PD-1 blockage delays murine squamous cell carcinoma development

Eduardo Bertoli Belai1, Carine Evelino de Oliveira2,3, Thais Helena Gasparoto, Rodrigo Nalio Ramos, Sergio Aparecido Torres, Gustavo Pompermaier Garlet, Karen Angélica Cavassani2, João Santana Silva2 and Ana Paula Campanelli*

Department of Biological Sciences and 1Department of Stomatology, Bauru School of Dentistry – University of São Paulo, Al. Octávio Pinheiro Brsolla, 9–75 – CEP, Bauru, São Paulo 17012–901, Brazil; 2Department of Pathology, Medical School, University of Michigan, Ann Arbor, MI 48109, USA and 3Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto – University of São Paulo, Ribeirão Preto, São Paulo 14049-900, Brazil

*To whom correspondence should be addressed. Tel: +55 14 3235 8271; Fax: +55 14 3235 8271; Email: apcampan@usp.br

Engagement of programmed death-1 (PD-1) with its two ligands [programmed death ligand-1 (PD-L1) and PD-L2] has been associated with the suppression of tumor-reactive T cells; however, the underlying mechanism for this T-cell dysfunction is not clear. We hypothesized that PD-1 and PD-L1 signals are, in part, responsible for squamous cell carcinoma (SCC) escape from immune antitumor regulation by modulation of the tumor environment. In the present study, we used a multistage model of SCC to examine the role of PD-1/PD-L1 activation during tumor development. Tumor sites presented an increased percentage of CD4+ and CD8+ T cells expressing PD-1 when compared with non-tumorigenic control mice, whereas the expression of PD-L1 was particularly increased in F4/80+ macrophages in tumor sites. Further, the systemic immune neutralization of PD-1 resulted in a decreased number and delayed incidence rate of papillomas followed by a differential expression of cytokeratins, suggesting that the PD-1–PD-L1 interaction contributes to the progression of SCC by downregulation of antitumor responses. In fact, blocking PD-1 increased the percentage of CD8+ and CD4+ T cells, and the levels of interferon-γ in the tumor sites. Our results indicated involvement of PD-1+ T cells in SCC development and in the modulation of the inflammatory immune response.

Introduction

Recent studies indicate that inflammation is a critical component of tumor progression, and is evident that the tumor microenvironment, which is orchestrated by inflammatory cells, plays a central role in immunologic response and tumor escape (1). In the presence of inflammation, regulatory T cells (Tregs) accumulate in tumor tissue and protect cancer cells from antitumor immunity (2); dendritic cells (DCs) induce tolerance rather than immunity (3); inhibitory signals, such as engagement of programmed death-1 (PD-1) with its two ligands [programmed death ligand-1 (PD-L1) and PD-L2], regulate both the effectiveness of tumor antigen cross-priming and the function of effector cells at tumor sites (4); tumor cells can express PD-L1 that inhibit T-cell activation (5,6). In this context, modulation of immune responses in the tumor microenvironment is a critical mechanism attributed to tumor evasion. Many studies were directed at the analyses of inhibitory signals, including the PD-L1/PD-1 pathway, in the function of immune effector cells and immune evasion by cancer cells (4–7).

PD-1 expression is induced by T-cell receptor signaling and is increased on activated CD4+ and CD8+ T cells (8,9). PD-1 activation limits the activity of T cells in the periphery during an inflammatory response (10). The ligands for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC) belong to the B7/CD28 family of ligands (11). Consistent with the inhibitory role of PD-1, PD-1-deficient mice develop a severe autoimmune disease (12–15), atherosclerosis (16), allograft vascular disease (17) and encephalomyelitis (18). Several groups have reported that engagement of PD-1 by PD-L1 or PD-L2 results in inhibition of T-cell proliferation or altered cytokine production (12,13,19).

Several lines of evidence also implicate the PD-1/PD-L1 pathway in T-cell exhaustion in cancer (20). PD-1 expression is found on tumor-infiltrating CD8+ T cells in multiple solid tumors (21–23), and PD-L1 blockade potentiates an antitumor immune response (21). Some human studies show that the presence of PD-L1-positive tumor cells is directly correlated with poorer prognosis and inversely correlated with CD8+ T cells infiltrating tumors (24–26). Our group recently demonstrated that PD-1 is detected in 20–40% of T cells in human tumor samples from patients with oral squamous cell carcinoma (SCC) (27). Although these reports have suggested a direct correlation between PD-1/PD-L1 and tumor escape, the involvement of these molecules in SCC has not been explored.

The development of SCC, one of the most common human cancers worldwide, is accompanied by a massive infiltration of mononuclear cells into the tumor, leading to local and systemic production of cytokines, chemokines and other immune mediators, including nitric oxide (28–30). In addition, progressive alterations in cytokeratins (CKs) expression are closely associated with the development of a variety of skin cancers, including SCC (31,32). CK1 and CK10 are expressed in well-differentiated SCC in association with keratinization (33–35). Notably, in poorly differentiated SCC or SCC areas, there is inappropriate expression of simple epithelial keratins CK8/CK18 and CK19, and sometimes even CK7 (33).

Tumor-specific immune responses are normally not enough to eradicate the tumors. The paucity of immunological danger signals necessary for immune activation, the increased concentration of immunosuppressive factors, including cytokines and cells, in the tumor microenvironment indicate that immune regulation has an active role in cancer progression (36). The identification of negative immunoreceptors on T cells, receptors that dampen the ability of T cells to proliferate, provide a novel therapeutic target that may lead to the enhanced tumor-specific T-cell immunity in vivo. To our knowledge, reports addressing the direct involvement of PD-1 in tumor progression in a model of murine SCC, which mimics the characteristics of human SCC, have not been studied. Our results showed that the immune neutralization of PD-1+ cells resulted in decreased papilloma incidence associated with CD4+ and CD8+ T cells infiltrate, higher levels of interferon-γ (IFN-γ) and decreased levels of transforming growth factor-β (TGF-β) in the tumor lesions. These findings support the role of PD-1 blockade as a promising component of immunotherapy against SCC.

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

Abbreviations: Ab, antibody; CK, cytokeratin; DC, dendritic cell; DMBA, 7,12-Dimethylbenz[a]anthracene; IFN-γ, interferon-γ; IgG, immunoglobulin G; LN, lymph node; PCNA, proliferating cell nuclear antigen; PD-1, programmed death-1; PD-L1, programmed death ligand-1; PMA, phorbol 12-myristate 13-acetate; SCC, squamous cell carcinoma; TGF-β, transforming growth factor-β.

1These authors contributed equally to this work.
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 killed in 2 week intervals until a 16 week endpoint after 7,12-Dimethylbenz[a]anthracene (DMBA) (Sigma–Aldrich, St Louis, MO) topical application was reached. All animal experiments were approved by the Animal Research Ethics Committee of the Bauru School of Dentistry, University of São Paulo.

DMBA/PPA-induced skin carcinogenesis

Eight weeks old mice (four groups of eight mice each) had their dorsal skin shaved and were topically treated with a single dose of DMBA (125 μg in 200 μl of acetone) and biweekly doses of phorbol 12-myristate 13-acetate (PMA) (200 μl of a 10−3 M solution in acetone) (Sigma–Aldrich) for 16 weeks as described previously (37). 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 16 weeks after initiation and were processed as described below.

Antibodies and treatment

The blockage of PD-1 was performed by intraperitoneal injection of 500 μg/ml hamster anti-mouse PD-1 (J43) (BD Biosciences) in the fourth and eighth weeks after the first DMBA application. Controls received 500 μg/ml of normal hamster immunoglobulin G (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 40 min at 37°C in RPMI 1640 medium containing 50 μg/ml of a collagenase CI enzyme blend (Boehringer Ingelheim Chemicals, Petersburg, VA), as described previously (38). The tissues were subsequently dissociated for 4 min in the presence of RPMI 1640 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 (BD Biosciences). The leukocytes compose a tumor-draining lymph nodes (LNs), axial and brachial LNs from healthy control mice were determined by dissociation, cell counting and immunostaining. The viability of the leukocytes was evaluated by Trypan blue exclusion, and these cells were used for cell activation and immunolabeling assays.

Antibodies and flow cytometry analysis

For immunostaining, PerCP-, PE- and fluorescein isothiocyanate-conjugated antibodies (Abs) against CD3 (17A2), CD4 (H129.19), CD8 (RPA-T8 and HIT8a), CD19 (ID3), PD-1 (J43), PD-L1 (MH5), PD-L2 (TY25), DC (3D3D1) and F4/80 (BM8), and the respective goat and rat isotype controls were used (BD Biosciences). Samples were run on a FACSort flow cytometer, and the data was analyzed using CellQuest software (BD Biosciences).

Histological analysis

Tissue samples were collected from tumor sites and fixed with 10% formalin for 6 h at room temperature. The tissues were subsequently dehydrated in ethyl alcohol, followed by washes in xylol, and were embedded in paraffin. Each sample was sectioned into slices 4–7 μm thick that were dried onto slides and stained with hematoxylin and eosin. Slides were examined in a blinded fashion by a pathologist and classified as papillomas or carcinoma. Carcinoma specimens were graded as well, moderately or poorly differentiated.

Immunohistochemistry

A Vector Kit (Vectastain Elite ABC kit, Vector Labs, Burlingame, CA) was used for immunohistochemical analysis of all markers. In brief, 4 μm tissue sections were cut, and slides were baked in a 58°C oven overnight. Sections were deparaffinized in xylene (Fisher Scientific, Pittsburgh, PA) for 15 min, washed twice in ethanol, and endogenous peroxidase activity was blocked with 3% H2O2/methanol for 30 min. Sections were incubated with Molecro® milk powder for 20 min to block non-specific Ab binding sites. Sections were then incubated with the following primary Abs: CK1 (GeneTex, San Antonio, TX), filaggrin (Conviron, Princeon, NJ), forcin (Covance), CK5 and 8 (Chemicon, Temecula, CA) and proliferating cell nuclear antigen (PCNA) (Millipore, Billerica, MA). Sections were incubated with a primary Ab overnight at 4°C, followed by incubations with peroxidase-labeled anti-rabbit or mouse IgG for 30 min. Staining was completed with a 10 min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Appropriate positive and negative control samples were used. Tissue staining was quantified using image analysis software (LEICA LAS Image Analysis; LEICA Microsystems, Wetzlar, Germany).

Cytokine assays

Interleukin-10, TGF-β and IFN-γ levels were quantified by enzyme-linked immunosorbent assay, according to the manufacturer’s instructions (BD Biosciences), as described previously (37). The concentrations of each cytokine were dosed as pg/ml, and the results were normalized and expressed as mg per protein.

Statistical analysis

The results are expressed as the mean ± SD or mean ± standard error of the mean 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). Log-rank (Mantel–Cox) test was used to determine whether the anti-PD-1-treated mice had better outcomes than the wild-type mice. Values of P < 0.05 were considered statistically significant.

Results

Characterization of PD-1+ cells in tumor of mice with SCC

We first evaluated the percentage of PD-1+, PD-L1+ and PD-L2+ positive cells in the skin of mice with or without SCC. After 16 weeks of the carcinogenesis, the number of CD8+PD-1+ and CD4+PD-1+ T cells present in the tumor tissues (2.2 ± 1.9 × 103 and 2.4 ± 1.9 × 103, respectively) of SCC mice were elevated when compared with the skin of non-tumorigenic control mice (0.5 ± 0.2 × 103 and 0.6 ± 0.9 × 103, respectively) (Figure 1B).

Since macrophages are crucial component of the leukocyte infiltrate in solid tumors (39), we next analyzed the percentage of total F4/80+ macrophages expressing PD-L1 and PD-L2 in the tumor sites from SCC 16 weeks after carcinogenic induction. PD-L1+ macrophages were elevated in the tumor tissues (12.9 ± 0.9 × 103) in comparison with skin tissues (0.06 ± 0.007 × 103) of non-tumorigenic control mice (Figure 1C). PD-L2+ macrophages were elevated in the tumor tissues (1.9 ± 0.8 × 103) of SCC mice in comparison with skin (0.01 ± 0.003 × 103) of non-tumorigenic control mice (Figure 1C). These findings support our hypothesis that PD-1/PD-L1 activation is involved in the regulation of immune response during SCC.

Effect of PD-1 inhibition on the SCC development in mice

Next, we assessed the role of PD-1+ cells during the development of SCC by immune neutralization of PD-1 molecules. The mice were followed for 20 weeks and we did not observe any difference in the mortality between both groups (data not shown). The number and rate of appearance (tumor incidence and initiation rate) of papillomas were delayed in anti-PD-1-treated mice (Figure 2A). Papillomas were found in 100% of IgG-treated mice 10 weeks after carcinogenic induction, whereas papillomas were found after 12 weeks in anti-PD-1-treated mice (Figure 2A). Moreover, the papilloma average number was lower in anti-PD-1-treated mice when compared with IgG-treated mice (Figure 2B).

These results were confirmed by macroscopic analysis that demonstrated treatment with anti-PD-1 delayed the carcinogenic process because the number and rate of appearance of papillomas were significantly lower in anti-PD-1-treated mice (Figure 2C and D). Histological analysis revealed a pronounced presence of inflammatory infiltrates and a lack of epithelial islet formation in anti-PD-1-treated mice compared with control groups (non-tumorigenic control mice and IgG-treated mice) (Figure 3A–C). Tumor differentiation was assessed to determine the effects of PD-1 blockade in tumor grade. The results show that 75% (9/12) anti-PD-1-treated mice developed benign tumors (papilloma), 8% (1/12) poorly differentiated SCC and 17% (2/12) well-differentiated SCC (Table 1). In contrast, 83% (10/12) IgG-treated mice developed well-differentiated SCC and only 17% papilloma (Table 1). Additionally, anti-PD-1-treated mice

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showed a lower degree of tumor invasiveness with smaller areas of tumor involvement over the same time course (Figure 3B).

Thus, our results indicate that the presence of PD-1+ cells affected skin tumor initiation and development.

Since the alterations of CKs expression are closely associated with the development of SCC, we analyzed the effects of PD-1 blockage on the expression of CKs in tumor sites and normal skin (Figure 3D, G, J, M and P). Positive immunostaining for CK1, loricrin, filaggrin, CK5-8 and PCNA were observed in the normal skin (non-tumorigenic control mice). The expression of CK1, loricrin and filaggrin was higher in anti-PD-1-treated mice (Figure 3D, G and J) when compared with samples from IgG-treated mice (Figure 3F, I and L). Low expression of CK5-8 was observed in SCC lesions from anti-PD-1-treated mice (Figure 3N), in contrast to the higher expression of CK5-8 in SCC lesions from IgG-treated mice (Figure 3O). No significant differences in PCNA expression was observed between the groups. It has been demonstrated that CK5

Fig. 1. SCC induced by DMBA/PMA generated an increase in the PD-1, PD-L1 and PD-L2 population in the tumor microenvironment. SCC mice were treated according to a chemical carcinogenic protocol using DMBA and PMA for 16 weeks. (A) The gating strategy utilized to determine T-cell population. (B) The number of CD4+PD-1+ and CD8+PD-1+ lymphocytes in skin was determined using flow cytometry. (C) F4/80+ macrophages were gated and coexpression of PD-L1 and PD-L2 in the skin was determined. Data from three independent experiments with eight mice per group. *P < 0.05.
and CK8 are overexpressed in various cancer cells including human breast carcinoma, lung SCC and gastric adenocarcinoma (40–45). These results suggest that the absence of PD-1 activation impairs the progression to SCC, which is correlated with the expression of specific CKs.

**Effect of PD-1 inhibition on the antitumor immune response**

The most conclusive readout that allows measurement of the *in vivo* relevance of signaling by PD-1 is the antitumor immune response characterized by lower numbers of papillomas, delay in the development of SCC, intense inflammatory infiltrates and a differential expression of CKs in tumor lesions. We therefore investigated the inflammatory infiltrate and the profile of cytokines in the tumor microenvironment 16 weeks after carcinogenesis. Our results showed that 12 ± 3.9 × 10⁶ leukocytes were present in the tumor samples (Figure 4A) of anti-PD-1-treated mice and 8 ± 0.3 × 10⁷ in the tumor samples of IgG-treated mice (Figure 4A). Of these leukocytes, 54 ± 11.7% represented T cells (CD3⁺) in the tumor samples from anti-PD-1-treated mice (Figure 4B). Of these T cells, 40 ± 10.1% were CD4⁺ and 14 ± 2.2% were CD8⁺ (Figure 4C). IgG-treated mice had a significantly lower percentage of CD3⁺ T cells (34.9 ± 10.4%) infiltrating tumor lesions, and significantly lower percentages of CD4⁺ and CD8⁺ T cells infiltrating tumor lesions, compared with anti-PD-1-treated mice (Figure 4C). We found that anti-PD-1-treated mice had a higher percentage of B cells (CD19⁺) (15.3 ± 4.6%) infiltrating tumor samples than the control group (3.6 ± 1.5%) (Figure 4C). Interestingly, DCs (30.1 ± 1.2%) and macrophages (86.7 ± 1.1%) were found infiltrating tumor samples at higher number from IgG-treated mice than from anti-PD-1-treated mice (Figure 4C). In addition, the blockage of PD-1 significantly decreased the percentage of CD4⁺PD-1⁺ T cells and in SCC lesions (Figure 4B).

We then analyzed the phenotype of the leukocyte subpopulations in the LNs from anti-PD-1-treated mice and control groups (Figure 4D). We found that the frequencies of CD3⁺ T cells in anti-PD-1-treated mice were similar to those detected in IgG-treated mice (Figure 4D). We also analyzed the population of macrophages and found significant differences between the anti-PD-1-treated mice (38 ± 8.8%) and IgG-treated mice (75.9 ± 15%) (Figure 4D). Similarly to macrophages, DCs (CD11c⁺CD11b⁺) were increased in the LNs from IgG-treated mice (13.6 ± 0.1%) when compared with anti-PD-1-treated mice (1.16 ± 0.03%) (Figure 4D). These data indicate that PD-1 blockage improved the accumulation of CD4⁺ and CD8⁺ T cells and B cells on tumor lesions, which could be associated with better antitumor immune responses.

Based on these observations, we next analyzed how PD-1 activation regulated the levels of cytokines during SCC. For this, we quantified the levels of interleukin-10, TGF-β and IFN-γ in tumor samples and LNs from anti-PD-1-treated mice and control IgG-treated mice (Figure 5A and B). Our data showed that samples from anti-PD-1-treated mice contained significantly elevated levels of IFN-γ when compared with samples from IgG-treated mice (Figure 5A). Conversely, we found lower levels of TGF-β in lesions and LNs from anti-PD-1-treated mice when compared with IgG-treated mice (Figure 5A and B). These data suggest that blockage of PD-1 could result in a predominantly Th1 tumor environment increasing IFN-γ and decreasing TGF-β, which is crucial for the protective antitumor responses.

**Discussion**

The PD-1/PD-L1 pathway is an important inhibitory pathway in the tumor microenvironment (7). PD-1 expression on tumor-infiltrating CD8⁺ T cells and increased expression of PD-L1 on tumor cells correlates with poor prognosis in patients with different types of cancers, including breast, ovarian, pancreatic, gastric, kidney and bladder cancers (46). In the present study, we found that PD-1–PD-L1 activation contributes to the SCC development revealed by increased numbers of papillomas per mouse followed by a differential expression of CKs, and a direct effect on the regulation of antitumor immune responses.

In SCC, the PD-1/PD-L1 pathway seems to be an important immunosuppression mechanism. For example, Malaspina et al. (27) found that the expression of PD-L1 was intense in oral SCC and moderate in
premalignant condition like actinic cheilitis lesion sites. Using a chemically induced tumor model, we observed that, after anti-PD-1 treatment, the total number of leukocytes was significantly increased. In particular, the number of CD8+ and CD4+ T cells was increased in tumor tissues after anti-PD-1 treatment. The increased frequency of CD8+ T cells in the tumor microenvironment could underlie the decrease of tumor incidence observed in anti-PD-1-treated mice in SCC models.

An important finding in the current study was the increased number of CD19+ B cells in the tumor and LNs of anti-PD-1-treated mice. B cells have a critical tumor-promoting role in SCC development (47) as B-cell-deficient mice exhibit resistance to several histologically diverse primary syngeneic tumors. Decreased tumor implantation and progression in B-cell-deficient mice were found to be associated with enhanced antitumor Th1 cytokines and cytotoxic T-lymphocyte responses (48). Thus, the accumulation of B cells may explain why PD-1 blockade in vivo had only a partial antitumor effect.

Our observation that anti-PD-1 treatment decreased the number of F4/80+ cells (i.e. macrophages) and DCs in the tumor and LNs...
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Table I. Histologic analysis of tumors from IgG-treated and anti-PD-1-treated mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>IgG-treated (%)</th>
<th>Anti-PD-1-treated (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papilloma</td>
<td>2/12 (17%)</td>
<td>9/12 (75%)</td>
<td>P = 0.0140</td>
</tr>
<tr>
<td>SCC</td>
<td>10/12 (83%)</td>
<td>3/12 (25%)</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>0/12 (0%)</td>
<td>1/12 (8%)</td>
<td>P = 0.5060</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>0/12 (0%)</td>
<td>0/12 (0%)</td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>10/12 (83%)</td>
<td>2/12 (17%)</td>
<td></td>
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</table>

Fig. 4. PD-1+ cells blockade caused enhancement of lymphocyte infiltration. (A) The absolute number of leukocytes in the tumor lesions was determined by flow cytometry and calculated according to the absolute numbers of cells found in the tissues. (B) Histogram exhibit PD-1 expression on CD4+ T cells (gray line, non-tumorigenic control mice; black line, anti-PD-1-treated mice; thin line, IgG-treated mice). The phenotypes of leukocyte populations in the tumor lesions (B) and LNs (C) from non-tumorigenic control mice (open bars), IgG-treated mice (gray bars) or anti-PD-1-treated mice (closed bars) were analyzed through flow cytometry. Data from three independent experiments with eight mice per group. Error bar represents mean ± SD. *P ≤ 0.05.
is consistent with the hypothesis that the blockade of PD-1 leads to a more effective antitumor immune response. Macrophages are recognized for having the potential not only to elicit tumor and tissue destructive reactions but also to promote tumor progression and metastasis (49–51). The decreased frequency of macrophage cells in the tumor microenvironment could be a direct consequence of the control of tumor development observed in anti-PD-1-treated mice. It is largely known that the binding of PD-1 with their ligands (PD-L1 and PD-L2) induces the apoptosis of immune cells (52). Thus, the high rate of apoptotic cells observed in the microenvironment may be related to macrophage infiltration in tumor (53), contributing to the modulation of the local immune response. In addition, PD-1 expression has recently been identified on DCs, where it suppresses innate immunity against infectious disease (54). A recent study shows that DCs in the ovarian tumor microenvironment express both PD-1 and PD-L1 and use the PD-L1/PD-1 inhibitory pathway in mediating immune suppression (46). Thus, the presence of PD-1 cells in the tumor microenvironment in general may regulate the local immune response, leading to tumor growth by inhibiting CD8+ T cells and natural killer cells, but also orchestrates responses in tumors that facilitate their escape from immune attack and inhibit angiogenesis within the tumor (58). In the other hand, TGF-β signaling is known to function as a tumor promoter (59). In human cancer, the overexpression of TGF-β correlates with tumor progression, metastasis, angiogenesis and poor prognostic outcome (60,61). In addition, the expression of TGF-β correlates with an increase in the conversion frequency from papilloma to SCC at later stages of skin carcinogenesis (62). Collectively, our findings suggest that PD-1 activation is directly linked to SCC, thereby providing potential new therapeutic strategies for this malignancy.

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