Cancer Immunol Immunother (2011) 60:965–974
DOI 10.1007/s00262-011-1007-5

ORIGINAL ARTICLE

Enhanced programmed death 1 (PD-1) and PD-1 ligand (PD-L1) expression in patients with actinic cheilitis and oral squamous cell carcinoma

Tatiana Salles de Souza Malaspina · Thaís Helena Gasparoto · Maria Renata Sales Nogueira Costa · Edgard Franco de Melo Jr · Maura Rosane Valério Ikoma · José Humberto Damante · Karen Angélica Cavassani · Gustavo Pompermaier Garlet · João Santana da Silva · Ana Paula Campanelli

Received: 31 December 2010 / Accepted: 9 March 2011 / Published online: 27 March 2011

Abstract PD-1 and PD-L1 can be involved in tumor escape, and little is known about the role of these molecules in oral tumors or pre-malignant lesions. In the present study, we investigated the expression of PD-1 and PD-L1 in the blood and lesion samples of patients with actinic cheilitis (AC) and oral squamous cell carcinoma (OSCC). Our results showed that lymphocytes from peripheral blood and tissue samples exhibited high expression of PD-1 in both groups analyzed. Patients with AC presented higher percentage as well as the absolute numbers of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ lymphocytes in peripheral blood mononuclear cells (PBMC) than healthy individuals, while patients with OSCC presented an increased frequency of $CD8^+PD1^+$ in PBMC when compared with controls. On the other hand, increased frequency of $CD4^+$ and $CD8^+$ T cells expressing $PD\text{-}1^+$ accumulate in samples from OSCC, and the expression of PD-L1 was intense in OSCC and moderate in AC lesion sites. Lower levels of IFN-γ and higher levels of TGF-β were detected in OSCC samples. Our data demonstrate that PD-1 and PD-L1 molecules are present in blood and samples of AC and OSCC patients. Further studies are required to understand the significance of PD-1 and PD-L1 in oral tumors microenvironment.

Keywords PD-1 · Oral squamous cell carcinoma · Actinic cheilitis

T. S. de Souza Malaspina · T. H. Gasparoto · G. P. Garlet · A. P. Campanelli (✉)
Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, Bauru, SP CEP 17012-901, Brazil
e-mail: apcampan@usp.br

J. H. Damante
Department of Stomatology, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil

M. R. S. N. Costa · E. F. de Melo Jr
Dermatology and Research Division, Institute Lauro de Souza Lima, Bauru, SP, Brazil

M. R. V. Ikoma
Amaral Carvalho Hospital, Jaú, SP, Brazil

J. S. da Silva
Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

K. A. Cavassani
Department of Pathology, Medical School, University of Michigan, Ann Arbor, MI, USA

Introduction

The modulation of immune responses in tumor sites is a critical mechanism attributed to tumor evasion. Soluble factors and membrane-bound molecules have been found to be up-regulated in tumor sites, which potentially inhibit immune responses [1, 2]. Current data suggest that PD-1: PD-L1 pathway regulates the organ-specific tolerance in normal tissue and may contribute to immune evasion by cancer cells [3–5].

Programmed death 1, or PD-1, is a member of the extended CD28/CTLA-4 family of T cell regulators [6]. Its structure suggests that PD-1 negatively regulates TCR signals [6]. PD-1 is expressed on the surface of activated T cells, B cells, and macrophages, suggesting that compared to CTLA-4, PD-1 more broadly, negatively regulates immune responses [7]. PD-L1 (programmed death-1 ligand 1) is expressed on resting B cells, T cells, macrophages, and dendritic cells (DCs) and the expression of PD-L1 is

further up-regulated on these cells by various stimuli including inflammatory cytokines such as IFN-γ, IL-12, GM-CSF, and IL-4 [7]. Engagement of PD-1 by PD-L1 leads to the inhibition of T cell proliferation and downregulation of cytokines production (i.e., IL-2 and IFN-γ) [3]. Consistent with the inhibitory role of PD-1, PD-1-deficient mice develop a severe autoimmune disease [3, 8–11]. Genetic studies have shown that PD-1 gene polymorphisms are associated with autoimmune diseases, including lupus erythematosus and rheumatoid arthritis [12, 13]. PD-1 and PD-L1 are highly expressed in different tumors, like lymphomas, as well as pancreas and renal cell carcinoma [14–16]. The presence of tumor PD-L1 positive cells is directly correlated with poorer prognosis and inversely correlated with $CD8^+$ T cells infiltrating tumors [15, 16]. In animal studies, PD1 blockade potentiates an antitumor immune response [6]. Although these reports have suggested a direct correlation between PD-1: PD-L1 and tumor escape, the information regarding the involvement of these molecules in pre-malignant lesions and lesions from patients with oral squamous cell carcinoma has not been described.

Actinic cheilitis (AC) is an oral pre-malignant lesion characterized by discrete chronic infiltrate and cellular abnormalities that might develop into oral squamous cell carcinoma (OSCC). Oral squamous cell carcinoma (OSCC) is characterized by a high degree of local invasion and high rate of metastasis to the cervical lymph nodes, directly affecting the prognosis of the patients [17–19]. OSCC may arise at different sites in the oral tissues, and is known that patients who present lip OSCC usually have a lower rate of regional lymph node metastasis and mortality [17–19]. Such differences are influenced by T lymphocyte function in the tissue samples, and we have shown that OSCC samples are largely infiltrated by T lymphocytes with regulatory phenotype [20].

Based on the importance of PD-1 in the regulation of tumor immune response, we explored the presence of $PD1^+$ cells in the peripheral blood and lesions from patients with OSCC and AC. The results showed increased expression of PD-1 on peripheral blood cells from AC and OSCC patients than healthy subjects. AC patients had significantly higher number of T $CD4^+PD\text{-}1^+$ and T $CD8^+PD\text{-}1^+$ cells than healthy subjects. In lesion sites, a higher number of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ cells were observed in OSCC sample when compared with AC sample, being significantly higher for $CD4^+PD\text{-}1^+$ subset. The analysis of different tumor sites demonstrated that tongue from OSCC had significantly higher percentage of $CD4^+PD\text{-}1^+$, $CD8^+PD\text{-}1^+$, and non-lymphocytes $PD\text{-}1^+$ cells than lip OSCC. Moreover, OSCC lesions presented lower levels of IFN-γ and higher levels of immunomodulatory cytokine TGFβ than healthy gingival tissues, supporting the correlation between PD1/PDL1 and the modulation of anti-tumor responses.

Materials and methods

Patients with OSCC, AC, and healthy volunteers

Tissue samples and peripheral blood mononuclear cells from 39 patients with a diagnosis of OSCC and 22 with AC were used in the present study. Specimens of OSCC were obtained from lip tumors ($n = 9$), tongue tumors ($n = 3$), and other sites ($n = 3$). PBMCs were obtained from patients with OSCC (29 men and 10 women; age ranged 41–96 years, mean age $= 58.42 \pm 2.25$ years old), patients with AC (17 men and 5 women; age ranged 31–86 years, mean age $= 63 \pm 4$ years old). Ten age-matched healthy volunteers (7 men and 3 women; age ranged 27–74 years) completed the samples. All patients had active disease at the time of phlebotomy and surgery. Tumors were classified as well, moderately, or poorly differentiated according to the WHO classification of histological differentiation grade. All patients presented undergone surgical resection of their tumors with a curative intent, alone or combined with radiotherapy and chemotherapy. In contrast, actinic cheilitis specimens were obtained when biopsy was indicated because therapeutic strategies had failed. All subjects signed an informed consent releasing the use of specimens (tissues and blood) for research purposes. The consent was approved by the ethical committee of Bauru School of Dentistry.

Antibodies

For immunostaining, PerCP, PE, APC, and FITC-conjugated antibodies against CD3 (UCHT1), CD4 (RPAT4), CD8 (RPA-T8), CD19 (HIB 19), PD-1 (MIH4), PD-L1 (MIHI), CD14 (M5E2) and the respective mouse and rat isotype controls were used (BD Biosciences). PE-conjugated streptavidin was purchased from Invitrogen Life Technologies.

Isolation of leukocytes

All procedures were conducted as described previously [20]. Peripheral blood was harvested with heparin (50 U/ml) from healthy subjects, AC, and OSCC patients. PBMC were isolated using Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation, washed, counted, and labeled with specific Abs for phenotypic analysis in flow cytometer or for purification of T cell subpopulations. To characterize the leukocytes present in the tumor site, the tumor samples from patients were collected and incubated

1 h at 37°C in RPMI 1640 medium, containing 50 μg/ml collagenase CI enzyme blend (Boehringer Ingelheim Chemicals). Next, the tissues were dissociated, for 4 min, in the presence of RPMI 1640 containing 10% serum and 0.05% DNase (Sigma–Aldrich) using a Medmachine (BD Biosciences), according to the manufacturer's instructions. The tissue homogenates were filtered using a 30-μm cell strainer (Falcon; BD Biosciences). The leukocyte's viability was evaluated by Trypan blue exclusion and used for cell activation or immunolabeling assays.

Flow cytometry analysis

For immunostaining, PerCP, PE-conjugated, and FITC-conjugated Abs against CD3 (UCHT 1), PerCP-CD4 (RPAT4), PerCPCD8 (RPA-T8), FITC-CD14 (M5E2), PE-CD19 (HIB 19), (BD Biosciences), FITC-PD-1 (J116), and PE-PD-L1 (MIH1) (eBiosciences, San Diego, USA), and the respective mouse and rat isotype controls were used (BD Biosciences). The cell acquisition was performed on a FACSort flow cytometer using CellQuest software (BD Biosciences). The absolute values of leukocyte/lesions and leukocyte/10 ml of peripheral blood subsets were calculated through percentage obtained by FACS, and the amount of cells was determined in Neubauer chamber.

Immunohistochemical analysis

Frozen tissue sections (5 μm) were obtained and fixed with cold acetone for 10 min. The slides were placed in a humidified chamber, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min followed by incubation with PBS plus 5% (w/v) nonfat milk (Nestle®). The slides were washed with PBS and incubated overnight with mouse IgG anti-human PD-1, CD4, and CD8 (all from BD Biosciences) or normal IgG (controls) diluted 100 times in PBS with 3% (w/v) nonfat milk. After successive rinsing with PBS, the sections were incubated for 30 min with biotin-labeled secondary antibodies followed by PBS washing and incubation with avidin-peroxidase link, and finally rinsed before chromogenic substrate application (3,3′-diaminobenzidine, Vector Laboratories). The slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Canada Balsan.

Cytokine assays

The supernatants of lesion samples were obtained by disaggregation through treatment with RPMI 1640 medium containing 0.25% collagenase (Worthington) and frozen at −70°C until analysis. The total protein concentration was measured using Quick Start™ Bradford Protein assay kit (Bio-Rad, CA, USA). IFN-γ, TGF-β, and IL-10 were quantified in the samples by the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using commercial capture and biotinylated detection antibodies (BD Pharmingen Corp., San Diego, CA), and the respective human recombinant cytokines (diluted in PBS) as standards, according to the manufacturer's instructions. The concentrations of the cytokine were dosed as pg/ml, and the results were normalized and expressed as mg/protein. Healthy gingival tissues, removed for orthodontic treatments, were used as cytokine controls in this analysis.

Statistical analysis

Data obtained from flow cytometry were expressed as standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by the Tukey's multiple comparison tests (PRISM Software; GraphPad). All values were considered significantly different at $P < 0.05$.

Results

PD-1 expression in PBMC from patients with OSCC and AC

First, we analyzed the phenotype of circulating subpopulations of lymphocytes in PBMC from actinic cheilitis ($n = 10$), oral squamous cell carcinoma ($n = 8$), and healthy controls subjects ($n = 11$). We found that the frequencies of B cells ($CD19^+$), $CD4^+$ T cells, and $CD8^+$ T cells in patients with OSCC and AC were similar to those detected in normal donors (Fig. 1c). However, our data showed significant difference in the percentage of $CD4^+$T cells expressing PD-1 between AC patients (36.6 ± 13.3%) compared with controls subjects (2.81 ± 0.4%) (Fig. 1d). Although we did not see any significant difference in the percentage, the absolute numbers of $CD8^+$ T cells expressing PD-1 ($6.6 \pm 1.7 \times 10^5$) were significantly higher in OSCC patients than in control subjects ($1.7 \pm 0.9 \times 10^5$) (Fig. 1e). Increased numbers of $CD4^+PD1^+$ and $CD8^+PD1^+$ T cells were recovered from blood of AC patients than normal donors (Fig. 1e). These data establish that AC patients, unlike from healthy controls, present higher rate of $PD1^+$ cells in the peripheral blood.

Characterization of the inflammatory infiltrate in AC and OSCC lesions

Since the inflammatory infiltrate surrounding the tumor is essential to dictate the anti-tumor responses, we next analyzed the inflammatory infiltrate in OSCC and AC lesions. Our results showed that the cell infiltrate is significantly

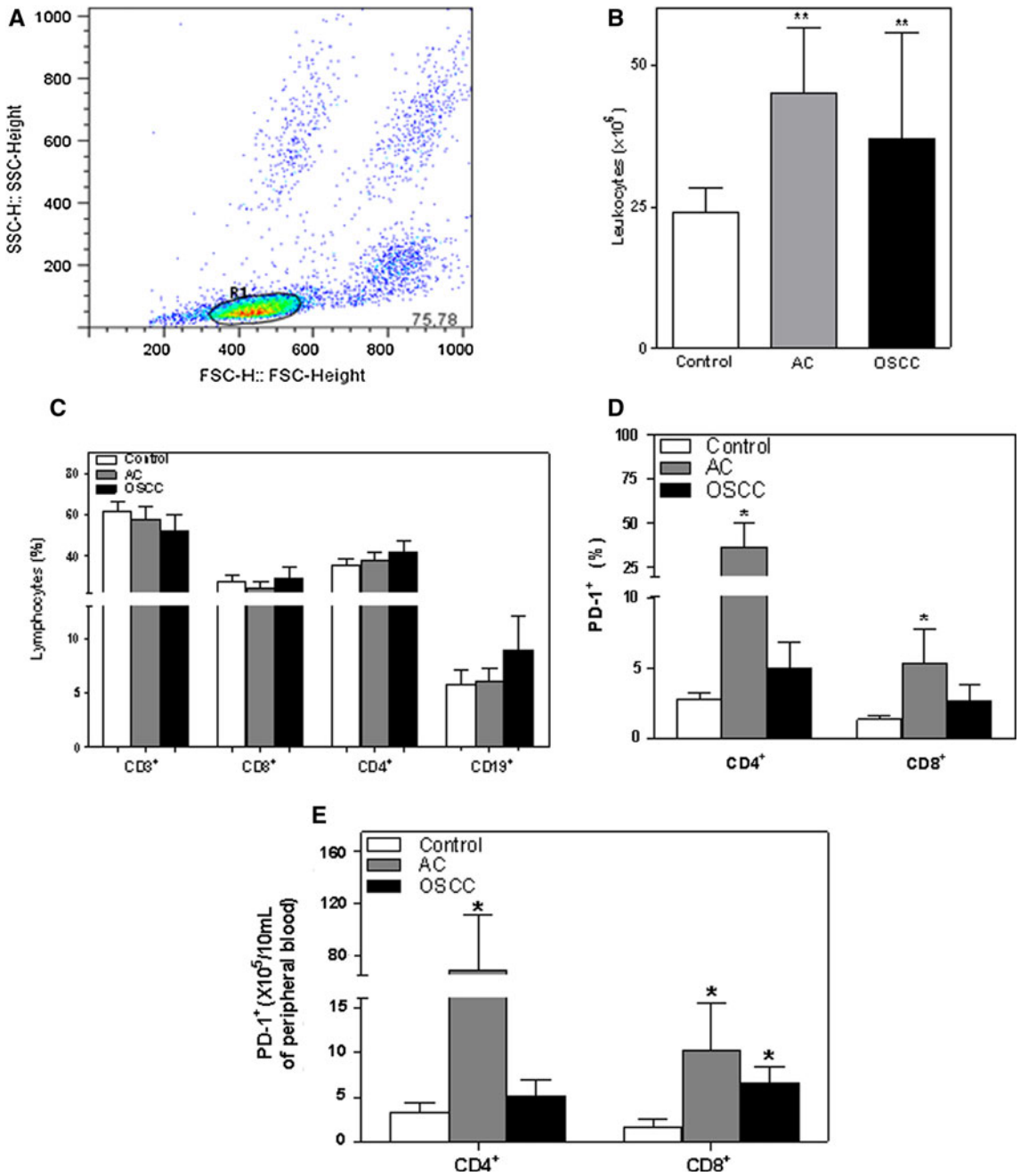

Fig. 1 Characterization of leukocytes derived from PBMC from patients with actinic cheilitis (AC), oral squamous cell carcinoma (OSCC), and control subjects. PBMC from control subjects (control, $n = 11$, *opened bars*), actinic cheilitis (AC, $n = 10$, *gray bars*), and oral squamous cell carcinoma patients (OSCC, $n = 8$, *closed bars*) were characterized by flow cytometry. **a** Representative forward (FSC) and side (SSC) *scatter dot plot* of PBMC lymphocytes (R1). **b** Number of leukocytes obtained from peripheral blood of healthy individuals, AC, and OSCC patients. **c** Cells gated in R1 were analyzed in relation to the expression of CD3, CD8, CD4, and CD19 molecules. **d** Each *bar* represents the percentage or **e** absolute numbers of gated $CD4^+$ and $CD8^+$ T cells expressing PD1. The results are expressed as the mean ± SEM for volunteers and patients samples tested individually. $^*P < 0.05$ and $^{**}p < 0.01$ compared with controls

lower in AC lesions. Indeed, AC lesions presented $0.6 \pm 0.05 \times 10^6$ leukocytes per sample while $6.7 \pm 3.8 \times 10^6$ leukocytes were found in OSCC infiltrate (Fig. 2b). $CD3^+$ cells represented the main population of the leukocytes infiltrating both OSCC (42 ± 7.9%) and AC (35 ± 19%) lesions (data not shown). Gating on $CD3^+$ cells, 54.8 ± 5.4% were $CD4^+$, 44.2 ± 4.8% were $CD8^+$ in OSCC lesions, 62 ± 11.7% were $CD4^+$, and 38 ± 10.8% were $CD8^+$ in AC samples (Fig. 2d). Similar percentages of $CD19^+$ and $CD14^+$ cells were present in

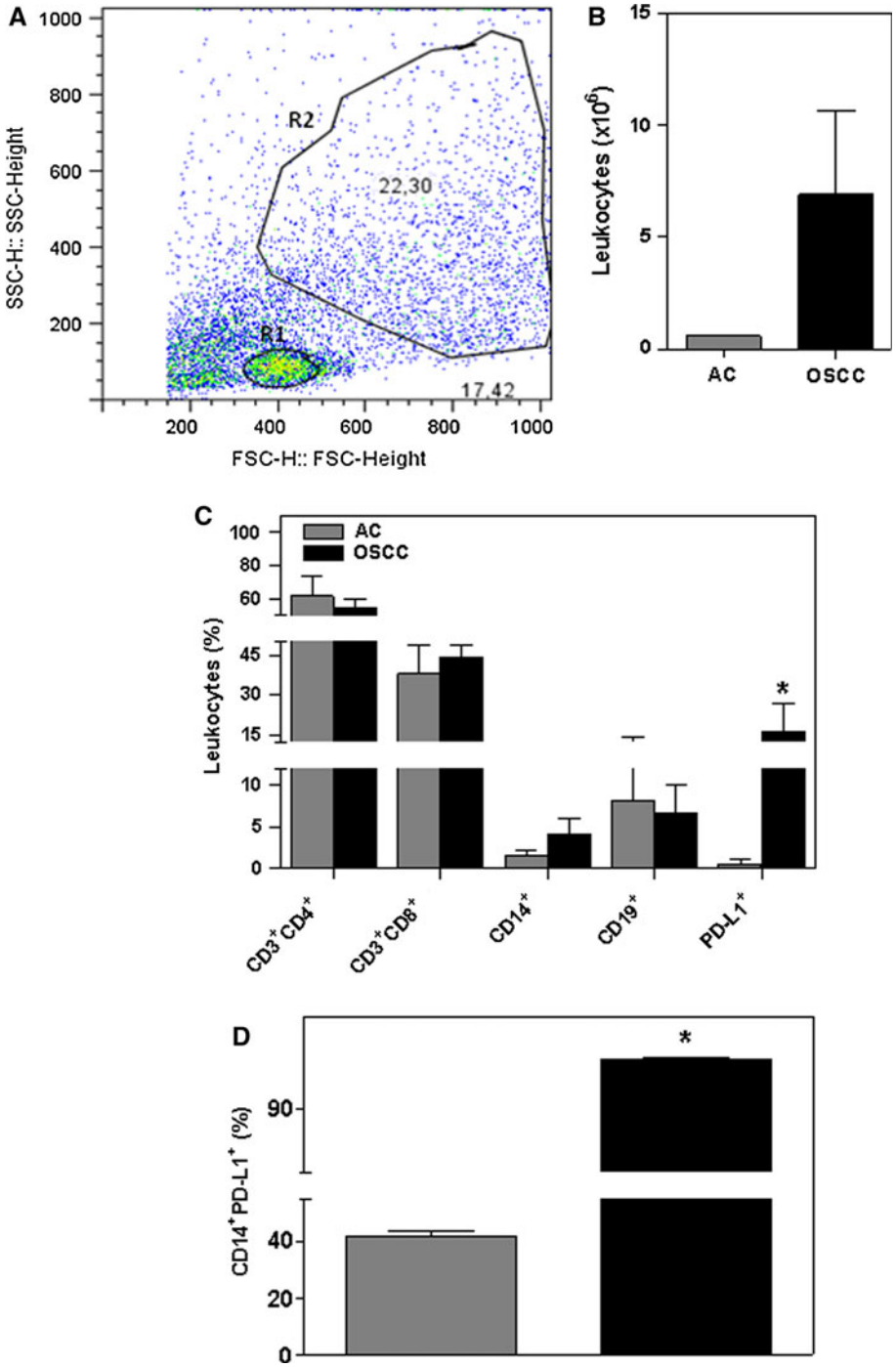

Fig. 2 Characterization of leukocytes derived from AC and OSCC lesions. Mononuclear cells isolated from AC ($n = 4$, *gray bars*) and OSCC ($n = 11$, *closed bars*) tumor samples were enzymatically digested with collagenase 0.25% and degraded using Medmachine, then, they were analyzed by flow cytometry. **a** Gate of lymphocytes (R1) and $CD3^{-}C14^{-}CD19^{-}$ cells (R2) from a representative patient. **b** Absolute numbers of leukocytes obtained from the lesions after digestion process. **c** Percentage of cells expressing CD3, CD8, CD4, CD19, CD14, and PD-L1. **d** Percentage of $CD14^{+}PD\text{-}L1^{+}$ cells from AC ($n = 4$, *gray bars*) and OSCC ($n = 11$, *closed bars*) lesions. The results are expressed as the mean ± SEM for patients samples tested individually. $^{*}P < 0.05$

AC and in OSCC (Fig. 2c). Interestingly, even though the most of the leukocytes gated in both R1 and R2 regions (Fig. 2a) of AC lesions did not express PDL-1, 16.1 ± 6.2% of leukocytes in OSCC expressed this molecule (Fig. 2c). In order to clarify which leukocyte populations infiltrating AC and OSCC lesions were expressing PD-L1, we verified that 97.4 ± 0.2% in OSCC lesions and 41.5 ± 3.5% in AC lesions were $CD14^{+}$ in both R1 and R2 regions (Fig. 2d). These data demonstrate that $CD14^{+}PD\text{-}L1^{+}$ cells infiltrated OSCC lesions more than AC lesions.

Characterization of $PD\text{-}1^{+}$ cells in AC and OSCC lesions

Since we found an increased expression of PD-1 in blood of OSCC patients, we also analyzed the expression of PD-1

in lesion samples from AC and OSCC patients. In contrast to PBMC, our data clearly showed that tumor samples contained elevated expression of PD-1 when compared with tissue from AC patients (Fig. 3b). As shown in the Fig. 3c, 21.1 ± 14.6% of the lymphocytes were $CD8^+PD\text{-}1^+$ T cells in OSCC whereas only 0.84 ± 0.6% of the $CD8^+$ T cells were $PD\text{-}1^+$ in AC lesions. Interestingly, more than 60% of lesion cells ($CD3^-CD14^-CD19^-$) (from R2 gate) (Fig. 3a) from both AC and OSCC lesions expressed PD-1 (Fig. 3c). Although no significant differences were detected between the percentages of $CD4^+PD1^+$ and $CD8^+PD1^+$ T cells from AC and OSCC (Fig. 3c), the number of $CD4^+PD1^+$ T cells was significantly higher in OSCC than AC sample (Fig. 3d). Immunohistochemistry analysis illustrated that in AC sample, PD-1 expression is limited to the connective tissue, but it is distributed in epithelial and in connective tissue in OSCC lesions (Fig. 3e). These data establish that OSCC tumor samples present higher number of $CD4^+PD1^+$ T cells.

PD-1 expression varies in the different OSCC sites

The enhanced expression of PD-1 in OSCC patients directed us to seek possible differences in the PD-1 expression according to the regions where the tumor is localized in oral cavity. For this, we assessed PD-1 expression in OSCC from lip, tongue, and other tissues (mucosa). Our results showed that mucosal lesions contained higher number of leukocytes (13.2 ± 9.2 × 10^6 cells/lesion) as compared with lip (7.6 ± 5.8 × 10^6 cells/lesion) and tongue lesions (6.7 ± 3.8 × 10^6 cells/lesion) (Fig. 4a). We analyzed the sub-population of T cells infiltrating (TILs) each type of OSCC and tumor cells, which can express PD-1. Despite the number of TILs was similar among all of lesions type (Fig. 4a); higher percentage of $CD4^+PD\text{-}1^+$ T cells was observed in tongue squamous cell carcinoma (SCC) (43.0 ± 22.5%) than that in lip (19 ± 6.5%) or mucosa SCC (17.9 ± 1.9%) (Fig. 4b). $CD8^+PD\text{-}1^+$ T cells were found in high percentages in tongue

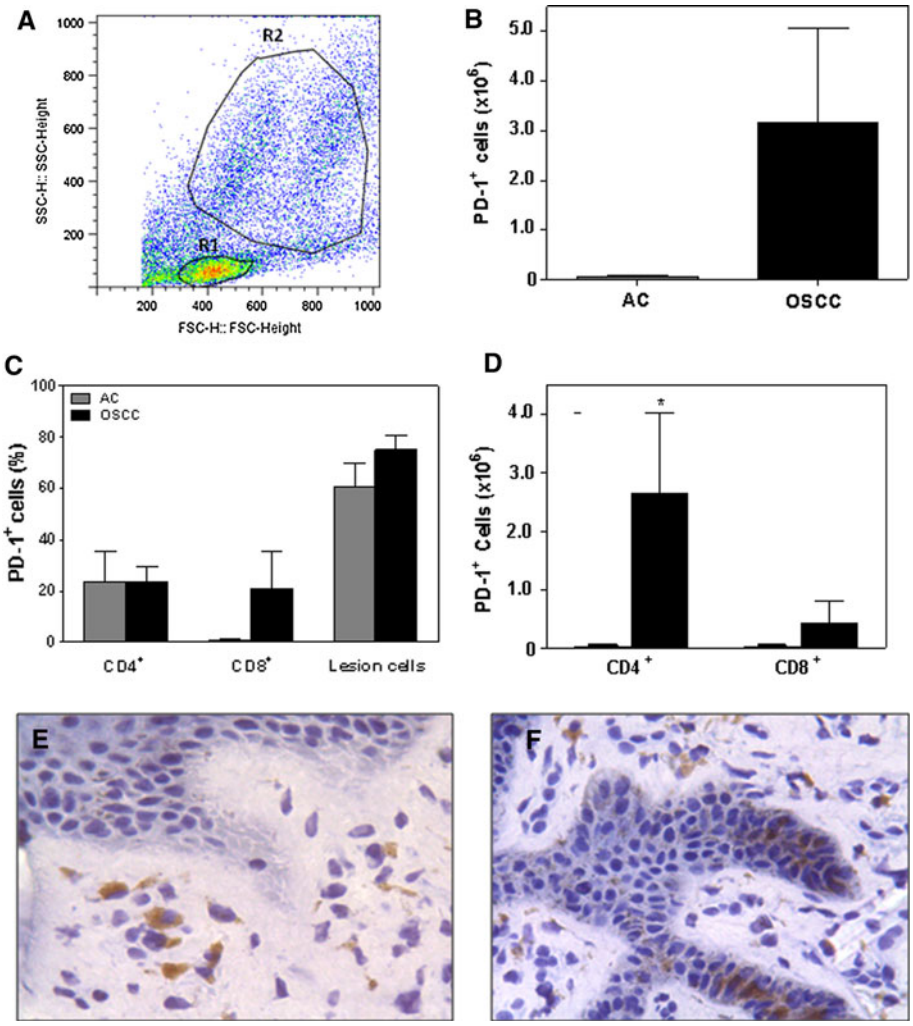

Fig. 3 PD-1 expression in lymphocytes derived from AC and OSCC lesions. Mononuclear cells isolated from AC ($n = 4$, *gray bars*) and OSCC ($n = 11$, *closed bars*) lesions were enzymatically digested with collagenase 0.25% and degraded using Medmachine, then they were analyzed by flow cytometry. **a** Gate of lymphocytes (R1) and $CD3^-C14^-CD19^-$ cells (R2) from a representative patient. **b** Percentage of total cells expressing PD-1. **c** Percentage of $CD4^+$, $CD8^+$ and $CD3^-C14^-CD19^-$ lesion cells and **d** Absolute numbers of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$. **e** Immunohistochemistry analysis of the PD-1 expression in a representative AC and **f** OSCC samples. The results are expressed as the mean ± SEM for patients. $^*P < 0.05$ compared AC and OSCC groups

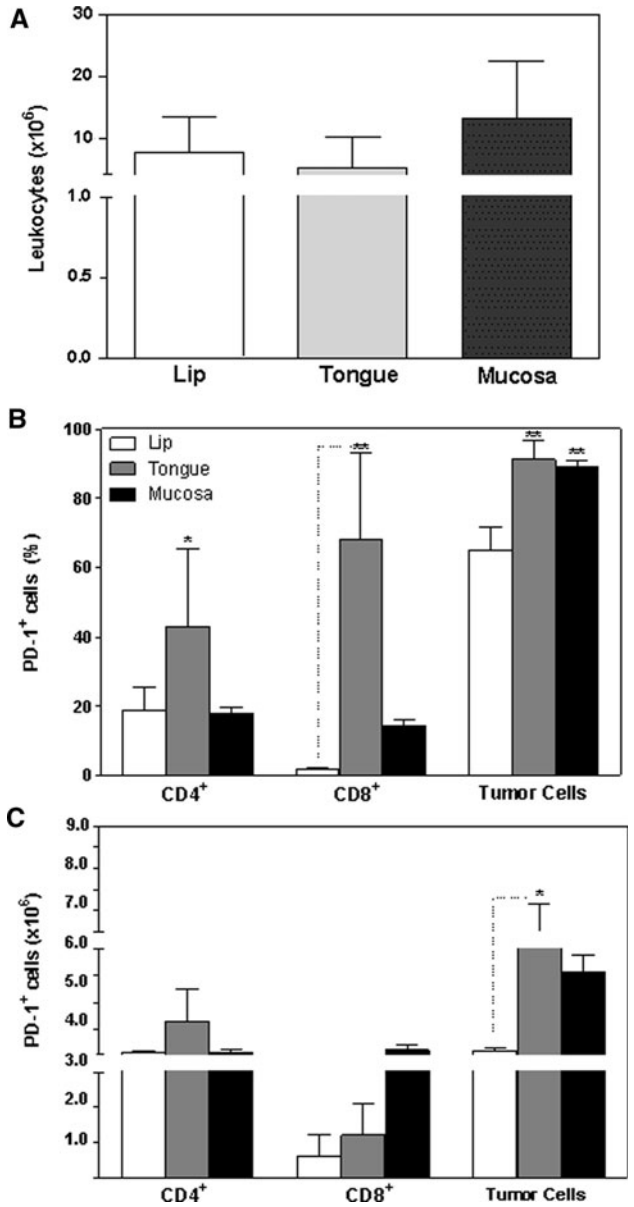

Fig. 4 PD-1 expression in different tumor sites. **a** Number of leukocytes obtained from lip ($n = 4$, *open bars*), tongue ($n = 3$, *gray bars*), and mucosal ($n = 4$, *closed bars*) squamous cell carcinoma. **b** Percentage or **c** absolute numbers of CD4, CD8 and tumor cells (gate R2) expressing PD-1. The results are expressed as the mean ± SEM for patients. $*p < 0.05$, $**p < 0.01$ when compared all three different OSCC groups

SCC (68.1 ± 25.1%), being significantly higher than that found in lip carcinoma (1.7 ± 0.7%) (Fig. 4b). Tongue SCC and mucosa SCC exhibited significantly higher percentage of the tumor cells PD-1$^+$ than lip SCC (Fig. 4b). When analyzed the number of tumor cells PD-1$^+$, data showed that tongue SCC had significant increased number than lip OSCC (Fig. 4c). In tongue SCC, where the tumor notoriously presents worse prognosis, we detected the higher levels of PD-1 expression. Tongue OSCC had higher number of PD-1$^+$ cells than lip SCC and mucosa SCC.

IFN-γ production in the lesions of AC and OSCC patients is decreased than in healthy subjects

Since the interaction of PD-1 and PD-L1 leads to inhibition of T cells proliferation and IFN-γ production, factors that are crucial for the anti-tumor responses [3], we determined the IFN-γ levels in OSCC and AC lesions. Our data showed that both AC and OSCC lesion samples contained low levels of IFN-γ, and they were significant only when OSCC was compared with healthy gingival tissue (Fig. 5a). Although AC lesions presented high levels of IL-10 (1,313 ± 665 pg/mg of AC tissue vs. 245.5 ± 108.6 pg/mg of tissue from control individuals), there were no significant differences among the tissues samples (Fig. 5b).

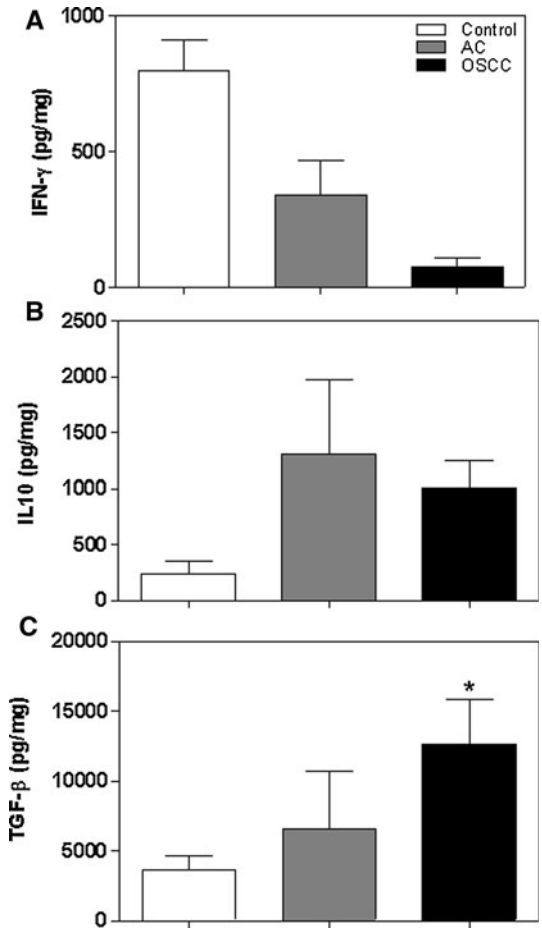

Fig. 5 Cytokine profiles of AC, OSCC lesions, and control tissue. AC ($n = 4$, *gray bars*) and OSCC ($n = 11$, *closed bars*) samples, and gingival tissue ($n = 3$, *opened bars*) from healthy control subjects were obtained. **a** IFN-γ, **b** IL-10, and **c** TGF-β were determined by ELISA. The results are expressed as the mean ± SEM from each patient analyzed individually. $*p < 0.05$ when compared all three different groups

OSCC lesions had increased levels of TGF-β in comparison with healthy gingival tissues (Fig. 5c). Therefore, lower levels of IFN-γ and higher levels of TGF-β were detected in OSCC lesions.

Discussion

Growing evidence suggests that inhibitory signals mediated by PD-1: PD-L1 could be associated with patient's cancer-specific survival rate [15, 21]. In the present study, we examined the expression of these molecules in blood and lesions of patients with actinic cheilitis (AC) and oral squamous cell carcinoma (OSCC). We report that OSCC patients had the highest expression of PD1 restricted to the tumor site; even though they had $CD8^+$ lymphocytes expressing PD-1 in the blood. On the other hand, AC patients had significant percentage and number of both $CD4^+$ and $CD8^+$ T cells expressing PD-1 in the blood but not infiltrating the lesions. Also, the proportion of circulating cells expressing PD-1 was higher in AC than in OSCC. The lower expression of PD-1 on peripheral lymphocytes from OSCC patients in relation to AC cells may be related to individual characteristics of immune response activation in OSCC subjects, or in an increase in the chemoattraction of $PD\text{-}1^+$ cells to the tumor site. The significant increase in PD-1 expression in the peripheral blood of AC and OSCC patients indicates alterations in the phenotype of circulating leukocytes, which would be directing these cells to a less responsive state. Because the level of PD-1 expression on T cells may regulate the activation threshold in T cells and their cytokine production [22], the level of functional exhaustion on T cells may also be variable among patients depending on the level of PD-1 expression. Here, the expression of PD-1 on PBMC correlates with a differential phenotype of T cells in AC and OSCC, since in OSCC patients only $CD8^+$ $PD\text{-}1^+$ cells was significantly increased. In part, these data are in accordance with those previous studies that observed high PD-1 expression in $CD4^+$ and $CD8^+$ T cells obtained from leukemia/lymphoma [23]. The accumulation of $PD\text{-}1^+$ cells in other tumor sites, like lymphomas and pancreas and kidney carcinomas have been recently described [14]. In fact, the results obtained in the present work revealed an accumulation of $CD4^+$ and $CD8^+$ T cells positives for PD-1 in OSCC lesions.

We detected a large proportion of tumor-infiltrating lymphocytes (TILs) expressing PD-1, a phenotypic comparison of CD4 and CD8 TILs with those found in circulating blood indicated that the expression of $PD\text{-}1^+$ on T cells point to differences between the groups. The number of $CD4^+PD\text{-}1^+$ cells was increased in OSCC than AC lesions, and only OSCC infiltrate presented significantly higher percentage of $CD4^+PD\text{-}1^+$ T cells. These differences are relevant especially in $CD4^+$ T cells subset, since these cells are crucial in anti-tumor immune responses [24, 25] and a limitation in the TILs activation leads to increased tumor growth and, consequently, bad prognostic of tumors [25–27]. Reinforcing this idea, we found significantly increased proportion of $PD\text{-}L1^+$ cells in the OSCC environment, but not in AC lesions. These data are compatible with invasion and malignancy features of such lesions [28, 29]. Besides, almost the totality of $CD14^+$ cells infiltrating OSCC lesions expressed PD-L1, and this fact could be contributing to ineffective response of T cells against the tumor cells and tumor escape [30–34]. We believe that PD-L1 expression might be a mediator of immune escape in oral tumors since our results showed squamous cell carcinoma $PD\text{-}1^+$ TILs in association with $PD\text{-}L1^+$ cells, suggesting that the pathway PD-1: PD-L1 could be involved with a immune evasion mechanism in oral cancer. These results open new perspectives to study this hypothesis in future experiments using an experimental murine model of skin carcinogenesis. Although PD-L1 has been identified in oral cancers [35–37], there are no data evaluating this molecule expression nor in AC lesion inflammatory infiltrate neither in these pre-malignant cells.

When we analyzed PD-1 expression in different oral carcinoma sites, we found higher levels in tongue SCC than in lip SCC, particularly on $CD8^+$ T lymphocytes and $CD3^-CD19^-CD14^-$ cells (R2 gated, please refer to Fig. 3a) present in the lesions. Similar data were previously observed in tongue SCC [38]. An explanation for this worse prognosis in tongue SCC could be the local lymphatic and blood supply of tongue, generating better nutrition and possibility of tumor invasiveness, since up to 66% of patients with primary tongue lesions have also neck disease at the time of diagnosis [39, 40]. In addition, lip SCC has a better prognosis than intraoral SCC, and perhaps it is due to that lesion, as well as AC, is related to the sun exposure while intraoral SCC lesions are more commonly associated with tobacco and alcohol consumption [29, 38, 39]. Further studies are necessary to better clarify such aspects of different OSCC and such potentially malignant disorders.

We observed that the IFN-γ production was reduced in lesion sites of both AC and OSCC, suggesting that inflammatory cells were functionally less active [41–43]. Collectively, these data indicate a cancer and a pre-malignant-induced immune suppression, especially considering that the diminishment of IFN-γ production was significant in the both AC and OSCC lesions. Perhaps, the inhibition of T cells, and even $CD14^+$ cells activation, through PD1: PD-L1 site was reducing IFN-γ secretion in the lesions [44]. According to this hypothesis, immune escape has been largely demonstrated and might be

mediated via TGF-β and engagement of PD-1 on the T cell [33]. TGF-β is a key regulator of epithelial cell proliferation, immune function, and angiogenesis [45, 46]. At sites where TGF-β is present (e.g., sites of immune privilege or inflammation), PD-L1 may promote the de novo generation of Treg cells [47]—a suppressor cell identified in OSCC tumor samples [reviewed by 20]. PD-1 also has emerged as pivotal player in the immune regulation, as well regulatory T cells (Tregs), in cancer patients and its blockade directly downregulated Foxp3 expression in Tregs [48].

Thus, to our knowledge, this is the first time that PD-1 and PD-L1 molecules are described as present in different phenotypes of lymphocytes in blood and lesions of patients with AC and OSCC. Possibly, the PD-1 expression may be used as a potential prognostic marker, as a biomarker, in oral tumors or in pre-malignant lesions.

Acknowledgments We thank Márcia Graeff, Marcimara Penitenti and Thiago José Dionísio for their technical assistance. We also would like to thank Professor Carlos Ferreira dos Santos for his helpful comments during the preparation of this research, FAPESP for financial support, and the patients for their donation of tissue and blood samples. This work was supported by FAPESP and CAPES.

References

1. Uppaluri R, Dunn GP, Lewis JS Jr (2008) Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in head and neck cancers. Cancer Immun 8:16
2. Gyrd-Hansen M, Meier P (2010) IAPs: from caspase inhibitors to modulators of NF-κB, inflammation and cancer. Nat Rev Cancer 10:561–574
3. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC et al (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192:1027–1034
4. Okazaki T, Honjo T (2006) The PD-1-PD-L pathway in immunological tolerance. Trends Immunol 27:195–201
5. Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu H, Tamada K, Pardoll DM, Chen L (2007) Interaction between B7–H1 and PD-1 determines initiation and reversal of T-cell anergy. Blood 110:180–185
6. Blank C, Mackensen A (2007) Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. Cancer Immunol Immunother 56:739–745
7. Okazaki T, Honjo T (2007) PD-1 and PD-1 ligands: from discovery to clinical application. Int Immunol 19:813–824
8. Chambers CA, Krummel MF, Boitel B, Hurwitz A, Sullivan TJ, Fournier S, Cassell D, Brunner M, Allison JP (1996) The role of CTLA-4 in the regulation and initiation of T-cell responses. Immunol Rev 153:27–46
9. Nishimura H, Nose M, Hiai H, Minato N, Honjo T (1999) Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 11:141–151
10. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, Mizoguchi A, Hiai H, Minato N, Honjo T (2001) Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 291:319–322
11. Okazaki T, Tanaka Y, Nishio R, Mitsuiye T, Mizoguchi A, Wang J, Ishida M, Hiai H, Matsumori A, Minato N, Honjo T (2003) Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice. Nat Med 9:1477–1483
12. Wang SC, Chen YJ, Ou TT, Wu CC, Tsai WC, Liu HW, Yen JH (2006) Programmed death-1 gene polymorphisms in patients with systemic lupus erythematosus in Taiwan. J Clin Immunol 26:506–511
13. Lin SC, Yen JH, Tsai JJ, Tsai WC, Ou TT, Liu HW, Chen CJ (2004) Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus. Arthritis Rheum 50:770–775
14. Yamamoto R, Nishikori M, Kitawaki T, Sakai T, Hishizawa M, Tashima M, Kondo T, Ohmori K, Kurata M, Hayashi T, Uchiyama T (2008) PD-1-PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. Blood 111:3220–3224
15. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, Nakamura S, Enomoto K, Yagita H, Azuma M, Nakajima Y (2007) Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res 13:2151–2157
16. Thompson RH, Dong H, Lohse CM, Leibovich BC, Blute ML, Cheville JC, Kwon ED (2007) PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. Clin Cancer Res 13:1757–1761
17. Antunes JL, Biazevic MG, De Araujo ME, Tomita NE, Chinellato LE, Narvai PC (2001) Trends and spatial distribution of oral cancer mortality in São Paulo, Brazil, 1980–1998. Oral Oncol 37:345–350
18. Vartanian JG, Carvalho AL, De Araújo Filho MJ, Junior MH, Magrin J, Kowalski LP (2004) Predictive factors and distribution of lymph node metastasis in lip cancer patients and their implications on the treatment of the neck. Oral Oncol 40:223–237
19. Massano J, Regateiro FS, Januario G, Ferreira A (2006) Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 102:67–76
20. Gasparoto TH, de Souza Malaspina TS, Benevides L, de Melo EJ Jr, Costa MR, Damante JH, Ikoma MR, Garlet GP, Cavassani KA, Silva JS et al (2010) Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment. Cancer Immunol Immunother 59:819–828
21. Ohigashi Y, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N, Mizuno T, Yoriki R, Kashizuka H, Yane K et al (2005) Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. Clin Cancer Res 11:2947–2953
22. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ (2007) The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 8:239–245
23. Shimauchi T, Kabashima K, Nakashima D, Sugita K, Yamada Y, Hino R, Tokura Y (2007) Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4+ T-cells in adult T-cell leukemia/lymphoma. Int J Cancer 121:2585–2590
24. Zitvogel L, Tesniere A, Kroemer G (2006) Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 6:715–727
25. Martorelli D, Muraro E, Merlo A, Turrini R, Rosato A, Dolcetti R (2010) Role of $CD4^+$ cytotoxic T lymphocytes in the control of viral diseases and cancer. Int Rev Immunol 29:371–402

26. Weber J (2010) Immune checkpoint proteins: a new therapeutic paradigm for cancer–preclinical background: CTLA-4 and PD-1 blockade. Semin Oncol 37:430–439
27. Erdman SE, Poutahidis T (2010) Cancer inflammation and regulatory T cells. Int J Cancer 127:768–779
28. Scully C, Bagan J (2009) Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. Oral Dis 15:388–399
29. Huber MA (2010) White oral lesions, actinic cheilitis, and leukoplakia: confusions in terminology and definition: facts and controversies. Clin Dermatol 28:262–268
30. Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677–704
31. Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M, Koren-Michowitz M, Shimoni A, Nagler A (2008) Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. Clin Cancer Res 14:3044–3051
32. Blank C, Gajewski TF, Mackensen A (2005) Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother 54:307–314
33. Zha Y, Blank C, Gajewski TF (2004) Negative regulation of T-cell function by PD-1. Crit Rev Immunol 24:229–237
34. Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu H, Tamada K, Pardoll DM, Chen L (2007) Interaction between B7–H1 and PD-1 determines initiation and reversal of T-cell anergy. Blood 110:180–185
35. Strome SE, Dong H, Tamura H, Voss SG, Flies DB, Tamada K, Salomao D, Cheville J, Hirano F, Lin W et al (2003) B7–H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. Cancer Res 63:6501–6505
36. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, Greenfield EA, Freeman GJ (2003) Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. J Immunol 170:1257–1266
37. Katou F, Ohtani H, Watanabe Y, Nakayama T, Yoshie O, Hashimoto K (2007) Differing phenotypes between intraepithelial and stromal lymphocytes in early-stage tongue cancer. Cancer Res 67:11195–11201
38. Neville BW, Day TA (2002) Oral cancer and precancerous lesions. CA Cancer J Clin 52:195–215
39. van der Waal I (2009) Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol 45:317–323
40. Davis CD, Emenaker NJ, Milner JA (2010) Cellular proliferation, apoptosis and angiogenesis: molecular targets for nutritional preemption of cancer. Semin Oncol 37:243–257
41. Ebelt K, Babaryka G, Frankenberger B, Stief CG, Eisenmenger W, Kirchner T, Schendel DJ, Noessner E (2009) Prostate cancer lesions are surrounded by FOXP3+, PD-1+ and B7–H1+ lymphocyte clusters. Eur J Cancer 45:1664–1672
42. Ebelt K, Babaryka G, Figel AM, Pohla H, Buchner A, Stief CG, Eisenmenger W, Kirchner T, Schendel DJ, Noessner E (2008) Dominance of CD4+ lymphocytic infiltrates with disturbed effector cell characteristics in the tumor microenvironment of prostate carcinoma. Prostate 68:1–10
43. Cianci R, Pagliari D, Pietroni V, Landolfi R, Pandolfi F (2010) Tissue infiltrating lymphocytes: the role of cytokines in their growth and differentiation. J Biol Regul Homeost Agents 24:239–249
44. Curran MA, Montalvo W, Yagita H, Allison JP (2010) PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci USA 107:4275–4280
45. White RA, Malkoski SP, Wang XJ (2010) TGFβ signaling in head and neck squamous cell carcinoma. Oncogene 29:5437–5446
46. Meulmeester E, Ten Dijke P (2011) The dynamic roles of TGF-β in cancer. J Pathol 223:205–218
47. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limón P (2010) The polarization of immune cells in the tumour environment by TGFbeta. Nat Rev Immunol 10:554–567
48. Wang W, Lau R, Yu D, Zhu W, Korman A, Weber J (2009) PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4+ CD25(Hi) regulatory T cells. Int Immunol 21:1065–1077