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CD4+ CD25+ regulatory T-cells role in tumor microenvironment of the squamous cell carcinoma

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Abstract

Introduction: Squamous cell carcinoma (SCC) is the most common skin cancer with a high rate of death. Different lymphocyte populations play an important role in modulating the immune response in the tumor microenvironment. The increase in the proportion of cluster of differentiation (CD)4+ CD25+ regulatory T-cell (Treg) lymphocytes is associated, in different studies, with the increase of the cell multiplication rate. **Aim:** To analyze the Treg lymphocyte subpopulations and to correlate the results with the presence of the CD8+ cytotoxic T-cell (Tc) lymphocyte population. **Materials and Methods:** Sixty primary skin SCC specimens were incubated with anti-CD8 (clone SP57) rabbit monoclonal antibody and anti-CD25 (clone 4C9) mouse monoclonal antibody. **Results:** The ratio of the intratumoral/peritumoral CD4+ CD25+ forkhead box protein p3 (Foxp3) lymphocytes was 0.46, emphasizing that at tumor margins, where tumor aggressiveness is higher, these lymphocytes subpopulations facilitate tumor progression. The comparative analysis of the tumor microenvironment profile revealed that in the case of intratumoral immune response, the number of Tc-type lymphocytes (CD8+) was 3.34 times higher compared to Treg lymphocytes ($p < 0.001$). In the peritumoral area, the number of Tc lymphocytes was 5.05 times higher compared to Treg lymphocytes ($p < 0.001$). **Conclusions:** Treg lymphocytes inhibition may cause the suppression of the antitumoral cell immune response in the tumor environment. We believe that Treg lymphocytes should represent a focus of interest for a new personalized therapy. New studies are needed to better understand the immune response in the tumor microenvironment.

Keywords: CD4+, CD25+, microenvironment, squamous cell carcinoma, tumor progression, therapy response.

Introduction

Squamous cell carcinomas (SCCs) represent one of the most frequent human solid tumors, also being reported as a major cause of mortality related to cancer [1]. Cutaneous SCC is the second most common skin cancer in Caucasians after basal cell carcinoma, usually occurring on sun-exposed/damaged areas of the body (head, neck, upper limbs) [2]. These tumors are extremely heterogeneous. Their origin is in the cells of the squamous layer of the epidermis being closely related to its ability to multiply and differentiate. The tumor cells can also originate from simple or pseudostratified epithelia through alteration or blockage of the cellular apoptosis mechanism [3].

Different lymphocyte populations play an important role in modulating the immune response in the tumor microenvironment. These lymphocytes are believed to hold double function, either inhibiting or promoting tumor growth and progression [4].

Some of the known reasons that what lead to this is the role played by the patient's immune system: a mechanism of altered antigen recognition or decreased ability to activate the host histocompatibility complex. Immunosuppression mediated by regulatory T-cells (Tregs) among other immune

cells is directly or indirectly involved in decreasing the protective immune response [5, 6].

The depletion of cluster of differentiation (CD)4+ CD25+ Tregs has been reported by several studies, resulting in a slower tumor growth rate [7]. The presence of secondary tumors, high tumor grade and advanced pathological tumor–node–metastasis (TNM) staging were correlated with the level of Tregs [8].

Recent studies consider that forkhead box protein p3 (Foxp3) expression could become a sensitive and specific marker of Tregs [9]. The Foxp3 belongs to forkhead family of transcription factors. The transcription factors are involved in the function of CD25+ Tregs control. Foxp3 expression in patients with malignant tumors is associated to a worse prognosis of the disease [10, 11].

Aim

The aim of our study was to randomly select resected specimens of patients with SCC of the skin and analyze the triggered immune response (CD4+ CD25+ Foxp3+). Furthermore, the current study tried to describe the features of the Treg lymphocyte subpopulations, focusing on the identification of possible correlations with the presence of the CD8+ cytotoxic T-cell (Tc) lymphocyte population.

Materials and Methods

The total number of cases included randomly in our study was 60 primary skin SCCs locally treated at the Marghita Municipal Hospital, Bihor County, Romania (between January 2015 and December 2016). All the cases included in the study belong to upper limbs, hand, and neck. Pathology specimens were processed in the Department of Pathology, Municipal Clinical Hospital, Oradea, Romania. The immunohistochemical tests were performed in the same laboratory using an automated method.

Tissue specimens underwent formalin fixation (neutral buffered 10% formalin) at 24 hours and 72 hours and were embedded in paraffin according to standard procedures. Immunohistochemistry analysis was performed on 4 μm -thick sections on a Ventana BenchMark GX (Ventana Medical Systems, Inc., Tucson, AZ, USA) with automated staining according to the manufacturer's instructions. Slides had the paraffin removed using EZprep solution (Ventana Medical Systems, Inc., Tucson, AZ, USA) at 90°C, and all reagents and incubation times were performed as recommended on the antibodies package inserts. Slides were developed using the OptiView DAB (3,3'-Diaminobenzidine) detection kit (Ventana Medical Systems, Inc., Tucson, AZ, USA). The counterstained was done with Hematoxylin and Blue-Dye staining [12–14].

The sections were incubated with anti-CD8 (clone SP57) rabbit monoclonal antibody, ready-to-use (RTU) and anti-CD25 (clone 4C9) mouse monoclonal antibody, RTU. For each case, positive control slides were used (with skin specimens obtained from healthy skin specimens from the same patients). Negative control was undertaken by omitting the primary antibody on the same section type. We determined the values of Treg lymphocytes (CD4+ CD25+ Foxp3+) from intra-tumoral and peritumoral areas and further on, compared these values to the ones obtained from normal skin specimens of the control group. The specimens were analyzed by two skilled pathologists and were double blinded. We used Leica DM3000 led microscope with intelligent automation and LAS EZ software (provided by Leica Biosystem) for capture of images and measurements.

Statistical analysis was performed using the Microsoft® Office Excel 2017 software with the χ^2 (*chi*-squared) test utilized for statistical relevance. The results, expressed *via* mean \pm standard deviation (SD), were statistically significant at a *p*-value less than 0.05.

The study was approved by the Ethics Committee of the Faculty of Medicine and Pharmacy, University of Oradea.

Results

According to our data, within the tumor bed, the number of CD4+ CD25+ Foxp3 lymphocytes was smaller compared to the area near the tumor where the cytotoxic activity was much higher (3650 *vs.* 7884). The mean values of the two areas studied were 104 \pm 83 [95% confidence interval (CI): 26–243] CD4+ CD25+ Foxp3 intratumoral lymphocytes *vs.* 225 \pm 159 (95% CI: 66–1008) peritumoral lymphocytes ($p < 0.001$, *chi*-squared test). The intratumoral/peritumoral CD4+ CD25+ Foxp3 lymphocytes ratio was 0.4.

In the distribution cases curve graph, on the one hand can notice higher variations in the number of CD4+ CD25+ Foxp3 lymphocytes in the peritumoral area, and on the other hand a small variation in the intratumoral area. Some

extreme cases can also be observed, where the number of Treg lymphocytes was abnormally high (Figure 1). Furthermore, the number of lymphocytes expressing the Foxp3 transcription factor (Treg lymphocytes) varies both intratumorally and peritumorally, as per Figures 2 and 3 (low number of Treg lymphocytes with an additional important lymphocytic presence). Figure 4 shows solid nests of SCC with a rich and diffuse stromal lymphocytic infiltrate. CD4+ CD25+ Foxp3+ lymphocytes population described in Figure 4 was lower in comparison to denser lymphocytic infiltrate depicted in Figure 5.

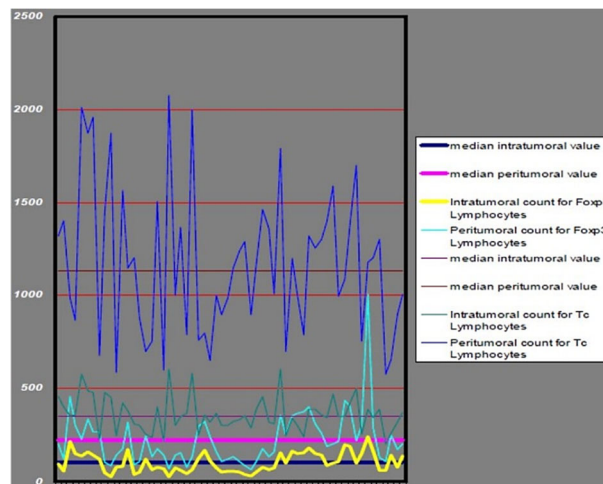


Figure 1 – The distribution cases curve graph of the immune profile. Foxp3: Forkhead box protein p3; Tc: Cytotoxic T-cell.

The comparative analysis of the tumor microenvironment profile reveals that in the case of intratumoral immune response, the number of Tc-type lymphocytes (CD8+) was 3.34 times higher compared to Treg lymphocytes ($p < 0001$, *chi*-squared test). In the peritumoral area, the number of Tc lymphocytes was 5.05 times higher compared to Treg lymphocytes ($p < 0001$, *chi*-squared test) (Figure 6).

The present study did not reveal any statistical correlation between the immune response in the tumor area and the degree of histopathological (HP) differentiation of SCC.

Discussions

To our best knowledge, this is the first study in a Romanian cohort of patients analyzing the impact of CD4+ CD25+ lymphocytes in tumor progression in the setting of SCC. Numerous studies have been performed to better understand the role of the cell-mediated immune response mediated by T-type lymphocytes *via* tumor-progression inhibition or promotion. Many theories, not all accepted, state that cancer cells escape immune surveillance mechanisms [15].

In the meta-analysis performed by Shang *et al.*, the conclusion is that irrespective of the immune response a Treg lymphocytes-population will be encountered, regardless of whether we refer to the inflammatory response associated with the tumors or not. The efficacy of the immune response does not depend on the presence of a lymphocyte population subtype but on the proportions of these types of immune cells [16]. In colon and rectal carcinomas, in the case of an increased CD8/Treg ratio, the overall mortality ratio is reduced by 70%. One explanation could be the inclusion of Cyclophosphamide

in the chemotherapeutic treatment of these patients. Certain reports claim that Cyclophosphamide reduces the number

of Tregs, thus encouraging the antitumoral immune response [17].

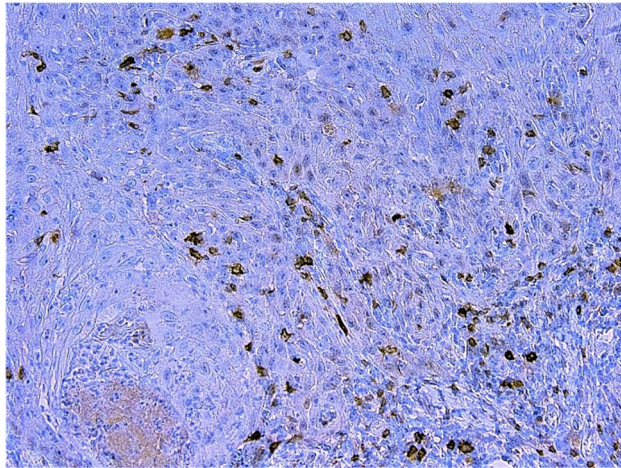


Figure 2 – Low number of Treg lymphocytes (brown) in the tumor bed of squamous cell carcinoma (100×). Treg: Regulatory T-cell.

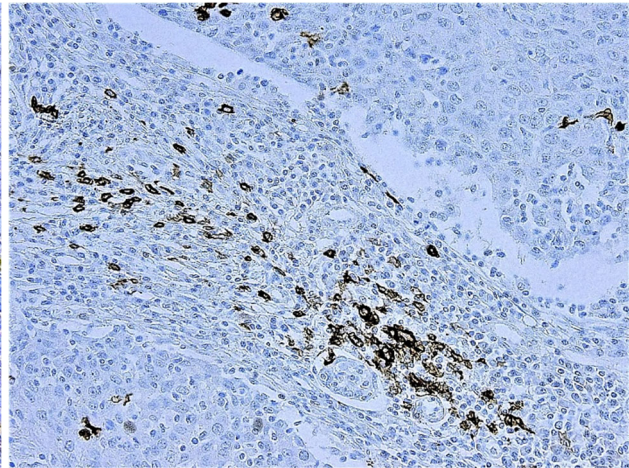


Figure 3 – Islands of squamous cell carcinoma that have at the periphery a limited number of Treg lymphocytes (brown) compared to other subclasses of lymphocytes (100×). Treg: Regulatory T-cell.

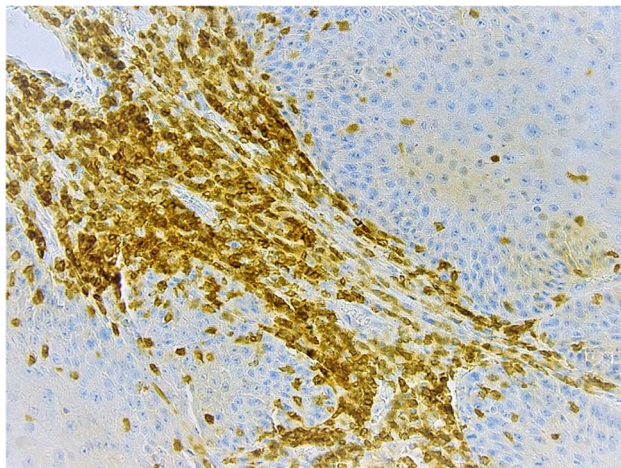


Figure 4 – Two islands of squamous cell carcinoma including diffuse infiltration of Treg lymphocytes (100×). Treg: Regulatory T-cell.

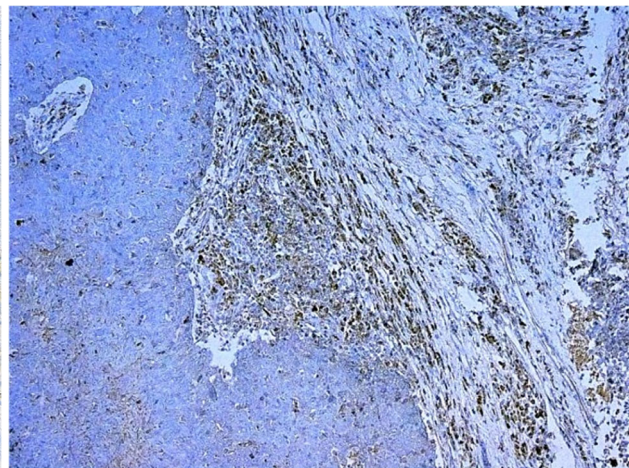


Figure 5 – On the right side, there is a tumor bed and at the level of the contact interface, there is a high number of Treg lymphocytes (brown) (100×). Treg: Regulatory T-cell.

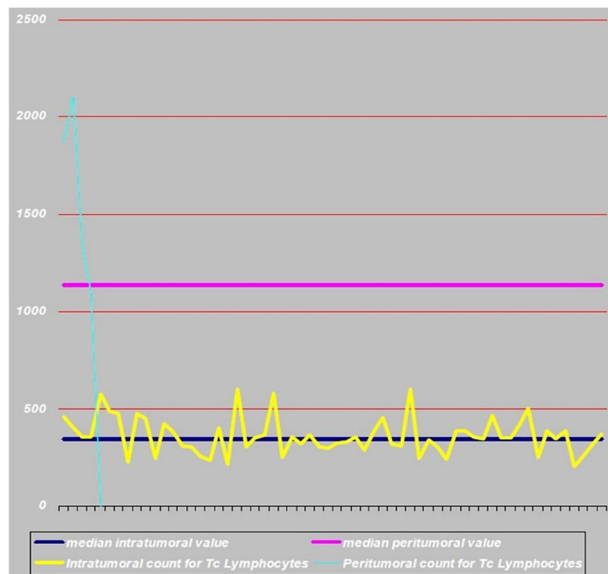


Figure 6 – The comparative analysis of the tumor microenvironment immune profile. Tc: Cytotoxic T-cell.

There is a general acceptance for the theory that CD4+ T-helper type 1 (Th1) and CD8+ T-type lymphocyte-mediated immune response has a tumor progression inhibition function, and that lymphocytes belonging to the CD4+ Foxp3+ Treg population favor tumor development and progression, by releasing numerous cytokines involved in local immunosuppression. The ratio between CD8+ and Treg, as well as a higher ratio of CD8+ to CD4+ (in favor of the cytotoxic lymphocytes) is associated with prolonged survival. This data could be explained by the antagonistic action between Treg and CD8+ T-cells, when facing the tumor microclimate [18]. Our results are in the line with the upper statement and show lymphocyte subpopulations (CD4+ CD25+ Foxp3) could facilitate tumor progression at tumor margins, where tumor aggressiveness is considered higher.

A similar conclusion was stipulated by Jordanova *et al.*, regarding cervical cancer: the CD8+/Treg ratio is the only predictive factor in this type of cancer. Another interesting finding of the study is that there are an increased number of intraepithelially CD8+ Tc-lymphocytes in cervical

carcinoma patients presenting with loss of expression of human leukocyte antigen (HLA), compared to patients who have only low HLA expression. The hypothesis is that CD8⁺ activation can also be achieved alternatively, *via* a non-classical pathway, in the absence of HLA at the tumor site [19].

The association between an increased number of CD8⁺ T-lymphocytes and an increased overall survival rate was also found in other studies regarding ovarian carcinoma, colorectal carcinoma, endometrial carcinoma, or malignant melanoma [20–22]. For these malignancies, the analysis of the Treg lymphocytes (CD4⁺ CD25⁺ Foxp3) number has been proposed to be used as a predictive factor for recurrence. When analyzing the intratumoral area, the number of lymphocytes expressing a modified *Foxp3* gene (Treg), present a sinusoidal distribution, with small variations, compared to the median values calculated from the entire specimens. In the intratumoral area are seeing much higher variations in the distribution case curve. In cervical carcinoma, a low CD4⁺/Treg ratio is associated with higher metastatic rates in the lymph nodes [23].

Treg lymphocytes inhibit the activation of both types of CD4⁺ and CD8⁺ lymphocytes and may lead to the suppression of the antitumoral cell immune response in the tumor microenvironment. Based on the hypothesis that an increase of the immunoregulatory function of these cells can trigger an increased immune and inflammatory response, we can consider that Tregs can help prevent and/or delay inflammation correlated to tumor progression.

The ratio between the number of CD8⁺/CD4⁺ CD25⁺ Foxp3 lymphocytes in the two areas (included in our study) showed a 35% increase in the value of the ratio between the two lymphocyte subpopulations in favor of the peritumoral area. Our hypothesis from the data obtained in the study is that the mechanism of tumor progression would be at the level of the intratumoral area. In an article published by Sinicrope *et al.*, an increased number of intratumoral Treg lymphocytes is associated with a higher tumor HP grading. The conclusion of the study is that a low CD3⁺/Treg ratio is associated with a poor prognosis in colorectal cancer [18].

The analysis of our data shows the importance of the presence of a higher number of Treg lymphocytes (CD4⁺ CD25⁺ Foxp3) in the intratumoral area compared with the cytotoxic cellular immune response (CD8⁺ lymphocytes). In the peritumoral area, CD8⁺ lymphocytes number was higher in comparison to CD4⁺ CD25⁺ Foxp3 (39780 *vs.* 7884). In our study, when comparing the normal distribution of the cellularity of lymphocytes found in the pathology specimens, we have noticed a significant discrepancy between the number of CD8⁺ and CD4⁺ CD25⁺ Foxp3 lymphocytes in the peritumoral area. Variations in the distribution curve of CD8⁺ lymphocytes were very large, with high oscillations between the maximum and minimum values. Except for a limited number of specimens, where there was a certain parallelism, in most of the remaining specimens, we have noticed that these two curves are reversely proportionate. Similar results have been reported concerning a low CD8⁺/CD25⁺ ratio being associated with a poorer prognosis for patients with ovarian carcinoma or hepatocarcinoma [24–26]. Other studies have shown that in hepatocarcinoma and malignant lymphoma there

is an inversely proportional correlation between the number of CD8⁺ and CD25⁺ lymphocytes [27].

When analyzing the immune response in an experimental model of sarcoma in mice, the number of Treg lymphocytes, in absolute value, or the number of T-effector lymphocytes, in absolute value, was not relevant, but rather the ratio between these two lymphocyte subpopulations could inhibit tumor cellularity [27–30].

☒ Conclusions

It is to our best belief that Treg lymphocytes could further represent a focus of interest for novel targeted therapies, also for cutaneous SCC. New studies are needed to better understand the immune response in the tumor microenvironment, in order implement this hypothesis in the clinical setting.

Conflict of interests

The authors declare that they have no conflict of interests. All authors read and approved the final manuscript.

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