Immunohistochemical analysis of FoxP3+ regulatory T cells in lower lip squamous cell carcinomas

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Abstract: The aim of this study was to determine the number of FoxP3+ regulatory T (Treg) cells in the microenvironment of lower lip squamous cell carcinomas (LLSCCs) and to correlate the findings with clinicopathological parameters (tumor size/extent, regional lymph node metastasis, clinical stage, and histopathological grade of malignancy). Fifty cases of LLSCC were selected. Lymphocytes exhibiting nuclear immunostaining for FoxP3 were quantified in 10 microscopic fields at the deep invasive front of LLSCCs. The results were analyzed statistically using the nonparametric Mann-Whitney test and Fisher's exact test. FoxP3+ lymphocytes were observed in all cases studied. The number of these cells tended to be higher in smaller tumors, tumors without regional lymph node metastasis, and tumors in early clinical stages, but the difference was not statistically significant (p > 0.05). Low-grade tumors contained a larger number of FoxP3+ lymphocytes than high-grade tumors (p = 0.019). Tumors with an intense inflammatory infiltrate exhibited a larger number of Treg cells (p = 0.035). On the other hand, the number of FoxP3+ lymphocytes was smaller in tumors arranged in small cell clusters (p = 0.003). No significant differences in the number of FoxP3+ lymphocytes were observed according to the degree of keratinization (p = 0.525) or nuclear pleomorphism (p = 0.343). The results suggest the participation of Treg cells in immune and inflammatory responses in the microenvironment of LLSCCs. These cells may play a more important role in early stages rather than in advanced stages of lip carcinogenesis.

Keywords: Carcinoma, Squamous Cell; Lip; T-Lymphocytes, Regulatory; Immunohistochemistry.

Introduction

Lower lip squamous cell carcinoma (LLSCC) is one of the most common malignant head and neck tumors, accounting for up to 66.5% of cases of oral cancer.[1] Although located in an anatomical region that favors an early diagnosis, an important number of LLSCC cases are identified in advanced stages when regional lymph node metastases are already present.[2] In these cases, the 5-year survival can decrease from 80% to only 25%, indicating a poor prognosis.[3,4] Despite the relatively high frequency of LLSCC and the important impact of regional lymph node metastasis on

the prognosis of this tumor, various features related to its progression remain incompletely understood.

Studies have shown that the interactions between neoplastic cells and cells of the immune system can modulate tumor progression and development.[5,6] In this process, neoplastic cells recruit different cell types that are able to suppress the immune response in the tumor microenvironment, particularly regulatory T (Treg) cells.[5] Treg cells are derived from the lymphocyte lineage that constitutively expresses CD4, CD25, and the nuclear transcription factor – forkhead box P3 (FoxP3). The latter factor, which is essential for the differentiation of T lymphocytes into Treg cells and their immunosuppressive functions,[6,7] has been indicated as an excellent marker for the identification of these cell types.[6,8]

Treg cells can suppress a variety of cells of the immune system through the release of inhibitory cytokines, cytolysis, modulation of antigen-presenting cell function, and metabolic interruption, including B lymphocytes, Natural Killer cells, CD4^{+} and CD8^{+} T lymphocytes, monocytes, and dendritic cells.[9] The production of inhibitory cytokines such as interleukin 10 (IL-10), IL-35, and transforming growth factor beta (TGF-β) is one of the main mechanisms of immunosuppression used by Treg cells.[10]

Several studies have associated a larger number of Treg cells in the tumor microenvironment with a reduced antitumor immune response and consequent poor prognosis.[6,11] In squamous cell carcinoma (SCC) of the oral cavity, a larger number of Treg cells have been associated with the presence of regional lymph node metastasis,[12] advanced clinical stage,[12] and low overall survival[13] and disease-free survival rates.[8] By contrast, other studies demonstrated an association between a larger number of Treg cells and a better prognosis for SCC of the oral cavity.[14,15] Studies investigating Treg cells in LLSCC are sparse[16,17] and do not permit categorically establishing the role of this T cell subpopulation in the progression of this cancer.

Therefore, the objective of this study was to evaluate the number of Treg cells in the microenvironment of LLSCC by immunohistochemistry and to correlate the findings with clinical and pathological parameters (tumor size/extent, regional lymph node metastasis, clinical stage, and histopathological grade of malignancy) in order to gain insight into the mechanisms underlying the progression of LLSCC.

Methodology

Fifty cases of LLSCC obtained from the archives of the Napoleão Laureano Hospital, João Pessoa, State of Paraíba, Brazil, were selected for this study. Data regarding patient gender and age, tumor size/extent, presence of regional lymph node metastases, distant metastases, and clinical stage (TNM) were collected from medical records. The parameters listed in the sixth edition of the TNM Classification of Malignant Tumors were used for clinical staging.[18] Only cases of LLSCC derived from surgical resections, with paraffin blocks containing sufficient material for histopathological and immunohistochemical analyses, were included in the sample. Tumors of patients who had been submitted to radiation therapy or chemotherapy, cases of recurrent tumors, and cases with incomplete clinical data were excluded. The study was approved by the local ethics committee (Protocol No. 631.357).

Histopathological analysis

For morphological analysis, 5-μm thick sections were obtained from paraffin-embedded biological material. These sections were mounted on glass slides and submitted to routine hematoxylin-eosin staining. Under a light microscope (Leica DM 500, Leica Microsystems Vertrieb GmbH, Wetzlar, Germany), two previously trained examiners histologically graded the specimens at the tumor invasive front according to the system proposed by Bryne et al.[19] This system consists of attributing scores to the following parameters: degree of keratinization, pattern of invasion, nuclear pleomorphism, and inflammatory infiltrate. The scores are summed to obtain the final score of malignancy of each case. According to Silveira et al.,[20] tumors with a final score ≤ 8 were classified as low grade and tumors with a final score ≥ 9 were classified as high grade.

Immunohistochemistry

Sections (3 μm) were cut from formalin-fixed and paraffin-embedded material and mounted on glass slides prepared with organosilane adhesive. The tissue sections

were deparaffinized and immersed in 3% hydrogen peroxide for blockade of endogenous peroxidase. The sections were washed in phosphate-buffered saline (PBS) and submitted to antigen retrieval in citrate solution, pH 6.0, in a steamer. The sections were then incubated with the primary anti-FoxP3 antibody (Ref. Ab22510, Abcam, Cambridge, UK; dilution 1:150) in a moist chamber for 60 min. After two washes in PBS, the sections were treated with a polymer complex (ADVANCE™ HRP, Dako, Carpinteria, USA) at room temperature. Peroxidase activity was visualized by immersing the sections in diaminobenzidine (Liquid DAB+, Dako, Carpinteria, USA), which results in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and mounted with a coverslip.

Tonsil specimens were used as positive control. Sections in which the primary antibody was replaced with 1% bovine serum albumin in buffer served as negative control.

Immunohistochemical analysis

Two previously trained examiners, who were unaware of the clinical and pathological data of the cases, performed the immunohistochemical analysis under a light microscope (Leica DM 500, Leica Microsystems Vertrieb GmbH, Wetzlar, Germany). Treg cells were analyzed quantitatively using a method adapted from Schwarz et al.[21] First, at 100X magnification, 10 areas of highest anti-FoxP3 immunoreactivity were selected along the tumor invasive front. At 400X magnification, each area was photographed (ICC 50HD, Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) and the images obtained were transferred to the ImageJ® program (Image Processing and Analysis in Java, National Institute of Mental Health, Bethesda, USA). Lymphocytes exhibiting nuclear immunostaining for FoxP3 were then counted in each photographed field. The values obtained per field were summed and the total number of FoxP3+ lymphocytes was determined. This number was used to calculate the mean number of Treg cells per case.

Statistical analysis

The results were analyzed statistically using the Statistical Package for the Social Sciences (version 17.0; SPSS, Inc., Chicago, USA). The distribution of FoxP3+ lymphocyte counts was evaluated using the Kolmogorov-Smirnov test, which indicated absence of a normal distribution. Therefore, the nonparametric Mann-Whitney test was used to compare median Treg cell numbers according to the different clinical and pathological parameters analyzed. Possible associations between the histopathological grade of malignancy of the tumors and the clinical parameters were evaluated using Fisher's exact test. A level of significance of 5% ($p < 0.05$) was adopted for all statistical tests.

Results

Clinical and morphological analyses

Thirty-eight (76%) out of the 50 LLSCC cases studied were diagnosed in men, with a male-to-female ratio of 3.2:1. Patient age ranged from 26 to 86 years, with a mean of 62.1 years. With respect to tumor size/extent and regional lymph node metastasis, there was a higher frequency of cases classified as T2 (n = 28; 56%) and N0 (n = 25; 50%). Distant metastases were not detected in any of the 50 cases studied. The most frequent clinical stages were stage III (n = 19; 38%) and stage II (n = 15; 30%) (Table).

Regarding histopathological grade, high-grade tumors predominated in the sample (n = 29; 58%) (Table). Most LLSCCs without regional lymph node metastasis (n = 17; 81%) were classified as low grade ($p < 0.001$). On the other hand, most patients in stages III and IV (n = 22; 84.6%) had high-grade tumors ($p < 0.001$).

Immunohistochemical analysis

FoxP3+ lymphocytes were detected along the tumor invasive front in all LLSCC cases evaluated (Figures 1A and 1B), with mean numbers ranging from 0.6 to 53.2 (median: 12.45). With respect to tumor size/extent, the median number of FoxP3+ lymphocytes was 14.7 (range: 2.1 – 53.2) in tumors classified as T1 and 10.8 (range: 0.6 – 37.9) in tumors classified as T2-T4 (Figure 2), with no significant difference between groups ($p = 0.244$). Regarding regional lymph node metastasis, the median number of FoxP3+ lymphocytes was 7.4 (range: 0.6–30.8) in LLSCCs with metastases and 15.2 (range: 0.8–53.2) in tumors without metastases (Figure 2). The nonparametric Mann-Whitney test revealed no statistically significant difference between groups ($p = 0.079$).

Table. Distribution of cases of lower lip squamous cell carcinoma according to clinicopathological parameters.

Clinicopathological parameters	n (%)
Gender	
Male	38 (76.0)
Female	12 (24.0)
Tumor	
T1	15 (30.0)
T2	28 (56.0)
T3	4 (8.0)
T4	3 (6.0)
Node	
N0	25 (50.0)
N1	20 (40.0)
N2	5 (10.0)
N3	0 (0.0)
Metastasis	
M0	50 (100.0)
M1	0 (0.0)
Clinical stage	
Stage I	9 (18.0)
Stage II	15 (30.0)
Stage III	19 (38.0)
Stage IV	7 (14.0)
Histopathological grade	
Low-grade	21 (42.0)
High-grade	29 (58.0)

Analysis of FoxP3+ lymphocytes according to the clinical stage of LLSCC showed a median number of 15.05 (range: 0.8–53.2) in early stages (I/ II) and of 7.9 (range: 0.6–37.9) in advanced stages (III/ IV) (Figure 2). This difference was not statistically significant (nonparametric Mann-Whitney test, p = 0.187). The median number of FoxP3+ lymphocytes was 16.0 (range: 0.6–53.2) in low-grade LLSCCs and 6.6 (range: 0.8–37.9) in high-grade tumors (Figure 2). This difference was statistically significant (p = 0.019) between groups.

Figure 3 illustrates the number of FoxP3+ lymphocytes according to the individual parameters of the histopathological grading system. No significant differences in the median numbers of Treg cells were observed according to the degree of keratinization (p = 0.525) or degree of nuclear pleomorphism (p = 0.343) of the tumors. Regarding the pattern of invasion, LLSCCs with clearly defined infiltrative borders or those arranged in cords, bands, or solid trabeculae exhibited a larger median number of FoxP3+ lymphocytes than tumors arranged in small cell clusters (p = 0.003). Furthermore, LLSCCs containing an intense inflammatory infiltrate exhibited a larger median number of Treg cells than tumors with moderate or absent inflammatory infiltrate (p = 0.035).

Discussion

The interactions between tumor cells and cells of the immune system can influence cancer progression.[22]
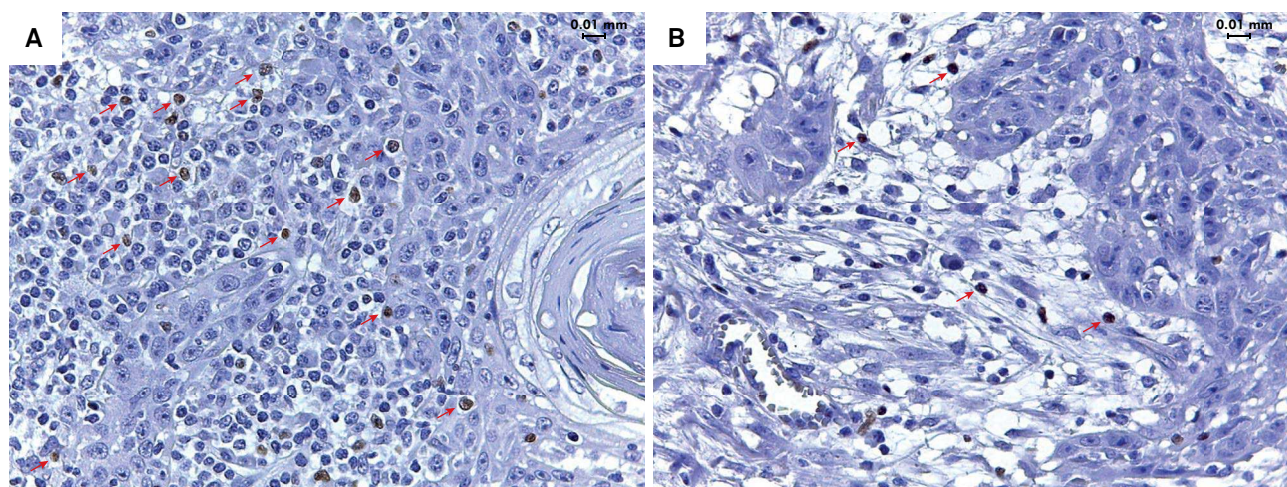

Figure 1. A) FoxP3+ lymphocytes (arrows) at the invasive front of low-grade LLSCC exhibiting marked inflammatory infiltrate (Advance, original magnification 400X). B) FoxP3+ lymphocytes (arrows) at the invasive front of high-grade LLSCC exhibiting slight inflammatory infiltrate (Advance, original magnification 400X).
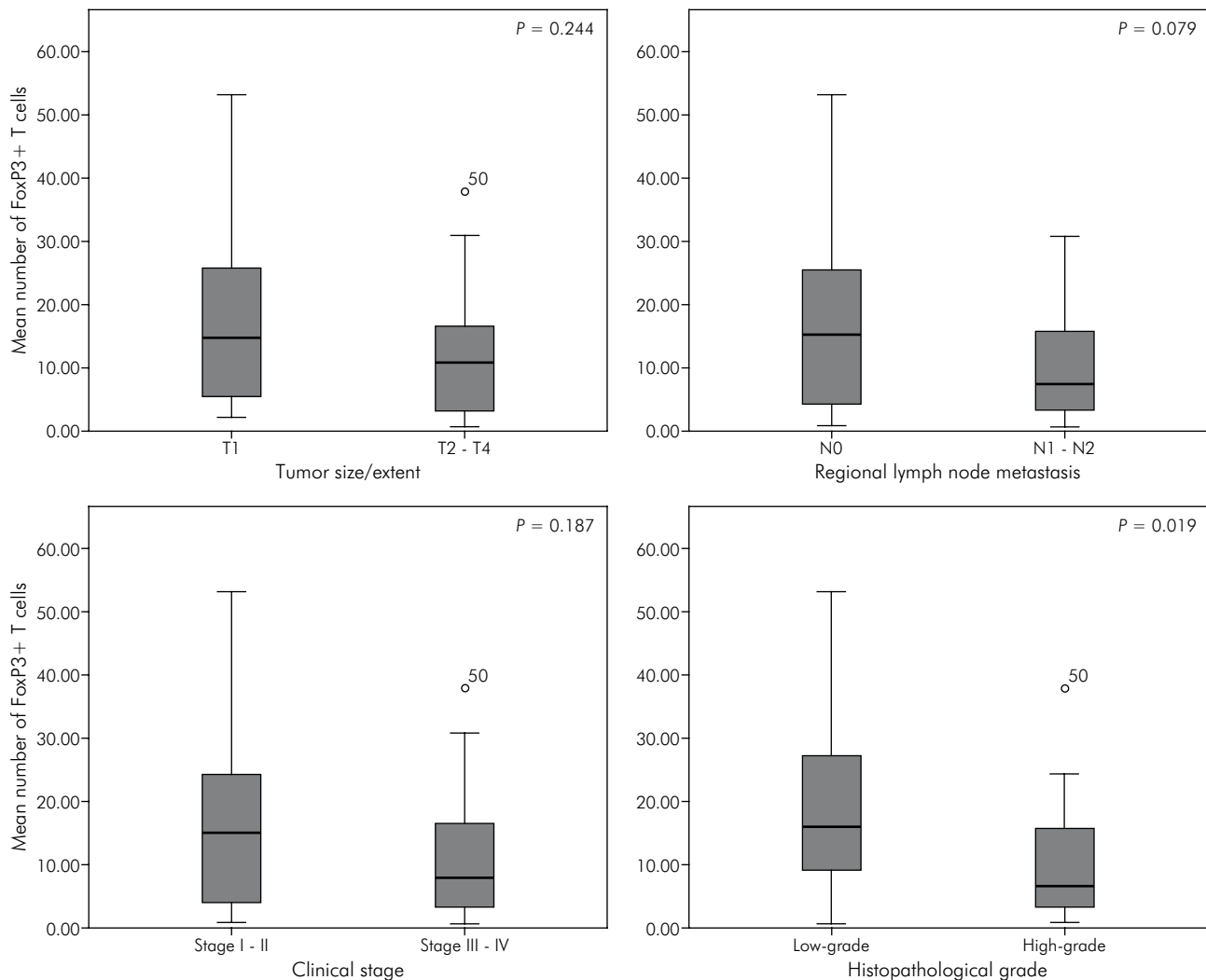
Figure 2. Box plot charts illustrating the number of FoxP3+ lymphocytes in LLSCCs according to tumor size, regional lymph node metastasis, clinical stage, and histopathological grade of malignancy.

In this respect, depending on the profile of immune cells and secreted chemical mediators, the tumor-associated inflammatory infiltrate can exert an antitumor or protumor effect.[13,15] Therefore, research into the cellular and molecular features of the immune response to tumors has intensified in recent years in an attempt to obtain a better understanding of the process of tumor progression, to identify more reliable prognostic factors, and to eventually define potential targets for immunotherapeutic strategies.[6,13,22,23]

Within the context of immune responses to tumors, studies have highlighted the participation of Treg cells, which can suppress the functions of different immune system cells.[5,9] In this respect, an increase in the number of Treg cells in the tumor microenvironment has been

associated with a poorer prognosis of head and neck carcinomas, including SCC of the oral cavity.[8,11,13] On the other hand, there are studies reporting a better prognosis of oral SCCs that contain a larger number of Treg cells.[14,15] A PubMed search for LLSCC revealed only two studies on Treg cells.[16,17] One of these studies included a relatively small sample of LLSCCs (n = 6)[17] and the other one did not associate the number of these cells with clinical and pathological findings.[16] Thus, the role of Treg cells in the progression of LLSCC remains poorly understood.

FoxP3+ lymphocytes were observed in all LLSCC cases studied. Taken together with previous studies investigating Treg cells in these tumors,[16,17] these findings corroborate the presence of this T cell subpopulation in the microenvironment of LLSCC. In the present study,
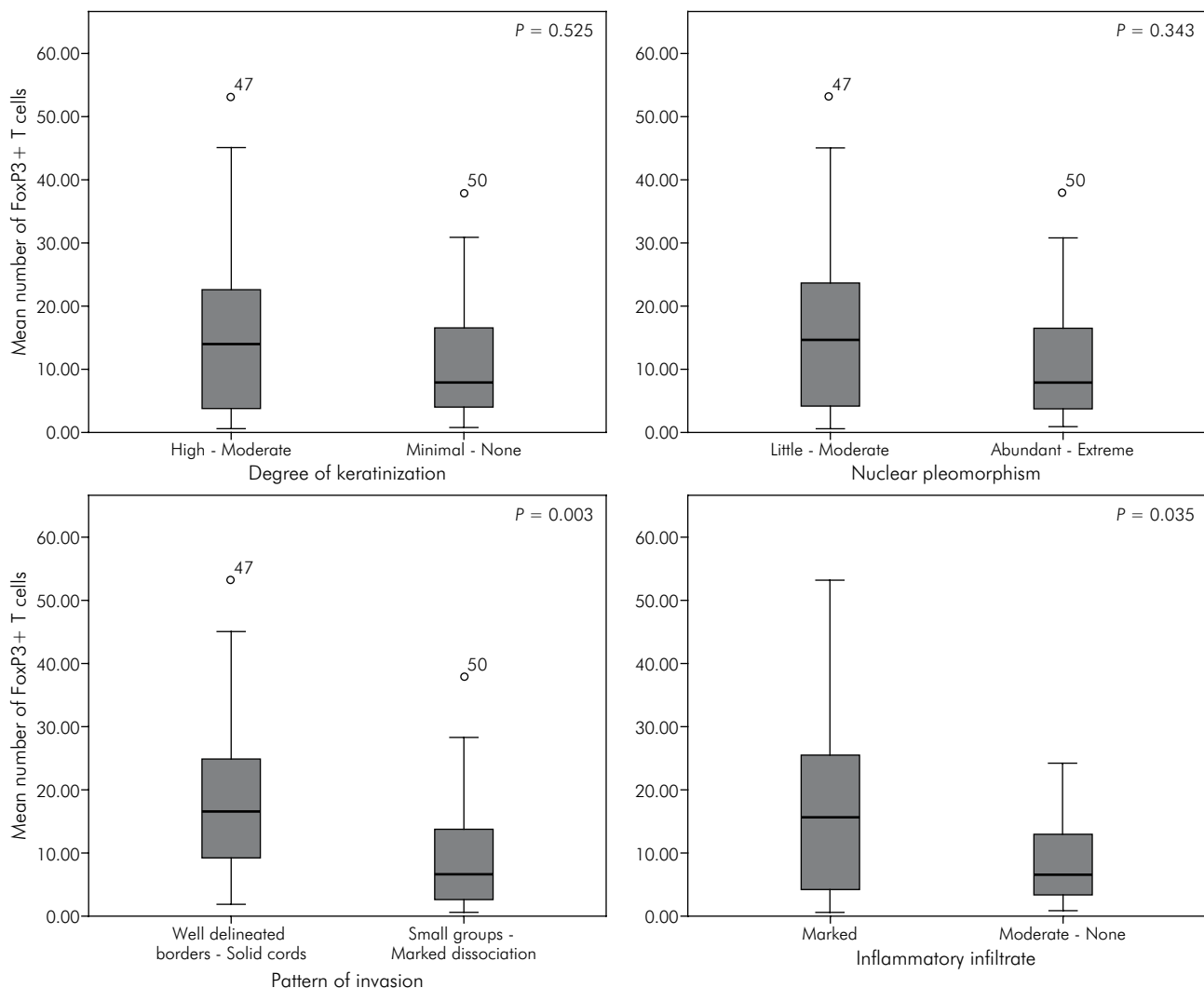
Figure 3. Box plot charts illustrating the number of FoxP3+ lymphocytes in LLSCCs according to degree of keratinization, nuclear pleomorphism, pattern of invasion, and inflammatory infiltrate.

despite the lack of statistical significance, the number of FoxP3+ lymphocytes tended to be higher in smaller tumors, tumors without regional lymph node metastasis, and early-stage tumors (p > 0.05). In agreement with these results, studies on SCC of the oral cavity demonstrated a larger number of Treg cells in the microenvironment of tumors of smaller size/extent[15] and in the peripheral blood of early-stage patients.[14] On the other hand, Gaur et al.[12] observed a larger number of Treg cells in the peripheral blood of patients with oral SCC who had regional lymph node metastases and were in advanced clinical stages.

Regarding histopathological features, a larger number of FoxP3+ lymphocytes were observed in low-grade LLSCCs (p = 0.019). Considering individual parameters, the identification of a larger number of Treg

cells in tumors with an intense inflammatory infiltrate deserves attention (p = 0.035). These tumors represented a higher proportion in the group of early-stage LLSCCs (data not shown). Although they used the World Health Organization grading system, Al-Qahtani et al.[22] also found a positive correlation between the intensity of inflammatory infiltration and the number of Treg cells in the microenvironment of SCCs of the oral cavity.

Taken together, the results of this study suggest an important role of Treg cells in the microenvironment of LLSCCs. The immunosuppressive activity of this subpopulation of T cells may be particularly necessary in the early stages of lip carcinogenesis, in which neoplastic cells are possibly more immunogenic. As observed in this study, early-stage LLSCCs are low-grade tumors

(p < 0.001) usually permeated by an intense inflammatory infiltrate. Therefore, neoplastic cells recruit a larger number of Treg cells to the tumor microenvironment in order to escape the antitumor immune response. In more advanced stages of lip carcinogenesis, neoplastic cells would have a decreased capacity to stimulate immune responses, with a consequent reduction in the number of Treg cells in the tumor microenvironment.

The results of a study investigating actinic cheilitis lesions, which are potentially malignant lesions that can progress to LLSCC, have shown that the participation of Treg cells is an early event in lip carcinogenesis. Using flow cytometry and proliferation assays, Gasparoto et al.[24] demonstrated the accumulation of functional Treg cells (CD4+CD25+FoxP3+) in actinic cheilitis. Furthermore, enzyme-linked immunosorbent assays (ELISA) revealed higher expression levels of IL-10 and TGF-β and lower levels of interferon gamma (IFN-γ) in actinic cheilitis when compared to the control group (healthy gingival tissue). According to Gasparoto et al.,[24] the accumulation of functional Treg cells in actinic cheilitis may inhibit the proliferation of T cells and produce a microenvironment with high levels of immunosuppressive cytokines, events that contribute to the persistence, recurrence, progression, and malignant transformation of these lesions.

In SCC of the oral cavity, a larger number of Treg cells in the tumor microenvironment have been associated with a poor prognosis, characterized by low overall survival[13] and low disease-free survival rates.[8] By contrast, other studies showed that larger numbers of Treg cells, both in the peripheral blood of patients[14] and in the tumor environment,[15] are associated with a better prognosis of oral SCCs. Similarly, studies on gastric adenocarcinomas[25] and head and neck carcinomas[7,26] demonstrated a relationship between a larger number of Treg cells in the tumor microenvironment and higher patient survival rates. In an attempt to explain these apparently contradictory findings, studies have suggested that Treg cells reduce the protumor effects of chronic inflammation, such as the release of angiogenic factors, mitogens, and proteases[5] which, in turn, stimulate tumor progression.[14]

As suggested earlier, in some cases, Treg cells may take part in predominant antitumor activities in the microenvironment of oral SCCs and LLSCCs. Evaluation of this possible scenario in these tumors requires the analysis of different types of cells of the immune system found in the tumor microenvironment, as well as of the secretion profile of chemical mediators. In this respect, it cannot be ruled out that other cells, such as M2 macrophages and myeloid-derived suppressor cells,[5] are the main cells responsible for suppression of the immune response in the microenvironment of advanced-stage oral SCCs and LLSCCs. Also within this context, studies suggest that changes in the balance of subpopulations of immune system cells are more important for the modulation of antitumor immunity than quantitative alterations in a single cell type.[12,13]

Conclusion

The results of this study suggest the participation of Treg cells in immune and inflammatory responses in the microenvironment of LLSCCs. The role of these cells may be more important in early stages than in advanced stages of lip carcinogenesis.

Acknowledgments

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