CD25+ T cell depletion impairs murine squamous cell carcinoma development via modulation of antitumor immune responses

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ABSTRACT

Squamous cell carcinoma (SCC) constitutes a microenvironment that could modulate the antitumor immune response. Also, tumor-infiltrating lymphocytes are believed to play complex regulatory roles in antitumor immunity against SCC. The presence of regulatory T cells (Tregs) has been associated with the suppression of tumor-reactive T cells. However, the underlying mechanism for this T cell dysfunction is not clear. We used a multistage model of SCC to examine the role of Treg cells during tumor development. 7,12-dimethylbenz[a]-anthracene/phorbol 12-myristate 13-acetate treatment and systemic depletion of Treg cells using an anti-CD25 monoclonal antibody (PC61) resulted in a decrease in the number and incidence of papilloma. Furthermore, CD25 depletion increased the proportion of CD8+ and CD4+ T cells that were isolated from tumor lesions. The levels of interleukin (IL)-1β, IL-10, IL-12, IL-13, interferon-γ, transforming growth factor-β and tumor necrosis factor-α, but not IL-17, were increased in the tumor microenvironment after Treg depletion. Therefore, our results indicated involvement of CD25+ T cells in SCC development and in the suppression of the inflammatory immune response.

Introduction

Squamous cell carcinoma (SCC) is one of the most common human cancers worldwide (1–3). Despite considerable advances in the understanding of the pathophysiology of SCC, therapeutic options are still limited. The development of this tumor is accompanied by an immune response, which leads to massive infiltration of inflammatory cells into the tumor environment, leading to local and systemic production of cytokines, chemokines and other mediators, such as nitric oxide (1–3).

Despite a vigorous response, the immune system is not capable of completely controlling tumor progression. This lack of control could be explained by an altered immune regulatory mechanism that may involve CD4+ CD25+ regulatory T cells (Tregs). Studies have shown a significantly elevated frequency of regulatory CD4+ CD25+ T cells in the peripheral blood of patients with head and neck SCC (1,2,4). Tregs constitute 5–10% of peripheral CD4+ T cells in mice and humans and are essential for maintaining homeostasis and peripheral tolerance to prevent autoimmune diseases and to limit chronic inflammatory processes. The main phenotype markers for this T-cell population are CD25, the transcription factor Foxp3, CD103, glucocorticoid-induced tumor necrosis factor receptor and the co-receptor CTLA-4 (5,6). To become suppressive (i.e., regulatory), CD4+ CD25+ T cells require activation through the T-cell receptor, and the suppressor activity of Tregs is to be primarily dependent on contact or also induced by soluble factors, such as transforming growth factor (TGF)-β and interleukin (IL)-10 (5). Although regulatory Foxp3+ cells had been shown to perform important function in the homeostasis maintenance (5,6), the depletion of Treg cells constitute a way to protect against human cancer, providing better specific immune response (7–9).

However, there have not been reports addressing the direct involvement of Treg cells in tumor progression in a model of murine SCC that mimics the characteristics of human SCC.

In this study, we investigated the involvement of CD4+ CD25+ T cells in murine SCC using monoclonal antibodies that are known to inhibit Treg function by blocking CD25 activity (10). Our results indicate that treatment with anti-CD25 resulted in decreased papilloma incidence and was associated with a significant increase in cytokine levels [IL-1β, IL-10, IL-12, IL-13, interferon (IFN)-γ, TGF-β and tumor necrosis factor (TNF-α)] and in the number of CD4+ and CD8+ T cells-infiltrating tumor lesions. These findings reveal a direct role of Tregs during SCC development and open new perspectives in the understanding about the importance of these cells in the modulation of the immune response in humans.

Materials and methods

Mice

Female Balb/c mice that were 6–8 weeks old were purchased from the Bauru School of Dentistry, University of São Paulo. Each mouse was housed in an isolated cage. Food and water were provided ad libitum. The mice were maintained on a 12 h light/12 h dark photocycle in a controlled temperature environment and were quarantined for a minimum of 1 week before the beginning of any treatment. Groups of mice were randomly sacrificed in 2 week intervals until 16 weeks after 7,12-dimethylbenz[a]-anthracene (DMBA) application. All animal experiments were approved by the Animal Research Ethics Committee of the Bauru School of Dentistry, University of São Paulo.

DMBA/phorbol 12-myristate 13-acetate-induced skin carcinogenesis

Eight-week-old mice (three groups of five mice each) had their backs shaved and were topically treated with a single dose of DMBA (25 μg in 200 μl of aceton) and biweekly doses of phorbol 12-myristate 13-acetate (PMA) (200 μl of a 10–4 M solution in aceton) for 16 weeks. The tumor incidence was recorded every other week through visual inspection. Papillomas were characterized by folded epidermal hyperplasia protruding from the skin surface, and carcinomas were usually endophytic tumors presenting as plaques with an ulcerated surface. Care of experimental animals was in accordance with institutional guidelines. Survival rates were determined in independent groups of animals. Tumors were collected at different time-points after initiation and were processed as described below.

Antibodies and treatment

An anti-CD25 (PC61) hybridoma was grown intraperitoneally in mineral oil-injected nude mice. The antibodies were purified from ascites by precipitation using ammonium sulfate (45% wt/vol) and were subsequently purified using a G protein column (Amersham Biosciences, Piscataway, NJ). Protein quantification was determined by the biinchoninic method. The blockage of CD25 was performed by intraperitoneal injection of 500 μg/ml mouse purified mAb every 30 days after the DMBA application. Controls received 500 μg/ml of normal rat IgG diluted in phosphate-buffered saline.

Isolation of leukocytes

To characterize the leukocytes present in the tumor site, biopsies of skin lesions from mice were collected and incubated for 1 h at 37°C in RPMI 1640 medium containing 50 μg/ml of a collagenease CI enzyme blend (Boehringer Ingelheim Chemicals). The tissues were subsequently dissociated for 4 min in the presence of RPMI 1640 medium with 10% serum and 0.05% DNase (Sigma–Aldrich) using a Medimachine (BD Biosciences), according to the manufacturer’s instructions. The tissue homogenates were filtered using a 30 μm cell strainer (Falcon; BD Biosciences). The viability of the leukocytes

Abbreviations: DMBA, 7,12-dimethylbenz[a]-anthracene; IFN, interferon; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; SCC, squamous cell carcinoma; TNF, tumor necrosis factor.
was evaluated by Trypan blue exclusion, and these cells were used for cell activation and immuno labeling assays (11).

Antibodies (Abs) and flow cytometry analysis
For immunostaining, PerCP-, phycoerythrin- and fluorescein isothiocyanate-conjugated Abs against CD3 (17A2), CD4 (H129.19), CD8 (RPA-T8 and HIT8a), CD14 (rmC5-3), CD19 (ID3) and CD25 (PC61) and the respective goat and rat isotype controls were used (BD Biosciences). The intracellular detection of Foxp3 (3G3) and TGF-β (BD Biosciences) in leukocytes obtained from lesions and lymph nodes was performed using Cytofix/Cytoperm and Perm/Wash buffer from BD Biosciences according to the manufacturer’s instructions. Briefly, the cells were labeled with Abs to the cell surface, such as fluorescein isothiocyanate anti-CD25 and PerCP-conjugated anti-CD4. Following the staining of surface markers, the cells were fixed, permeabilized and stained with phycoerythrin-labeled anti-mouse Foxp3 (MACS Miltenyl Biotech), phycoerythrin-labeled anti-mouse TGF-β or the isotype control (12). Samples were acquired on a FACSort flow cytometer, and the data were analyzed using CellQuest software (BD Biosciences).

Histological analysis
Tissue samples were collected from tumor sites and fixed with 10% (vol/vol) formalin for 6 h at room temperature. The tissues were subsequently dehydrated in ethyl alcohol, followed by waxes in xylol and were embedded in paraffin. Each sample was sectioned into slices 5–7 µm thick that were dried onto slides and stained with hematoxylin and eosin.

Immunofluorescence analysis and confocal microscopy
Slides for double immunofluorescence staining were post fixed with 4% paraformaldehyde and blocked with protein-block assay diluent (BD Company). After being washed in phosphate-buffered saline, the slides were incubated with the primary antibody, washed again and incubated with the appropriate fluorochrome-conjugated (Texas Red or fluorescein isothiocyanate) secondary antibodies. After being washed, the slides were mounted using mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories) to stain the nucleus and were analyzed by confocal microscopy. Images were captured with a Leica TCS SPE confocal laser system equipped with a 63 oil immersion plan apochromatic objective (1.3 CS) with differential interference contrast. LAS AF 2.5.1 software was used for image acquisition.

Cytokine assays
The supernatants of tumor samples were obtained by disaggregation through treatment with RPMI 1640 medium containing 0.25% collagenase (Worthington) and were frozen at −80°C until analysis. The total protein concentration was measured using a Quick StartTM Bradford Protein assay kit (Bio-Rad). The total amount of cytokines was quantified in the samples using a quantitative sandwich enzyme-linked immunosorbent assay using commercial capture and biotinylated detection antibodies (BD Pharmingen Corp., San Diego, CA) and the respective recombinant mouse cytokines (diluted in phosphate-buffered saline) as standards, according to the manufacturer’s instructions. IL-13 and IL-17 levels were determined using an immunoassay kit according to the manufacturer’s instructions. The concentrations of each cytokine were measured as pg/ml, and the results were normalized and expressed as mg/protein.

Statistical analysis
The results are expressed as the mean ± SD, and statistical analysis was performed using analysis of variance to compare multiple groups or by the parametric Student’s t-test for the comparison of two groups (GraphPad software 4). Linear regression was done to compare incidence and average of lesions differences between untreated and treated groups. P < 0.05 was considered to indicate statistical significance.

Results
Characterization of CD4⁺ CD25⁺ T cells in lesions and lymph nodes of mice with SCC
We evaluated the percentage of CD4⁺ CD25⁺ T-cells in the skin and lymph nodes of mice with SCC and control mice without SCC (Figure 1A–D). The results revealed higher percentages of CD4⁺ CD25⁺ T-cells in the tumor-draining lymph nodes and tumors of SCC mice (Figure 1C). CD4⁺ CD25⁺ T-cell levels were elevated in the tumor-draining lymph nodes (5.6 ± 1 × 10⁵) and tumor tissues (1.7 ± 0.7 × 10⁵) of SCC mice in comparison with lymph nodes (0.34 ± 0.25 × 10⁵) and skin tissues (0.06 ± 0.01 × 10⁵) of control animals (Figure 1A and C). CD4⁺ CD25⁺ T cells in DMBA-induced tumor mice exhibited a phenotype compatible with Treg cells, as they expressed significantly more Foxp3 in both tumor-draining lymph nodes and lesions (Figure 1B and D). Furthermore, we found a higher percentage of CD25⁺ CD4⁺ T cells expressing TGF-β in the tumor and tumor-draining lymph nodes (Figure 1E and F). These results indicate that CD4⁺ CD25⁺ T cells expressing Treg markers were found in higher frequency in lymph nodes of SCC than controls, and importantly these cells were found in the tumor sites indicating that Tregs are modulating the immune response surrounding the tumor cells.

Effect of CD25 inhibition on the development of tumors in mice with SCC
To assess the role of CD25⁺ cells in multistage tumorigenesis, skin tumors were initiated in anti-CD25-treated and control littermate mice by treatment with DMBA and PMA. We found that the survival rate was nearly identical in both anti-CD25-treated mice and Rat-IgG control mice (data not shown). The number and rate of appearance (tumor incidence and initiation rate) of benign papillomas were delayed in anti-CD25-treated mice compared with Rat-IgG mice (Figure 2A–F). The tumor incidence also was lower in anti-CD25-treated mice (Figure 2B and D) compared with Rat-IgG mice (Figure 2A and C). Papillomas were found in 100% of Rat-IgG mice 7 weeks after carcinogenic induction (Figure 2E); papillomas were found after 10 weeks in anti-CD25-treated mice (P < 0.001; Figure 2E). Besides, the papilloma average was decreased in anti-CD25-treated mice compared with Rat-IgG mice (Figure 2F).

In agreement with these data, macroscopic analysis demonstrated that the treatment with anti-CD25 delayed the carcinogenic process because the number and rate of appearance of papillomas was significantly lower in anti-CD25-treated mice (Figure 2B and D). Histological analysis revealed a pronounced presence of inflammatory infiltrate and a lack of epithelial islet formation in anti-CD25-treated mice compared with control mice (8 weeks post-DMBA/PMA) (Figure 3A and B). Additionally, anti-CD25-treated mice showed a lower degree of tumor invasiveness with a slight area of tumor involvement over the same period of time (Figure 3A–D). Thus, our results indicated that the presence of CD25⁺ cells had crucial role in the SCC development.

Effect of CD25 inhibition on the modulation of the antitumor immune response in the tumor microenvironment
The most conclusive readout that allows measurement of the in vivo relevance of signaling by CD25 is the antitumor immune response. We therefore investigated the inflammatory infiltrate and the profile of cytokines in the tumor microenvironment. Our results showed that 49.6 ± 15.6 × 10⁵ leukocytes were present in the tumor samples of anti-CD25-treated mice and 10.3 ± 2.5 × 10⁵ in the tumor samples of control animals (Rat-IgG) (Figure 4A). Of these leukocytes, 84 ± 1.5% represented T cells (CD3⁺) in the tumor samples from anti-CD25-treated mice (data not shown). Of these T cells, 39.7 ± 1.9 × 10⁵ were CD4⁺ and 9.92 ± 1.45 × 10⁵ were CD8⁺ (Figure 4B). Control mice (Rat-IgG-treated) had significantly lower numbers of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells-infiltrating tumor (Figure 4B). Similar number of CD19⁺ B lymphocytes was isolated from the tumor sample of untreated and anti-CD25-treated mice (Figure 4B). Interestingly, CD14⁺ leukocytes were found at higher levels infiltrating tumor samples from control mice (Rat-IgG) (8.6 ± 1.3 × 10⁵) than from anti-CD25-treated mice (3.97 ± 0.96 × 10⁵) (Figure 4B). When analyzed CD4⁺ CD25⁺ Foxp3⁺ lymphocytes, we found significantly increased number in control than anti-CD25-treated mice (Figure 4B).

We then analyzed the phenotype of the leukocyte populations in the lymph nodes from anti-CD25-treated and control mice (Figure 4C). We found that the frequencies of B cells (CD19⁺) in anti-CD25-treated mice were similar to those detected in the control group (Figure 4C). Among gated CD3⁺ T cells, we found that lymph nodes from anti-CD25-treated mice had a higher number of CD8⁺ T cells (15.6 ± 4.7 × 10⁵) as well as CD4⁺ T cells (15.4 ± 1.6 × 10⁵) than the control group (5.85 ± 2.9 × 10⁵) (Figure 4C). We also analyzed the population of CD14⁺ cells and found significant
Fig. 1. SCC induced by DMBA/PMA generated an increase in the T regulatory population in mice. SCC mice were treated according to a chemical carcinogenic protocol using DMBA and PMA for 16 weeks. Tumor tissues and lymph nodes from SCC and healthy control mice were obtained, and the proportion of CD4^+CD25^+ T lymphocytes was determined using flow cytometry. (A) The absolute numbers of CD4^+CD25^+ T cells from lymph nodes and (B) tumor samples of SCC or skin from control mice were established. (A and B) Representative pictures of CD4^+CD25^+ and CD4^+CD25^- T cells isolated of tumor-draining lymph nodes and tumor samples, and percentages of Foxp3 expression in those populations were determined. (C and D) Immunofluorescence images revealed the presence of CD4^+CD25^+ and CD25^-TGF-β^+ cells in tumor samples. *P < 0.05 and **P < 0.01 (Student's t-test).
differences between the anti-CD25-treated mice (2.5 ± 0.4 $\times 10^7$) and the control group (11.75 ± 4.7 $\times 10^7$) (Figure 4C). Our data showed that CD4+CD25+Foxp3+ cells were at lower number in lymph nodes from anti-CD25-treated group (6.9 ± 0.6 $\times 10^7$) than untreated group (1.9 ± 0.14 $\times 10^7$) (Figure 4C). We confirmed such data by immunofluorescence analysis using anti-CD4 antibodies into tumor samples (Figure 4D). These data clearly indicate that anti-CD25 treatment induces changes in the tumor microenvironment of SCC mice increasing CD3+CD4+ and CD3+CD8+ T-infiltrating cells.

To determine if treatment with anti-CD25 could also impact the production of cytokines in the tumor microenvironment, we quantified the levels of IL-1β, IL-10, IL-12, IL-13, IFN-γ, TGF-β, TNF-α and IL-17 in tumor samples from anti-CD25-treated mice and control littermate mice (Figure 5). Our data clearly show that samples from anti-CD25-treated mice contained significantly elevated amounts of IL-1β, IL-10, IL-12, IL-13, IFN-γ, TGF-β and TNF-α compared with the samples from the control group (Rat-IgG-treated mice) (Figure 5). Conversely, IL-17 levels in lesions from anti-CD25-treated mice were lower than those in lesions from the control group (Figure 5F). These data indicate a balance among Th1, pro-inflammatory and suppressor cytokines in anti-CD25-treated lesions probably providing an efficient immune response against the tumors.

**Discussion**

The suppressor activities mediated by Tregs (i.e., cytokines and/or contact-dependent mechanisms) have been shown to be an important obstacle in successful antitumor immunotherapy (4–10,13,14). Additionally, several authors have shown a higher number of Tregs in peripheral blood and tumor tissue in mice and humans than in healthy individuals (4,15,16). Our results indicate that a significant number of CD4+CD25+ T cells expressing Foxp3 were found in the lymph nodes and tumors of SCC mice. Tumor-infiltrating lymphocytes were found to be CD4+CD25+Foxp3+ and CD4+CD25+TGF-β+, consistent with the results of previous studies (17,18).

It is probable that Tregs have a pivotal role in cancer immune surveillance because of their ability to suppress tumor-associated immunity and to promote immune escape by the tumor (19–21). Studies previously showed that treatment of mice with anti-CD25 antibodies, which are known to inhibit Treg functions (10,22), elicits an effective antitumor response, resulting in a decrease in tumor growth providing the in vivo antitumor activity (8,10,13). Although we did not verify differences in the survival rate or weight gain with the CD25 depletion in SCC mice, mAb treatment resulted in a significant reduction in the ratio and average number of papillomas. In addition, there was
a decrease in tumor invasiveness and an enhancement of leukocyte infiltration in lesion tissues. These results are consistent with those of previous studies showing that depletion of CD25<sup>+</sup> cells influences solid tumor development in several animal models (23,24).

Tregs can suppress the proliferation of T cells and they can also control the magnitude of the effector functions of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, such as IFN-𝛾 production and cytotoxic activity, particularly in the context of tumors (5,16,25,26). Using a chemically-induced tumor model, we observed that after anti-CD25 treatment, the total number of leukocytes was significantly increased. In particular, CD4<sup>+</sup> and CD8<sup>+</sup> tumor-infiltrating lymphocytes were increased in tumor tissues after CD25<sup>+</sup> cell depletion. The increased frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the tumor microenvironment could be a direct consequence of the control of tumor incidence observed in anti-CD25-treated mice in SCC models. Although we did not address the ability of Tregs to regulate cytotoxic T lymphocytes in vitro, our data suggest that Tregs control CD4<sup>+</sup> and CD8<sup>+</sup> T cell migration in vivo and is consistent with the hypothesis that Treg cells can also influence immune responses by modulating the recruitment and function of different cell types (26).

Our observation that anti-CD25 treatment decreases the number of CD14<sup>+</sup> cells (i.e. macrophages) in the tumor is consistent with the hypothesis that the depletion of Treg cells leads to a more effective antitumor immune response. Tumor-associated macrophages are recognized for having the potential both to elicit tumor and tissue destructive reactions and to promote tumor progression, thus promoting cancer progression and metastasis (27–30). The decreased frequency of macrophage cells in the tumor microenvironment could be a direct consequence of the control of tumor incidence observed in anti-CD25-treated mice. Thus, the presence of Treg cells in the tumor microenvironment may regulate the local immune response, leading to tumor development by inhibiting the migration and activity of CD8<sup>+</sup> T cells and enhancing macrophage migration to the tumor site in SCCs.

CD25 molecule is expressed on the cell surface of T lymphocytes and precursor of activated B lymphocytes (31) and, also, in a mature subpopulation of dendritic cells (32). Although the administration of CD25 antibodies could eliminate activated CD25<sup>+</sup> T lymphocytes, we verified that anti-CD25 blocking did not influence B cell frequency neither in lymph nodes nor in tumor samples. In contrast, we observed increase in the T-cell population in both tissues. In agreement with our results, other studies revealed that anti-CD25 method is a critical approach for Treg depletion (nor inactivation) in tumor microenvironment (reviewed in ref. 16), transplantation (33) and infection models (34). This protocol is sustainable because Treg cells, compared with activated T cells, show a higher expression of CD25 molecule that preferentially can be bound to anti-CD25 antibodies, leading Treg cells to death or anergy. More recently, authors described that T-reg depletion by PC61 antibodies (~70% of circulating Tregs) is mediated by phagocytes via fragment crystallizable receptors and also showed that non-deleted remaining cells presented CD25low or CD25neg expression (35). Here, we are not excluding that other cell expressing CD25 (non-Tregs) could also be deleted after PC61 treatment, however, after anti-CD25 antibodies treatment, we clearly also showed a significant decrease in frequency CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells, indicating a strongly evidence that Treg cells are the major target of anti-CD25 antibodies.

Higher levels of certain classes of cytokines in the tumor microenvironment from anti-CD25-treated mice might provide one explanation for the control of tumor occurrence. Although the role of TNF-𝛼 is controversial, this cytokine is essential for the activation and maturation of CD8<sup>+</sup> cytotoxic T lymphocyte effectors (36), suggesting that increased TNF-𝛼 level in the tumor microenvironment may direct an effective antitumor immune response. This study showed that...
CD25+ cell depletion resulted in a decrease in IL-17 production in the tumor microenvironment and correlated with less severe disease. Other studies have shown that IL-17 may facilitate the establishment and promote the progression of different types of tumors including SCC (37–42). The IL-17 pathway can have pro or antitumor effects in different settings. In ovarian carcinoma, CD4+ T cell-derived IL-17 can mediate the recruitment of myeloid cells into tumors and enhance tumor growth (27). However, other studies have indicated that IL-17 not only mediates the recruitment of tumor-associated macrophages but also enhances their protumoral properties through an IL-6–STAT3 circuit (27). The influence of CD25+ cells on regulating IL-17 levels remains to be elucidated. Additionally to lower levels of IL-17, we also detected increased levels of IL-13 in tumor lesions from anti-CD25-treated mice. IL-13 has been demonstrated to exert a potent antitumor activity in a mouse model of oral SCC (41). In this way, IL-17 might have a poor role in the protector immune response against SCC. In fact, such a thing has been recently verified (42).

In this study, we also detected significantly elevated production of IL-1β in tumor tissues after CD25 depletion. IL-1β is synthesized as an inactive precursor (pro-IL-1β) and then cleaved by the IL-1β-converting enzyme, caspase-1, into active IL-1β (43). Perhaps, IL-1β has a protector role in the murine SCC. However, this result remains to be elucidated. We also verified that both IFN-γ and IL-12 were dramatically increased in tumor lesions from anti-CD25-treated mice. IL-12 is known to have potent antitumor activity by inducing IFN-γ production and activating effector cells, such as CD8+ T cells and natural killer cells. This cascade leads to a protective immune response against cancer (44). The activation of these cytokines pathway may play a role in enhancing the antitumor immune response during the carcinogenic process in anti-CD25-treated mice in SCC tumor model. In addition, these data are consistent with those of previous studies showing an increase in the number of CD4+ T cells and the level of IL-2 secretion after Treg inactivation (45,46).

On the other hand, we found higher IL-10 and TGF-β levels in tumor lesions from anti-CD25-treated mice compared with control mice. Although elevated production of both those cytokines had been detected by Tregs purified from human oral SCC (4), our data in the present study reflect microenvironment of the tumor lesions after CD25 depletion, and those cytokines could be being secreted by several cell types other than Tregs, establishing cytokine balance and avoiding an exacerbated inflammation which facilitate tumor progression (47–49).

We conclude that anti-CD25 treatment was effective in positively to modulate the immune response in SCC tumor mice. Our present observations, in a mouse model, could be strongly correlated with a higher frequency of Treg cells in blood and tumor tissue in patients with oral SCC as described in our previous findings (4). Regarding the
prospective approaches of immunotherapy against tumor in patients, the mechanisms mediated by Treg cells in SCC need to be explored more fully. However, our results strongly suggest that the blockage of Treg impairs the progression of SCC tumors by modulating the immune response around the tumors and might be a potential target to be implemented in immunotherapeutic protocols where patients are routinely exposed to carcinogenic environment such as industrial settings. Moreover, the success of this treatment will depend on the efficacy of depletion (phase and dose) and consequently the microenvironment in which the response is made (9, 16). As mentioned above, these mechanisms need to be explored more fully, however the present study clearly shows that the presence of Treg cells have a critical role in modulating the antitumor immune responses and consequently the SCC development.

**Funding**


**Acknowledgements**

We are grateful to Prof. Denise Tostes Oliveira for help with histopathological analysis and to Bruno Alvares Viscelli for histopathological slides.

**Conflict of Interest Statement:** None declared.

**References**


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**Fig. 5.** Cytokine levels increased in the tumor microenvironment after CD25+ T depletion. Tumor-induced mice received peritoneal injections of anti-CD25 monoclonal antibody (500 μg/ml) every 30 days at 4 and 8 weeks post-DMBA. IL-10 (A), TGF-β (B), IL-12 (C), IFN-γ (D), IL-17 (F), TNF-α (G) and IL-11 (H) levels were analyzed in the supernatants from cultures as described in the Materials and methods. Results are expressed as the mean ± standard error of the mean from each individual mouse analyzed. *P* < 0.05 and **P** < 0.01 when compared with anti-CD25-treated and with Rat-IgG mice with SCC, respectively.


Received March 13, 2011; revised January 10, 2012; accepted February 12, 2012