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Evaluation of Cd8+ and natural killer cells defense in oral and oropharyngeal squamous cell carcinoma

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ABSTRACT

Purpose: The aim of this study was to evaluate the population of CD8+ and natural killer (NK) cells in samples of oral (OSCC) and oropharyngeal (OPSCC) squamous cell carcinoma.
Patients and methods: Fifty-four cases squamous cell carcinoma (42 OSCC and 12 OPSCC) were immunohistochemically treated by CD8 and CD57 monoclonal antibodies. It was evaluated the relationship of CD8+ and NK cells with tumor size, lymph node metastasis (LNM), clinical staging (CS), overall survival (OS) and disease-free survival (DFS).
Results: Only CD8 was higher expressed in both tumors T1 and T2 than T3 and T4, as well as in tumours without LNM and with CS II or III ($P < 0.05$). There was no association with OS and DFS of both biomarkers.
Conclusions: These findings suggest that the differential CD8+ cells infiltration in OSCC and OPSCC might reflect a distinctive tumor microenvironment with a favorable local cytotoxic immune response against neoplastic cells.

1. Introduction

Oral cancer is one of the major threats to public health in the developed world and increasingly in the developing world (Singh et al., 2013). Oral squamous cell carcinoma (OSCC) is the sixth most common type of cancer and is a huge problem of public health worldwide; besides, it is associated with important mortality and morbidity (Anaya-Saavedra et al., 2008; Chen et al., 2008, 2017; Pontes et al., 2011; de Matos et al., 2012). An occurrence of almost half million new cases and 200,000 deaths annually have been estimated. Cancer arises from the uncontrolled dissemination and spread of clones of transformed cells, which should be recognized by the immune system before transforming into a tumor. Although it has been demonstrated that the immune system reacts to many tumors and at least slows down the progression, it is not yet known how immune reactions are used to destroy tumors in a specific manner (de Matos et al., 2012; dos Santos Pereira et al., 2014; Xu et al., 2016).

Recent advances in tumor immunology have revealed that clinical cancers develop after escaping host immunosurveillance and, as a result, established cancers are either of low immunogenicity or have the capacity to attenuate host antitumor immunity. Established solid cancers are infiltrated to varying degrees by immune cells (tumor-infiltrating lymphocytes – TILs), and this phenomenon was suggested to be a manifestation of host immune response against cancer cells. However, the clinical outcome does not appear to be dependent on the total number of TILs, but rather on various types of TILs (Watanabe et al., 2010; Xu et al., 2016; Fang et al., 2017).

In this context, CD8+ cells that represent an important subpopulation of cytotoxic T-lymphocytes and NK cells are the most likely effector cells for an efficient anti-tumor immunity (Katou et al., 2007; DeNardo et al., 2008; Zancope et al., 2010; Fang et al., 2017). These cells can participate in the immunologic surveillance, recognizing, and killing potentially malignant cells, which express peptides from oncogenic viral proteins or mutant cellular proteins, presented with class I major histocompatibility molecules (MHC) (Zancope et al., 2010). Upon reduction or absence of MHC class I molecules by neoplastic cells, the specialized innate immune

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lymphocytes, NK cells, can play an important role in anti-tumor immunity.

Accumulating evidence has shown that the presence of TILs is significantly correlated with prognosis of various solid tumors (Donnem et al., 2016; Nguyen et al., 2016; Feng et al., 2017). Strong peritumoural TILs infiltration has been observed in squamous cell carcinoma of the head and neck patients with lower tumour stage and less invasive growth also in patients with squamous cell carcinoma of the head and neck (Balermpas et al., 2016). However, mixed findings have been reported with regard to the prognostic relevance of TILs infiltration according to the tumour site. These differences could be attributed to the heterogeneity in population and treatment (Distel et al., 2009; Balermpas et al., 2014; Zhou et al., 2018).

Despite the lack of direct evidence that cells of the immune system can protect against the development and progression of cancer, clinical observations and experimental studies suggest their activity in the response against tumor cells of different origins. Unfortunately, little is known about the pathomechanisms and mutual interactions between tumor cells and the autologous immune cells i.e. the impact of neoplastic cells in modulating the function of cells involved in the immunological processes, the activity of NK cells and CD8+ lymphocytes and the regulation and determination of antitumor cellular immune response in patients with oropharyngeal squamous cell carcinoma (OPSCC) and oral squamous cell carcinoma (OSCC) (Starska et al., 2011; Lopes et al., 2017).

Such studies could increase considerably the knowledge about the relationship between immunologic responses and the development of OSCC and OPSCC. This may contribute to improve the therapies against cancer and support the development of new treatments. Thus, this study aimed to analyze the immunoexpression of CD8+ and NK cells in OSCC and OPSCC, and their relationship with survival data, lymph node metastasis, tumor size and clinical staging.

2. Material and methods

2.1. Study characterization

The present study was submitted to the Research Ethics Committee of the Norteriograndense League Against Cancer (CEP/LNRCG) for evaluation, and was approved under nº 170/170/2010, and the study protocol was conformed to the ethical guidelines of the Declaration of Helsinki.

The samples of this retrospective study consisted of incisional biopsy specimens from 74 patients with primary OSCC and OPSCC from 2004 to 2008 treated exclusively with radiotherapy and chemotherapy. These samples were obtained from the Hospital Dr. Luiz Antônio da Liga Norte-Riograndense Contra o Câncer, Brazil. The following exclusion criteria were adopted for this research: prior radiotherapy and no material from diagnostic incisional biopsy. Then, after applied the criteria described above, the final sample of this experiment was composed of 54 patients. Clinical data (gender, age, tumor location, tumor size, lymph node metastasis and clinical staging) and follow-up information (survival data and death) were obtained from medical records. This study was approved by the Institutional Ethics Committee for human subjects.

The HPV status of the samples was not included in this study. As the prevalence of HPV in head and neck SCCs is very low in Brazil, comprising around 3.5%–10% of total cases (Hauck et al., 2015; López et al., 2014), its investigation is not yet routinely performed in Brazilian public hospitals.

2.2. Immunohistochemistry

Sections of 3 μm from routinely processed paraffin embedded blocks were deparaffinized and dehydrated. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. The sections were immersed in Tris/EDTA (pH 9.0) for 3 min at 21 °C for antigen retrieval. Sections were then blocked by incubation with 3% normal goat serum for 20 min. CD8+ T and NK cells were identified using anti-CD8 antibody (TB01, Dako, Carpinteria, CA, USA) at 1:100 and anti-CD57 antibody (C8144B, Dako) at 1:100. The slides were incubated with the primary antibodies for 60 min. The slides were counterstained with Mayer hematoxylin and cover-slipped with Permount (Thermo Fisher Scientific, Waltham, MA). Normal human tonsil tissue was used as a positive control. As negative control, the primary antibody was substituted by bovine serum albumin.

2.3. Cell counting and statistical analysis

The immunohistochemical expression of CD8 and CD57 in OSCC and OPSCC intratumoral specimens was analyzed considering the amount of immunopositive cells. This evaluation was made twice at different times by a single examiner blinded to the patient diagnosis. Cells were counted under light microscopy in 5 random histologic fields, with a magnification of × 400, adapted from Reichert et al. (2002).

Digital images were loaded on IMAGE J (National Institutes of Health, Bethesda, MD) to count the number of immunostained cells, and the percentage of positive cells was classified according as follows: less than 50% positive immunostaining cells and ≥50% positive immunostaining cells. The patterns of NK+ and CD8+ TILs locations in OSCC and OPSCC specimens were analysed in stroma.

It was evaluated the relationship of CD8+ and CD57 + cells with tumor size, lymph node metastasis (LNM) and clinical staging (CS) by chi-squared test. Overall survival (OS) and disease-free survival (DFS) were analyzed by Kaplan–Meier method. The level of statistical significance was accepted at $P < 0.05$.

3. Results

Forty-two patients with OSCC and 12 with OPSCC were studied. The main clinical features of our series are summarized in Table 1.

Table 1
Main clinical findings n (%) of patients with OCSCC (n = 42) and OSCC (n = 12).

Parameter		SCC		Total	P[a]
		OSCC	OPSCC		
Age	≤60 years	20 (37.0%)	8 (14.8%)	28 (51.9%)	0.332
	>60 years	22 (40.7%)	4 (7.4%)	26 (48.1%)	
Gender	Females	15 (27.8%)	0 (0.0%)	15 (27.8%)	0.015
	Males	27 (50.0%)	12 (22.2%)	39 (72.2%)	
T stage	T1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.098
	T2	2 (3.7%)	3 (5.6%)	5 (9.3%)	
	T3	7 (13.0%)	2 (3.7%)	9 (16.7%)	
	T4	33 (61.1%)	7 (13.0%)	40 (74.1%)	
N stage	N0	20 (37.0%)	5 (9.3%)	25 (46.3%)	0.750
	N1	8 (14.8%)	2 (3.7%)	10 (18.5%)	
	N2	8 (14.8%)	4 (7.4%)	12 (22.2%)	
	N3	6 (11.1%)	1 (1.9%)	7 (13.0%)	
Metastasis	M0	42 (77.8%)	12 (22.2%)	54 (100.0%)	–
	M1	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Clinical staging	II – III	8 (14.8%)	2 (3.7%)	10 (18.5%)	0.851
	IV	34 (63.0%)	10 (18.5%)	44 (81.5%)	
Recidive	Absent	7 (12.9%)	7 (12.9%)	14 (25.8%)	0.007
	Present	35 (64.8%)	5 (9.2%)	40 (74.2%)	
Clinical outcome	Dead	34 (63.0%)	5 (9.2%)	39 (72.2%)	0.007
	Alive	8 (14.8%)	7 (12.9%)	15 (27.8%)	

[a] χ^2.

The clinical profile was classic: male patients with over 60 years in the majority. Most cases were T4 stage (74.1%), with LNM (53.7%), clinical staging IV (81.5%), with presence of recidive of the lesion (74.2%).

Immunohistochemical expression of CD57 and CD8 was evaluated. NK and CD8 positivity was observed as membranous staining. Positive NK and CD8 cells were sparse and scattered within mononuclear inflammatory infiltration in the tumor stroma (Fig. 1). All cases studied were positive for immunohistochemistry. CD8+ and NK + cells were diffusely distributed throughout the tumoral stroma and infiltrating tumor parenchyma (Fig. 1a–d). Data regarding frequency of distribution of immune expression of CD8- and NK-positive cells according to clinical parameters are presented in Table 2.

In addition, the log-rank test showed no difference in overall and disease-free survival between high (≥50% positive cells) and low (<50% positive cells) CD8+ (Fig. 2a,b) and NK + cells ($P > 0.05$) (Fig. 2c,d).

Although we observed a lower probability of local recurrence and death in the group of patients with more than 50% of NK + cells, we did not find a statistically significant difference.

4. Discussion

Squamous cell carcinoma is the most common malignancy of head and neck mucosa and the second most common skin cancer. In spite of the progress in the development of newer and more effective therapeutic alternatives, individual response to treatment remains an intriguing aspect in cancer patients (Chen et al., 2017). Therefore, one could infer that some unique features of the patient or tumor type would serve as predictors of treatment response.

Considering that T and NK lymphocytes play a major role in protecting the human body against cancer, we set out to investigate tumor response to radiotherapy and chemotherapy in OSCC and OPSCC cases based on a quantitative histological assessment of CD8+ and NK cells.

In the present work, CD8+ T cells and NK cells were identified by means of immunohistochemistry in all cases, but with clear variations in number. Patients with CD8+ cells indices lower than 50% showed larger tumors (T3 and T4), nodal metastasis and advanced clinical staging (IV) ($P < 0.05$). Pages et al. (2005) demonstrated that higher T cell density in the infiltrate with a high proportion of CD8+ T cells was associated with a significantly lower rate of tumor recurrence in patients with primary colorectal adenocarcinoma. Maleki et al. (2011) assessed CD8 immunoexpression in 40 cases of OSCC and noted that cases with lower CD8 expression associated significantly with more advanced clinical staging. Conversely, dos Santos Pereira et al. (2014) observed higher median CD8 expression in OSCC patients without nodal metastasis, while da Silveira et al. (2010) noted a trend toward lower CD8 expression in OSCC cases with nodal metastasis. Surprisingly, Zancope et al. (2010) found no difference in CD8 expression between OSCC cases with and without nodal metastasis.

Taken together, the scientific information presently available suggests that inflammatory cell infiltration in the tumor microenvironment may inhibit tumor growth. In this context, CD8+ cells represent an important sub-population of cytotoxic T lymphocytes that recognize tumor antigens bound to class I histocompatibility molecules (MHC). Class I MHC molecules carry protein peptides produced intracellularly and take them to the cell surface, thereby allowing specific recognition by T cells and by CD8 + cells' surface receptors. Once MHC class I molecules disappear in neoplastic cells, cells of the innate immune response and NK cells may play an important role in antitumor immunity (Zancope et al., 2010). Based on their results showing the presence of CD8 + and NK cells in tongue and lip SCC, Zancope et al. (2010) proposed that a tumor microenvironment densely populated by these cells may offer a better immune cytotoxic response against cancer cells. An improved cytotoxic response would then translate into a trend

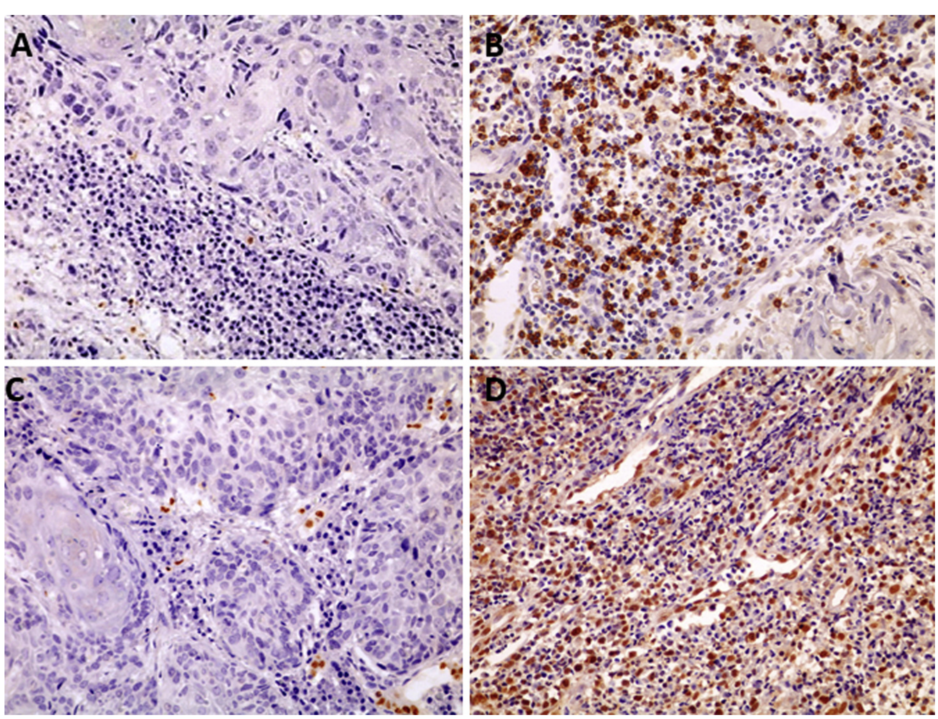

Fig. 1. Expressions of TCD8 and CD57 in OSCC tissues detected by immunohistochemistry. (a) SCC with <50% TCD8 positive cells (b) SCC with ≥50% TCD8 positive cells. (c) SCC with <50% NK (CD57) positive cells (d) SCC with ≥50% NK (CD57) positive cells.

Table 2
Frequency of distribution of immune expression of CD8-and NK-positive cells according to clinical parameters.

Parameter		CD8		P[a]	NK		P[a]
		<50%	≥50%		<50%	≥50%	
T stage	T1 and T2	1 (1.9%)	4 (7.40%)	0.010	2 (3.7%)	3 (5.6%)	0.702
	T3 and T4	37 (68.5%)	12 (22.2%)		24 (44.4%)	25 (46.3%)	
LNM	Absent	12 (22.2%)	14 (25.9%)	0.010	11 (20.4%)	15 (27.8%)	0.408
	Present	24 (44.4%)	4 (7.4%)		15 (27.8%)	13 (24.1%)	
Clinical staging	II – III	3 (5.6%)	7 (13.0%)	0.002	5 (9.3%)	5 (9.3%)	0.897
	IV	35 (64.8%)	9 (16.7%)		21 (38.9%)	23 (42.6%)	

LNM, Lymph Node Metastasis.
[a] χ^2.

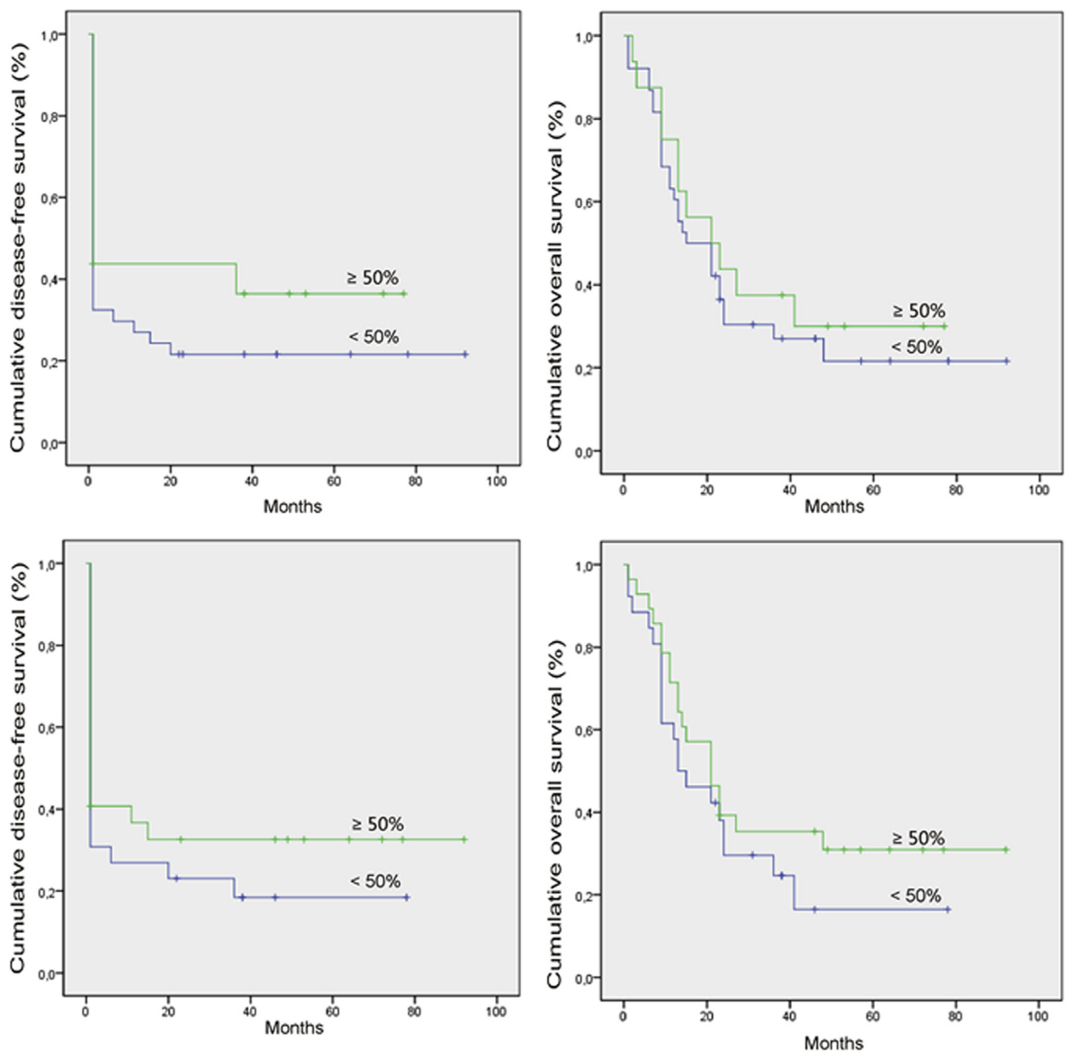

Fig. 2. (a) Kaplan–Meier survival curves, according to the immnuexpression of CD8+ cells. ($P = 0.2$) (b) Kaplan–Meier survival curves, according to the immnuexpression of CD8+ cells. ($P = 0.6$) (c) Kaplan–Meier survival curves, according to the immnuexpression of NK cells. ($P = 0.3$) (d) Kaplan–Meier survival curves, according to the immnuexpression of NK cells. ($P = 0.3$).

toward longer survival, an opinion that is also shared by Costa et al. (2010).

An intact immune system is critical to control carcinogenesis, in order for this to happen, T-lymphocytes must be able to infiltrate the tumor and mount appropriate responses (Lanitis et al., 2017) and higher numbers of CD3+, CD8+ and FOXP3+ tumor infiltrating lymphocytes are associated with a favorable outcome in several malignancies, including HNSCC (de Ruiter et al., 2017; Forster and Devlin, 2018). Regulatory T-cells are known to actively suppress immune responses and as such their presence within the tumor microenvironment may assist immune evasion in SCC. However, further understanding is required as although their presence is linked to a decreased survival in several tumor types (Forster and Devlin, 2018).

Eckert et al. reported that the tumor microenvironment was associated with an increased immune escape of the factors that cause immune suppression in OSCC (Eckert et al., 2016). Feng et al. (2017) evaluated simultaneously T cell frequency and assessing suppressive elements and defects in antigen-processing machinery. The authors observed that high numbers of CD8 T cells at the invasive margin correlated significantly with prolonged overall survival (OS), while the number of FoxP3 or PD-L1 cells did not. Compiling the number of FoxP3 or PD-L1 cells within 30 μm of CD8 T cells identified a significant association with a high number of suppressive elements close to CD8 T cells and reduced OS (Feng et al., 2017).

At least in our patient base, CD8 marking had no impact on the overall survival curve. In our point of view, the peritumoral inflammatory infiltrate influences local response and relapse rate first, and only after a significant impact on these aspects will it affect overall survival. Consistently, Watanabe et al. (2010) found an association between lower CD8 expression and shorter survival. Likewise, Zancope et al. (2010) proposed that some features of the peritumoral inflammatory infiltrate such as the presence of CD8+ and NK cells are associated with better prognosis and survival. That proposal is supported by the work of Liu et al. (2011) who analyzed the presence of CD8 + cells in the inflammatory infiltrate of breast cancer samples and reached the conclusion that the presence of these cells was an independent prognostic factor both for local tumor control and for overall survival.

The absence of a clear relation between the immunoexpression of CD8+ cells and survival might be related to cancer cells escaping from the immunological barriers. In an attempt to elucidate the differences in antitumor immune responses between primary and metastatic head and neck SCCs, Kumagai et al. (2010) assessed MRNA levels of CD markers and cytokines such as CD4, CD8, Interleukin-2, -4, -5, and interferon gamma. CD8 levels were similar in metastatic and non-metastatic tumors, as was the profile of Th1 cytokines. On the other hand, the clonality of T cell receptors (TCR) differed between primary and metastatic tumors. Thus, the authors suggested that antitumor immune response changes during metastasis and that tumor cells may escape the cytotoxic immune response mediated by CD8 + cells.

There are works proposing that the presence of inflammatory infiltrate cells is a negative prognostic factor for tumor development. One example is the report of Grabenbauer et al. (2006), who assessed CD3+, Granzyme B+ and CD8+ cells in rectal SCC patients. This was the only study identified in our literature review that associated a worse prognosis with cases showing an exuberant presence of inflammatory cells in the tumor environment. We believe that sample size ($n = 38$) may have influenced the study's outcomes.

Regarding CD57 immunohistochemical staining, there was a trend toward lower recurrence and longer survival in groups with large populations of NK cells, although not statistically significant. Clinical response to treatment was similar in both groups. The study by Agarwal et al. (2016) assessed the correlation of the expression of CD57 with 3 years survival in patients with OSCC. This study revealed that there was a significant correlation of CD57 labeling index with the status of life. There are similar studies conducted on OSCC, who also documented that increase in tumor infiltration by CD57 + lymphocytes results in a better prognosis (Fang et al., 2017; Lopes et al., 2017).

Yasuda et al. (2011) investigated a possible link between the density of CD8 and CD4 in advanced rectal tumor samples and the response to radiotherapy and neoadjuvant chemotherapy. An association between the presence of those cells in the tumor nest and a better response to preoperative treatment was established and, according to the authors, this was the first study to demonstrate a relationship between tumor radiosensitivity and lymphocytic inflammatory infiltrate in rectal tumors. Apparently, direct cytotoxic activity against tumor cells and indirect action via cytokine production could be the mechanism through which the beneficial result takes place (Xu et al., 2016).

Sridharan et al. (2016) prospectively collected longitudinal samples from head and neck squamous cell carcinoma patients receiving definitive radiation therapy. In this study, treatment not only increased circulating CD-8+ T-effector cells, but also myeloid-derived suppressor cells, regulatory T cells, and checkpoint receptor-expressing T cells, particularly PD-1+ T cells. The results suggested that fractionated chemoradiation leads to quantifiable effects in circulating immune mediators, including a balance of stimulatory and suppressive mechanisms. These results suggest future combinations with immune checkpoint blockade.

5. Conclusion

These findings suggest that the differential CD8+ cells infiltration in OPSCC and OSCC might reflect a distinctive tumor microenvironment with a favorable local cytotoxic immune response against neoplastic cells. The search for markers that influence or predict prognosis should continue through further research. Hopefully, science will get to the point of developing drugs that target antineoplastic immune response cells in a controlled manner and end up creating an inhospitable microenvironment for the development of squamous cell carcinoma.

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Conflicts of interest

All authors have declared no potential conflicts of interest.

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