumados a una alta proporción intratumoral de linfocitos $CD8^+/FOXP3,^+$ se asoció significativamente con tumores que expresaron PD-L1 en más del 10% de las células.

8. La infiltración de linfocitos $CD4^+$ y $CD8^+$ estromales o tumorales mostró una asociación inversa con la ausencia o la baja expresión de diversos marcadores de células troncales cancerosas (NANOG, SOX2 y Nestina).

9. Los carcinomas orales de células escamosas con elevadas densidades de linfocitos $FOXP3^+$ estromales o tumorales presentaron una alta expresión de PDPN.

6. REFERENCIAS

1. Ferris, R.L. Immunology and Immunotherapy of Head and Neck Cancer. *J. Clin. Oncol. 2015*, 3, 3293–3304.
2. He, K.F.; Zhang, L.; Huang, C.F.; Ma, S.R.;Wang, Y.F.;Wang,W.M.; Zhao, Z.L.; Liu, B.; Zhao, Y.F.; Zhang,W.F.; et al. $CD163^{+}$ tumor-associated macrophages correlated with poor prognosis and cancer stem cells in oral squamous cell carcinoma. *Biomed Res. Int. 2014*, 2014, 838632.
3. Zhou, HM., Zhang, JG., Zhang, X. *et al.*Targeting cancer stem cells for reversing therapy resistance: mechanism, signaling, and prospective agents. *Sig Transduct Target Ther* 6, 62 (2021).
4. Fujita, Y.; Okamoto, M.; Goda, H.; Tano, T.; Nakashiro, K.; Sugita, A.; Fujita, T.; Koido, S.; Homma, S.; Kawakami, Y.; et al. Prognostic significance of interleukin-8 and CD163-positive cell-infiltration in tumor tissues in patients with oral squamous cell carcinoma.*PLoS ONE 2014*, 9, e110378.
5. Alves, A.M.; Diel, L.F.; Lamers, M.L. Macrophages and prognosis of oral squamous cell carcinoma: A systematic review. *J. Oral Pathol. Med. 2018*, 47, 460–467.
6. Xiao M., Zhang J., Chen W., Chen W. M1-like tumor-associated macrophages activated by exosome-transferred THBS1 promote malignant migration in oral squamous cell carcinoma. *J. Exp. Clin. Cancer Res.* 2018; 37:143. doi: 10.1186/s13046-018-0815-2.
7. Evrard D., Szturz P., Tijeras-Raballand A., Astorgues-Xerri L., Abitbol C., Paradis V., Raymond E., Albert S., Barry B., Faivre S. Macrophages in the microenvironment of head and neck cancer: Potential targets for cancer therapy. *Oral Oncol.* 2019; 88:29–38.doi:10.1016/j.oraloncology.2018.10.040.
8. Mou W., Xu Y., Ye Y., Chen S., Li X., Gong K., Liu Y., Chen Y., Li X., Tian Y., et al. Expression of Sox2 in breast cancer cells promotes the recruitment of M2 macrophages to tumor microenvironment. *Cancer Lett.* 2015; 358:115–123. doi: 10.1016/j.canlet.2014.11.004.
9. Chen T., Chen J., Zhu Y., Li Y., Wang Y., Chen H., Wang J., Li X., Liu Y., Li B., et al. CD163, a novel therapeutic target, regulates the proliferation and stemness of glioma cells via casein kinase 2. *Oncogene.* 2019; 38:1183–1199. doi: 10.1038/s41388-018-0515-6.

10. Kubota K., Moriyama M., Furukawa S., Rafiul H.A.S.M., Maruse Y., Jinno T., Tanaka A., Ohta M., Ishiguro N., Yamauchi M., et al. $CD163^{+}CD204^{+}$ tumor-associated macrophages contribute to T cell regulation via interleukin-10 and PD-L1 production in oral squamous cell carcinoma. *Sci. Rep.* 2017; 7:1755. doi: 10.1038/s41598-017-01661-z.
11. Haque A.S.M.R., Moriyama M., Kubota K., Ishiguro N., Sakamoto M., Chinju A., Mochizuki K., Sakamoto T., Kaneko N., Munemura R., et al. $CD206^{+}$ tumor-associated macrophages promote proliferation and invasion in oral squamous cell carcinoma via EGF production. *Sci. Rep.* 2019; 9:14611. doi: 10.1038/s41598-019-51149-1.
12. Troiano G., Caponio V.C.A., Adipietro I., Tepedino M., Santoro R., Laino L., Lo Russo L., Cirillo N., Lo Muzio L. Prognostic significance of $CD68^{+}$ and $CD163^{+}$ tumor associated macrophages in head and neck squamous cell carcinoma: A systematic review and meta-analysis. *Oral Oncol.* 2019; 93:66–75. doi: 10.1016/j.oraloncology.2019.04.019.
13. Zou W., Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat. Rev. Immunol. 2008*; 8:467–477. doi: 10.1038/nri2326.
14. Lund F.E. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr. Opin. Immunol.* 2008; 20:332–338. doi: 10.1016/j.coi.2008.03.003.
15. Hadler-Olsen, E.; Wirsing, A.M. Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma: A systematic review and meta-analysis. *Br. J. Cancer 2019*, 120, 714–727.
16. Wang, T.; Niu, G.; Kortylewski, M.; Burdelya, L.; Shain, K.; Zhang, S.; Bhattacharya, R.; Gabrilovich, D.; Heller, R.; Coppola, D.; et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat. Med. 2004*, 10, 48–54.
17. da Silveira, E.J.; da Costa Miguel, M.C.; Lima, K.C.; de Almeida Freitas, R.; Queiroz, L.M.G. Analysis of local immunity in squamous cell carcinoma of the tongue and lower lip. *Exp. Mol. Pathol. 2010*, 88, 171–175.
18. Nguyen N., Bellile E., Thomas D., McHugh J., Rozek L., Virani S., Peterson L., Carey T.E., Walline H., Moyer J., et al. Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. *Head Neck. 2016*; 38:1074–1084. doi: 10.1002/hed.24406.

19. Shimizu S., Hiratsuka H., Koike K., Tsuchihashi K., Sonoda T., Ogi K., Miyakawa A., Kobayashi J., Kaneko T., Igarashi T., et al. Tumor-infiltrating $CD8^+$ T-cell density is an independent prognostic marker for oral squamous cell carcinoma. *Cancer Med. 2019*; 8:80–93. doi: 10.1002/cam4.1889.
20. Huang Z., Xie N., Liu H., Wan Y., Zhu Y., Zhang M., Tao Y., Zhou H., Liu X., Hou J., et al. The prognostic role of tumour-infiltrating lymphocytes in oral squamous cell carcinoma: A meta-analysis. *J. Oral Pathol. Med. 2019*; 48:788–798. doi: 10.1111/jop.12927.
21. de Ruiter E.J., Ooft M.L., Devriese L.A., Willems S.M. The prognostic role of tumor infiltrating T-lymphocytes in squamous cell carcinoma of the head and neck: A systematic review and meta-analysis. *Oncoimmunology. 2017*;6: e1356148. doi: 10.1080/2162402X.2017.1356148.
22. Borsetto D., Tomasoni M., Payne K., Polesel J., Deganello A., Bossi P., Tysome J.R., Masterson L., Tirelli G., Tofanelli M., et al. Prognostic Significance of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes in head and neck squamous cell carcinoma: A meta-analysis. *Cancers. 2021*;13:781. doi: 10.3390/cancers13040781.
23. Stasikowska-Kanicka, O.;Wa˛growska-Danilewicz, M.; Danilewicz, M. Immunohistochemical analysis of $Foxp3^+$, $CD4^+$, $CD8^+$cell infiltrates and PD-L1 in oral squamous cell carcinoma. *Pathol. Oncol. Res. 2018*, 24, 497–505.
24. Tao H., Mimura Y., Aoe K., Kobayashi S., Yamamoto H., Matsuda E., Okabe K., Matoto T., Sugi K., Ueoka H. Prognostic potentialof FOXP3 expression in non-small cell lung cáncer cells combined with tumor-infiltrating regulatory T cells. *Lung Cancer. 2012*;75:95–101. doi: 10.1016/j. lung can.2011.06.002.
25. Tang Y., Xu X., Guo S., Zhang C., Tang Y., Tian Y., Ni B., Lu B., Wang H. An increased abundance of tumor-infiltrating regulatory T cells is correlated with the progression and prognosis of pancreatic ductal adenocarcinoma. *PLoS ONE. 2014*;9:e91551. doi: 10.1371/journal.pone.0091551.
26. Sayour E.J., McLendon P., McLendon R., De Leon G., Reynolds R., Kresak J., Sampson J.H., Mitchell D.A. Increased proportion of $FoxP3^+$ regulatory T cells in tumor infiltrating lymphocytes is associated with tumor recurrence and reduced survival in patients with glioblastoma. *CancerImmunol. Immunother. 2015*;64:419–427. doi: 10.1007/s00262-014-1651-7.

27. de Leeuw R.J., Kost S.E., Kakal J.A., Nelson B.H. The prognostic value of FoxP3$^+$ tumor-infiltrating lymphocytes in cancer: A critical review of the literature. Clin. *Cancer Res. 2012*;18:3022–3029. doi: 10.1158/1078-0432.CCR-11-3216.
28. Martin-Hijano L., Sainz B., Jr. The interactions between cancer stem cells and the innate interferon signaling pathway. *Front. Immunol. 2020*;11:526. doi: 10.3389/fimmu.2020.00526.
29. Kindt N., Descamps G., Seminerio I., Bellier J., Lechien J.R., Mat Q., Pottier C., Delvenne P., Journé F., Saussez S. High stromal Foxp3-positive T cell number combined to tumor stage improved prognosis in head and neck squamous cell carcinoma. *Oral Oncol. 2017*;67:183–191. doi: 10.1016/j.oraloncology.2017.02.023.
30. Park K., Cho K.J., Lee M., Yoon D.H., Kim S.B. Importance of FOXP3 in prognosis and its relationship with p16 in tonsillar squamous cell carcinoma. *Anticancer Res. 2013*;33:5667–5673.
31. Zeng C., Kuang H., Fan W., Chen X., Yu T., Tang Q., Zhou Z., Liang F. Downregulation of FOXP3 in neutrophils by IL-8 promotes the progression of oral squamous cell carcinoma. *Oncol. Lett. 2019*;18:4771–4777. doi: 10.3892/ol.2019.10828.
32. Zhang B., Wu C., Zhang Z., Yan K., Li C., Li Y., Li L. CXCL12 is associated with FoxP3$^+$ tumor-infiltrating lymphocytes and affects the survival of patients with oral squamous cell carcinoma. *Oncol. Lett. 2019*;18:1099–1106. doi: 10.3892/ol.2019.10415.
33. Song J.J., Zhao S.J., Fang J., Ma D., Liu X.Q., Chen X.B., Wang Y., Cheng B., Wang Z. Foxp3 over expression in tumor cells predicts por survival in oral squamous cell carcinoma. *BMC Cancer. 2016*;16:530. doi: 10.1186/s12885-016-2419-6.
34. Yarchoan M., Ho W.J., Mohan A., Shah Y., Vithayathil T., Leatherman J., Dennison L., Zaidi N., Ganguly S., Woolman S., et al. Effects of B cell-activating factor on tumor immunity. *JCI Insight.* 2020;5:e136417. doi: 10.1172/jci.insight.136417.
35. Hladíková K., Koucký V., Bouček J., Laco J., Grega M., Hodek M., Zábrodský M., Vošmik M., Rozkošová K., Vošmiková H., et al. Tumor-infiltrating B cells affect the progression of oropharyngeal squamous cell carcinoma via cell-to-cell interactions with CD8$^+$ T cells. *J. Immunother. Cancer.* 2019;7:261. doi: 10.1186/s40425-019-0726-6.

36. Mizoguchi A.M., Mizoguchi E., Takedatsu H., Blumberg R.S., Bhan A.K. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity. 2002*;16:219–230. doi: 10.1016/S1074-7613(02)00274-1.
37. Valle S., Martin-Hijano L., Alcalá S., Alonso-Nocelo M., Sainz B., Jr. The ever-evolving concept of the cancer stem cell in pancreatic cancer. *Cancers. 2018*;10:33. doi: 10.3390/cancers10020033.
38. Wirsing, A.M.; Ervik, I.K.; Seppola, M.; Uhlin-Hansen, L.; Steigen, S.E.; Hadler-Olsen, E. Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma. *Mod. Pathol. 2018,* 31, 910–922.
39. Davis S.J., Divi V., Owen J.H., Bradford C.R., Carey T.E., Papagerakis S., Prince M.E.P. Metastatic potential of cancer stem cells in head and neck squamous cell carcinoma. *Arch. Otolaryngol. Head NeckSurg. 2010*;136:1260–1266. doi: 10.1001/archoto.2010.219.
40. de Vicente J.C., Rodríguez-Santamarta T., Rodrigo J.P., Allonca E., Vallina A., Singhania A., Donate-Pérez Del Molino P., García-Pedrero J.M. The emerging role of NANOG as a nearly cáncer risk biomarker in patients with oral potentially malignant disorders. *J. Clin. Med. 2019*;8:1376. doi: 10.3390/jcm8091376.
41. de Vicente J.C., Donate-Pérez Del Molino P., Rodrigo J.P., Allonca E., Hermida-Prado F., Granda-Díaz R., Rodríguez Santamarta T., García-Pedrero J.M. SOX2 expression is an independent predictor of oral cáncer progression. *J. Clin. Med. 2019*;8:1744. doi: 10.3390/jcm8101744.
42. Major A.G., Pitty L.P., Farah C.S. Cancer stem cell markers in head and neck squamous cell carcinoma. *StemCellsInt. 2013*;2013:319489. doi: 10.1155/2013/319489.
43. Grubelnik G., Boštjančič E., Grošelj A., Zidar N. Expression of NANOG and its regulation in oral squamous cell carcinoma. *Biomed. Res. Int. 2020*;2020:8573793. doi: 10.1155/2020/8573793.
44. Tsai L.L., Yu C.C., Chang Y.C., Yu C.H., Chou M.Y. Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. *J. Oral. Pathol. Med. 2011*;40:621–628. doi: 10.1111/j.1600-0714.2011.01015.x.

45. Chiou S.H., Yu C.C., Huang C.Y., Lin S.C., Liu C.J., Tsai T.H., Chou S.H., Chien C.S., Ku H.H., Lo J.F. Positive correlations of Oct-4 and Nanog in oral cáncer stem-like cells and high-grade oral squamouscell carcinoma. *Clin. Cancer Res. 2008*;14:4085–4095. doi: 10.1158/1078-0432.CCR-07-4404.
46. Curtarelli R.B., Gonçalves J.M., Dos Santos L.G.P., Savi M.G., Nör J.E., Mezzomo L.A.M., Rodríguez Cordeiro M.M. Expression of cáncer stem cell biomarker sin human head and neck carcinomas: A systematic review. *Stem. Cell Rev. Rep. 2018*;14:769–784. doi: 10.1007/s12015-018-9839-4.
47. Lee S.H., Oh S.Y., Do S.I., Lee H.J., Kang H.J., Rho Y.S., Bae W.J., Lim Y.C. SOX2 regulates self-renewal and tumorigenicity of stem-likecellsof head and neck squamous cell carcinoma. Br. J. *Cancer. 2014*; 111:2122–2130. doi: 10.1038/bjc.2014.528.
48. Lothian C., Lendahl U. An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. *Eur. J. Neurosci. 1997*;9:452–462. doi: 10.1111/j.1460-9568.1997.tb01622.x.
49. Ravindran G., Devaraj H. Prognostic significance of neural stem cell markers, Nestin and Musashi-1, in oral squamous cell carcinoma: Expression pattern of Nestin in the precancerous stages of oral squamous epithelium. *Clin. Oral Investig. 2015*;19:1251–1260. doi: 10.1007/s00784-014-1341-z.
50. Hu L., Zhang P., Mei Q., Sun W., Zhou L., Yin T. Podoplanin is a useful prognostic marker and indicates better differentiation in lung squamous cell cancer patients? A systematic review and meta-analysis. *BMC Cancer. 2020*;20:424. doi: 10.1186/s12885-020-06936-9.
51. De Vicente J.C., Santamarta T.R., Rodrigo J.P., García-Pedrero J.M., Allonca E., Blanco-Lorenzo V. Expression of podoplanin in the invasion front of oral squamous cell carcinoma is not prognostic for survival. *Virchows Arch. 2015*;466:549–558. doi: 10.1007/s00428-015-1746-3.
52. De Vicente, J.C.; Rodríguez-Santamarta, T.; Rodrigo, J.P.; Blanco-Lorenzo, V.; Allonca, E.; García-Pedrero, J.M. PD-L1 Expression in Tumor Cells is an Independent Unfavorable Prognostic Factor in Oral Squamous Cell Carcinoma. *Cancer Epidemiol. BiomarkersPrev. 2019*, 28, 546–554.
53. Kythreotou A., Siddique A., Mauri F.A., Bower M., Pinato D.J. PD-L1. *J. Clin. Pathol. 2018*;71:189–194. doi: 10.1136/jclinpath-2017-204853.

54. Remmele W., Schicketanz K.H. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs. Subjective grading (IRS) *Pathol. Res. Pract. 1993*;189:862–866. doi: 10.1016/S0344-0338(11)81095-2.
55. Amin, M.B. *AJCC Cancer Staging Manual*, 8th ed.; Springer: Chicago, IL, USA, 2017; pp. 79–94.
56. Lin J.Y., Li X.Y., Tadashi N., Dong P. Clinical significance of tumor-associated macrophage infiltration in supraglottic laryngeal carcinoma. *Chin. J. Cancer. 2011*;30:280–286. doi: 10.5732/cjc.010.10336.
57. Kouketsu A., Sato I., Oikawa M., Shimizu Y., Saito H., Tashiro K., Yamashita Y., Takahashi T., Kumamoto H. Regulatory T cells and M2-polarized tumour-associated macrophages are associated with the oncogenesis and progression of oral squamous cell carcinoma. *Int. J. Oral Maxillofac. Surg. 2019*;48:1279–1288. doi: 10.1016/j.ijom.2019.04.004.
58. Lu C.F., Huang C.S., Tjiu J.W., Chiang C.P. Infiltrating macrophage count: A significant predictor for the progression and prognosis of oral squamous cell carcinomas in Taiwan. *Head Neck. 2010*;32:18–25. doi: 10.1002/hed.21138.
59. Wen Z.F., Liu H., Gao R., Zhou M., Ma J., Zhang Y., Zhao J., Chen Y., Zhang T., Huang F., et al. Tumor cell-released autophagosomes (TRAPs) promote immunosuppression through induction of M2-like macrophages with increased expression of PD-L1. *J. Immunother. Cancer. 2018*;6:151. doi: 10.1186/s40425-018-0452-5.
60. Zhang X., Zeng Y., Qu Q., Zhu J., Liu Z., Ning W., Zeng H., Zhang N., Du W., Chen C., et al. PD-L1 induced by IFN-γ from tumor-associated macrophages via the JAK/STAT3 and PI3K/AKT signaling pathways promoted progression of lung cancer. *Int. J. Clin. Oncol. 2017*;22:1026–1033. doi: 10.1007/s10147-017-1161-7.
61. Tsukamoto M., Imai K., Ishimoto T., Komohara Y., Yamashita Y.I., Nakagawa S., Umezaki N., Yamao T., Kitano Y., Miyata T., et al. PD-L1 expression enhancement by infiltrating macrophage-derived tumor necrosis factor-α leads to poor pancreatic cancer prognosis. *Cancer Sci. 2019*;110:310–320. doi: 10.1111/cas.13874.
62. Lai Y.S., Wahyuningtyas R., Aui S.P., Chang K.T. Autocrine VEGF signalling on M2 macrophages regulates PD-L1 expression for immunomodulation of T cells. *J. Cell. Mol. Med. 2019*;23:1257–1267. doi: 10.1111/jcmm.14027.

63. Lin C., He H., Liu H., Li R., Chen Y., Qi Y., Jiang Q., Chen L., Zhang P., Zhang H., et al. Tumour-associated macrophages-derived CXCL8 determines immune evasion through autonomous PD-L1 expression in gastric cancer. *Gut. 2019*;68:1764–1773. doi: 10.1136/gutjnl-2018-316324.
64. Kasikara C., Kumar S., Kimani S., Tsou W.I., Geng K., Davra V., Sriram G., Devoe C., Nguyen K.N., Antes A., et al. Phosphatidylserine Sensing by TAM Receptors Regulates AKT-Dependent Chemoresistance and PD-L1 Expression. *Mol. Cancer Res. 2017*;15:753–764. doi: 10.1158/1541-7786.MCR-16-0350.
65. Chen J., Feng Y., Lu L., Wang H., Dai L., Li Y., Zhang P. Interferon-γ-induced PD-L1 surface expression on human oral squamous carcinoma via PKD2 signal pathway. *Immunobiology. 2012*;217:385–393. doi: 10.1016/j.imbio.2011.10.016.
66. Jiang C., Yuan F., Wang J., Wu L. Oral squamous cell carcinoma suppressed antitumor immunity through induction of PD-L1 expression on tumor-associated macrophages. *Immunobiology.2017*;222:651–657. doi:10.1016/j.imbio.2016.12.002.
67. Sumitomo R., Hirai T., Fujita M., Murakami H., Otake Y., Huang C.L. PD-L1 expression on tumor-infiltrating immune cells is highly associated with M2 TAM and aggressive malignant potential in patients with resected non-small cell lung cancer. *LungCancer. 2019*;136:136–144. doi: 10.1016/j.lungcan.2019.08.023.
68. Kondoh N., Mizuno-Kamiya M., Umemura N., Takayama E., Kawaki H., Mitsudo K., Muramatsu Y., Sumitomo S. Immunomodulatory aspects in the progression and treatment of oral malignancy. *Jpn. Dent. Sci. Rev. 2019*;55:113–120. doi: 10.1016/j.jdsr.2019.09.001.
69. Yuen G.J., Demissie E., Pillai S. B lymphocytes and cancer: A love-hate relationship. *Trends Cancer. 2016*;2:747–757. doi: 10.1016/j.trecan.2016.10.010.
70. Tobón G.J., Izquierdo J.H., Cañas C.A. B lymphocytes: Development, tolerance, and their role in autoimmunity-focusonsystemic lupus erythematosus. *Autoimmune Dis. 2013*:827254. doi: 10.1155/2013/827254.
71. Nielsen J.S., Sahota R.A., Milne K., Kost S.E., Nesslinger N.J., Watson P.H., Nelson B.H. $CD20^+$ tumor-infiltrating lymphocytes have an atypical $CD27^-$ memory phenotype and together with $CD8^+$ T cells promote favorable prognosis in ovarían cancer. *Clin. Cancer Res. 2012*;18:3281–3292. doi: 10.1158/1078-0432.CCR-12-0234.
72. Li Q., Grover A.C., Donald E.J., Carr A., Yu J., Whitfield J., Nelson M., Takeshita N., Chang A.E.J. Simultaneous targeting of CD3 on T cells and CD40 on B or

dendritic cells augments the antitumor reactivity of tumor-primed lymph node cells. *J. Immunol. 2005*;175:1424–1432. doi: 10.4049/jimmunol.175.3.1424.

73. Wang S.S., Liu W., Ly D., Xu H., Qu L., Zhang L. Tumor-infiltrating B cells: Their role and application in anti-tumor immunity in lung cancer. *Cell Mol. Immunol. 2019*;16:6–18. doi: 10.1038/s41423-018-0027-x.
74. Sharonov G.V., Serebrovskaya E.O., Yuzhakova D.V., Britanova O.V., Chudakov D.M. B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nat. Rev. Immunol. 2020*;20:294–307. doi: 10.1038/s41577-019-0257-x.
75. Pretscher D., Distel L.V., Grabenbauer G.G., Wittlinger M., Buettner M., Niedobitek G. 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. doi: 10.1186/1471-2407-9-292.
76. Taghavi N., Mohsenifar Z., Baghban A.A., Arjomandkhah A. $CD20^+$ Tumor Infiltrating B Lymphocyte in Oral Squamous Cell Carcinoma: Correlation with Clinicopathologic Characteristics and Heat Shock Protein 70 Expression. Pathol. *Res. Int. 2018*;2018:4810751. doi: 10.1155/2018/4810751.
77. Sautès-Fridman C., Petitprez F., Calderaro J., Fridman W.H. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat. Rev. Cancer. 2019*;19:307–325. doi: 10.1038/s41568-019-0144-6.
78. Colbeck E.J., Ager A., Gallimore A., Jones G.W. Tertiary lymphoid structures in cancer: Drivers of antitumor immunity, immunosuppression, or bystander sentinels in disease? *Front. Immunol. 2017*;9:1830. doi: 10.3389/fimmu.2017.01830.
79. Kroeger D.R., Milne K., Nelson B.H. Tumor-infiltrating plasma cells are associated with tertiary lymphoid structures, cytolytic T-cell responses, and superior prognosis in ovarian cancer. *Clin. Cancer Res. 2016*;22:3005–3015. doi: 10.1158/1078-0432.CCR-15-2762.
80. De Ruiter E.J., de Roest R.H., Brakenhoff R.H., Leemans C.R., de Bree R., Terhaard C.H.J., Willems S.M. Digital pathology-aidedassessmentof tumor-infiltrating T lymphocytes in advanced stage, HPV-negative head and neck tumors. *Cancer Immunol. Immunother. 2020*;69:581–591. doi: 10.1007/s00262-020-02481-3.

81. Kashima Y., Nishii N., Tachinami H., Furusawa E., Nagai S., Harada H., Azuma M. Orthotopic tongue squamous cell carcinoma (SCC) model exhibiting a different tumor-infiltrating T-cell status with margin-restricted $CD8^+$ T cells and regulatory T cell-dominance, compared to skin SCC. Biochem. Biophys. *Res. Commun. 2020*;526:218–224. doi: 10.1016/j.bbrc.2020.03.022.
82. 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*;58:3491–3494.
83. Menon A.G., Janssen-van Rhijn C.M., Morreau H., Putter H., Tollenaar R.A., van de Velde C.J., Fleuren G.J., Kuppen P.J. Immunesystem and prognosis in colorectal cancer: A detailed immunohistochemical analysis. *Lab. Investig. 2004*;84:493–501. doi: 10.1038/labinvest.3700055.
84. Hu C., Tian S., Lin L., Zhang J., Ding H. Prognostic and clinicopathological significance of PD-L1 and tumor infiltrating lymphocytes in hypopharyngeal squamous cell carcinoma. *Oral Oncol. 2020*;102:104560. doi: 10.1016/j.oraloncology.2019.104560.
85. Peske J.D., Woods A.B., Engelhard V.H. Control of CD8 T-cell infiltration into tumors by vasculature and microenvironment. Adv. Cancer Res. 2015;128:263–307.
86. Balermpas P., Rödel F., Weiss C., Rödel C., Fokas E. Tumor-infiltrating lymphocytes favor the response to chemoradiotherapy of head and neck cancer. *Oncoimmunology. 2014*;3:e27403. doi: 10.4161/onci.27403.
87. Kim T.K., Herbst R.S., Chen L. Defining and understanding adaptive resistance in cancer immunotherapy. *TrendsImmunol. 2018*;39:624–631. doi: 10.1016/j.it.2018.05.001.
88. Zhu Q., Cai M.Y., Weng D.S., Zhao J.J., Pan Q.Z., Wang Q.J., Tang Y., He J., Li M., Xia J.C. PD-L1 expression patterns in tumour cells and their association with CD8(+) tumour infiltrating lymphocytes in clear cell renal cell carcinoma. *J. Cancer. 2019*;10:154–1161. doi: 10.7150/jca.29052.
89. Stasikowska-Kanicka O., Wągrowska-Danilewicz M., Danilewicz M. $CD8^+$ and $CD163^+$infiltratingcells and PD-L1 immunoexpression in oral leukoplakia and oral carcinoma. *APMIS. 2018*;126:732–738. doi: 10.1111/apm.12881.
90. Thompson E.D., Zahurak M., Murphy A., Cornish T., Cuka N., Abdelfatah E., Yang S., Duncan M., Ahuja N., Taube J.M., et al. Patterns of PD-L1 expression and CD8

T cell infiltration in gastric adenocarcinomas and associated immune stroma. *Gut. 2017*;66:794–801. doi: 10.1136/gutjnl-2015-310839.

91. Sharma A., Rudra D. Emerging functions of regulatory T cells in tissue homeostasis. *Front. Immunol. 2018*;9:883. doi: 10.3389/fimmu.2018.00883.
92. Ghiringhelli F., Puig P.E., Roux S., Parcellier A., Schmitt E., Solary E., Kroemer G., Martin F., Chauffert B., Zitvogel L. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing $CD4^{+}CD25^{+}$regulatory T cell proliferation. *J. Exp. Med. 2005*;202:919–929. doi: 10.1084/jem.20050463.
93. Pedregal-Mallo D., Hermida-Prado F., Granda-Díaz R., Montoro-Jiménez I., Allonca E., Pozo-Agundo E., Álvarez-Fernández M., Álvarez-Marcos C., García-Pedrero J.M., Rodrigo J.P. Prognostic significance of the pluripotency factors NANOG, SOX2, and OCT4 in head and neck squamous cell carcinomas. *Cancers. 2020*;12:1794. doi: 10.3390/cancers12071794.
94. Ghazi N., Aali N., Shahrokhi V.R., Mohajertehran F., Saghravanian N. Relative expression of SOX2 and OCT4 in oral squamous cell carcinoma and oral epithelial dysplasia. *Rep. Biochem. Mol. Biol. 2020*;9:171–179. doi: 10.29252/rbmb.9.2.171.
95. Vijayakumar G., Narwal A., Kamboj M., Sen R. Association of SOX2, OCT4 and WNT5A expression in oral epithelial dysplasia and oral squamous cell carcinoma: An immune histochemical study. *Head NeckPathol. 2020*;14:749–757. doi: 10.1007/s12105-019-01114-1.
96. Piazzolla D., Palla A.R., Pantoja C., Cañamero M., Perez de Castro I., Ortega S., Gómez-López G., Dominguez O., Megías D., Roncador G., et al. Lineage-restricted function of the pluripotency factor NANOG in stratified epithelia. *Nat. Comm. 2015*;5:4226. doi: 10.1038/ncomms5226.
97. Swann J.B., Smyth M.J. Immune surveillance of tumors. *J. Clin. Investig. 2007*;117:1137–1146. doi: 10.1172/JCI31405.
98. Quan H., Shan Z., Liu Z., Liu S., Yang L., Fang X., Li K., Wang B., Deng Z., Hu Y., et al. The repertoire of tumor-infiltrating lymphocytes within the microenvironment of oral squamous cell carcinoma reveals immune dysfunction. *Cancer Immunol. Immunother. 2020*;69:465–476. doi: 10.1007/s00262-020-02479-x.
99. Wolf G.T., Chepeha D.B., Bellile E., Nguyen A., Thomas D., McHugh J. University of Michigan Head and Neck SPORE Program. Tumor infiltrating lymphocytes (TIL) and prognosis in oral cavity squamous carcinoma: A preliminary study. *Oral Oncol. 2015*;51:90–95. doi: 10.1016/j.oraloncology.2014.09.006.

100. Ono T., Azuma K., Kawahara A., Sasada T., Hattori S., Sato F., Shin B., Chitose S.I., Akiba J., Hirohito U. Association between PD-L1 expression combined with tumor-infiltrating lymphocytes and the prognosis of patients with advanced hypopharyngeal squamous cell carcinoma. *Oncotarget. 2017*;8:92699–92714. doi: 10.18632/oncotarget.21564.

101. De Meulenaere A., Vermassen T., Aspeslagh S., Zwaenepoel K., Deron P., Duprez F., Rottey S., Ferdinande L. Prognosticmarkers in oropharyngeal squamous cell carcinoma: Focus on CD70 and tumour infiltrating lymphocytes. *Pathology. 2017*;49:397–404. doi: 10.1016/j.pathol.2017.02.002.

102. Schipmann S., Wermker K., Schulze H.J., Kleinheinz J., Brunner G. Cutaneous and oral squamouscell carcinoma-dual immunosuppression via recruitment of FOXP3^{+}regulatory T cells and endogenous tumour FOXP3 expression? *J. Craniomaxillofac. Surg. 2014*;42:1827–1833. doi: 10.1016/j.jcms.2014.06.022.

103. Salama P., Phillips M., Grieu 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*;27:186–192. doi: 10.1200/JCO.2008.18.7229.

104. Homma Y., Taniguchi K., Murakami T., Nakagawa K., Nakazawa M., Matsuyama R., Mori R., Takeda K., Ueda M., Ichikawa Y., et al. Immunological impact of neoadjuvant chemoradiotherapy in patients with borderline resectable pancreatic ductal adenocarcinoma. *Ann. Surg. Oncol. 2014*;21:670–676. doi: 10.1245/s10434-013-3390-y.