

Regulatory T cells in the actinic cheilitis

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BACKGROUND: Actinic cheilitis (AC) is an oral potentially malignant lesion which is the counterpart of actinic keratosis of the skin and has potential to develop into squamous cell carcinoma. Regulatory T cells (Tregs) have a critical role in modulating the antitumor immune responses. The presence of regulatory T cells in potentially malignant lesions has not been described. We chose to investigate the involvement of regulatory T cells in potentially malignant lesions.

METHODS: The frequency, phenotype, and activity of CD4⁺CD25⁺ T cells isolated from blood and lesion of AC patients were analyzed by flow cytometry. Cytokines were quantified by ELISA. Data were compared with samples from healthy subjects.

RESULTS: The frequency and suppressor activity of circulating CD4⁺CD25⁺ T cells was similar in AC patients and control subjects. However, the frequencies of IL-10⁺ positive Tregs were higher in AC patients, and these cells inhibited interferon-gamma (IFN- γ) and increased interleukin (IL)-10 productions in co-cultures. Furthermore, CD4⁺CD25⁺ T cells accumulate in AC lesions. Lesion-derived regulatory T cells suppressed lymphocyte proliferation and pro-inflammatory cytokine production. Moreover, high levels of IL-10 and transforming growth factor- β (TGF- β), and low IFN- γ were detected in the potentially malignant lesions.

CONCLUSION: Therefore, our data show that Tregs accumulate in AC lesions, and these cells could be suppressing immune responses in a potentially malignant microenvironment.

Keywords: actinic cheilitis; IL-10; potentially malignant lesion; Tregs

Introduction

Actinic cheilitis (AC) is a potentially malignant lesion of the lip predominantly induced by chronic exposure to the ultraviolet (UV) sunlight (1). AC lesions are characterized by epithelial and connective tissue alterations with increased immune cell infiltration that has potential to develop oral squamous cell carcinoma (OSCC) (2, 3). However, the events leading to AC malignant transformation remain to be elucidated. Molecular markers and immunoregulatory events that could predict AC malignant potential are not well established (4).

Regulatory T cells (Tregs), particularly those of the Foxp3⁺ subtype, play an important role in immune homeostasis modulating the activation, proliferation, and effector function of conventional T cells in several immunological settings (5–7). Tregs also are involved in tumor escape because of their ability to suppress the effector immune response of lymphocytes against tumor antigens (6–11). Tregs limit the development of T helper type 1 (Th1) immune responses that drive CD8⁺ T cells and IFN-gamma-dependent antitumor immunity, and blockade of their activity provides effective therapy against cancer (12, 13). Malignant environment could also convert naïve peripheral CD4⁺Foxp3⁻ into Foxp3⁺ Tregs after activation by T-cell receptor (TCR) in the presence of TGF- β (14–16). Although these reports suggested a direct correlation between Tregs and the suppression of the immune response in tumor microenvironment, the presence and characteristics of Tregs in potentially malignant lesion have not been described. We hypothesize that Treg cells in a malignant environment impairs T-cells-mediated immune response, thereby leaving the development of SCC. To address this hypothesis, we investigate the presence, functional and phenotypic characteristics of Tregs in the peripheral blood (PBMC) and lesions from AC patients.

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Material and methods

Subjects and study design

We used peripheral blood mononuclear cells (PBMCs) from thirteen patients with a diagnosis of actinic cheilitis (age ranged 38–86 years and mean = 65.4 ± 1.8 years old), as well as 11 age-matched healthy volunteers (age ranged 27–74 years and mean = 58.4 ± 2.2 years old). To be considered eligible for inclusion in the study, a patient had to be over the age of 18 years and to have a histological analysis showing epithelial changes restricted to the lower two-thirds of the epithelium, comprising ‘mild’ (grade 1) and ‘moderate’ (grade 2) dysplasia (17). To confirm the clinical diagnosis, incisional biopsies were performed in areas of erythema, paleness, ulceration, or atrophy, and hematoxylin and eosin-stained sections were examined under a light microscope. Of the thirteen patients enrolled in the study, nine patients had a vermilionectomy indication as an therapy. Tissue control samples were obtained from esthetic or orthodontic surgical indication. All subjects signed an informed consent allowing the use of specimens (tissues and blood) for research purposes approved by Bauru School of Dentistry, University of São Paulo (Proc. #04/2006). We collected all blood samples from subjects with AC and controls at 9:00 a.m. in heparinized vacutainers (BD Biosciences, Milan, Italy) and processed them within the following 30 min.

Chemical reagents

Leukocytes obtained from blood and oral tissues were cultivated in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (all from Sigma-Aldrich, St. Louis, MO, USA). Phytohemagglutinin (PHA) and PE-conjugated streptavidin were purchased from Invitrogen Life Technologies. All cultures and co-cultures were performed in RPMI 1640 plus 10% fetal bovine serum (FBS), 2 mM-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (GIBCO BRL)—complete RPMI.

Histopathologic analysis

For histological analysis, excised tissue samples were fixed with 10% buffered formalin and processed using routine histological techniques. Tissue sections were stained with H&E and analyzed by light microscopy.

Flow cytometry

For immunostaining, PerCP, PE- and FITC-conjugated Abs against CD3 (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD19 (HIB 19), CD25 (M-A251), CD45RO (UCHL 1), CD152 (BNI3.1), CD103 (Ber-ACT8), CD69 (FN50), CCR4 (1G1), Foxp3 (PCH101) (BD Biosciences, San Diego, CA, USA), and respective mouse and rat isotype controls were used (BD). PE-conjugated mice monoclonal antibody (mAb), antihuman GITR (110416), and biotinylated anti-TGF-β1 (LAP, 27240) were purchased from R&D Systems. PE-conjugated anti-IL-10 (JES3-19F1) and biotinylated anti-TGF-β (4492) (R&D Systems, Minneapolis, MN, USA) were used for intracellular cytokine staining.

The cell acquisition was performed on a FACSort flow cytometer using and CellQuest software (BD Biosciences). Unconjugated anti-CD3 (UCHT 1) and anti-CD28 (CD28.2) (BD Biosciences) were used for polyclonal activation.

PBMC and lesion mononuclear cell isolation

Peripheral blood mononuclear cells from AC patients and health controls subjects were obtained by centrifuging whole blood through a Ficoll-Hypaque gradient (Sigma-Aldrich). The AC biopsies were collected from the lesions using a 4-mm biopsy punch, and digested in serum-free RPMI medium with 500 µg/ml liberase CI (Roche, Basel, Switzerland) for 1 h at 37°C. They were then macerated in medcons (BD Biosciences). The cell suspension was centrifuged through a Ficoll-Hypaque gradient (Sigma-Aldrich), and the mononuclear cells were isolated and quantified. The leukocytes viability was evaluated by Trypan blue exclusion.

CD4⁺CD25^{high} T-cell separation and cultures

CD4⁺CD25^{high} T cells were enriched using a CD4⁺CD25⁺ Treg isolation kit (Miltenyi Biotec), according to manufacturer's instructions. CD4⁺CD25⁺ T cells were isolated from PBMCs and AC lesions by a first step of negative sorting using a cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies and microbeads coupled to an antihapten monoclonal antibody (CD4⁺ T-cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). This was followed by a step of positive selection of CD25⁺ cells by microbead separation (CD25 microbeads; Miltenyi Biotec), a procedure yielding to 90% or more purity as assessed by flow cytometric counting of CD4⁺CD25⁺ cells.

Immunosuppression assay

In order to verify the suppressor activity of Tregs, CD4⁺CD25⁺T cells (1×10^5 cells/well) were first activated as previously described (18, 19). For Immunosuppression assay, the total PBMC from autologous individuals were stained with CFSE and cultured alone or in presence of CD4⁺CD25⁺ T cells (1×10^4 cells/well) with or without 1 µg/ml PHA (18).

T cell proliferation

Cells were cultured for 96 h at 37 C in a 5% CO₂ atmosphere, and CFSE⁺ cells were analyzed regarding staining dilution (proliferative response). For each sample, CFSE dilution was evaluated in PHA-stimulated cultures, in the presence or absence of different amounts of purified CD4⁺CD25⁺ cells. T-cells proliferation was characterized by sequential halving of CFSE fluorescence, generating equally spaced peaks on a logarithmic scale (19). Data represent the percentage of inhibition calculated on the PHA-induced proliferation of allogeneic T cells cultured with PHA in the absence of CD4⁺CD25⁺T cells.

Detection of cytokine by ELISA

Actinic cheilitis samples were thawed on ice and homogenized in a solution containing 2 mg of protease inhibitor (Boehringer I Mannheim, Indianapolis, IN, USA). Organ extracts were centrifuged to remove all particulate material.

IL-10, TGF- β , and IFN- γ levels were measured using ELISA kits (BD or R&D Systems), according to the manufacturer's instructions. The cytokine levels in skin homogenates were normalized to the protein levels measured using a Bradford assay. IL-10, TGF- β , and IFN- γ levels also were quantified in the supernatants immunosuppression assay.

Statistical analysis

Data obtained from flow cytometry and cells proliferation assay were expressed as the standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA followed by the Tukey's multiple comparison test (PRISM Software; GraphPad, La Jolla, CA, USA). *P* values ≤ 0.05 were considered statistically significant.

Results

Phenotypic and functional characterization of Treg cells in PBMC from AC patients

First, we analyzed lymphocytes profiles in PBMC samples from AC patients and healthy controls subjects (Fig. 1). There was no significant difference in the percentage of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD4⁺CD25⁺ cells in PBMC from patients and healthy subjects (Fig. 1A). Next, we used cell surface markers to define regulatory T cells. Patients and controls showed similar percentage of CD4⁺CD25⁺ T cells expressed CTLA-4, GITR, CD103, CD45RO, CD69, Foxp3, and CCR4 (data not shown); however, higher percentage of CD4⁺CD25⁺IL-10⁺T cells (26.8 \pm 8%) was detected in AC samples compared with control samples (9.4 \pm 3.8%) (Fig. 1B). Furthermore, we found no significant differences in suppression activity of CD4⁺CD25⁺ T cells from patients and controls (Fig. 1B). However, only CD4⁺CD25⁺ cells from AC patients inhibited PHA-stimulated IFN- γ secretion and induced increased levels of IL-10 (Fig. 1C).

CD4⁺CD25⁺ T cells isolated from AC lesions present regulatory profile and exert suppressive activity

Histological analysis revealed that AC lesions presented hyperkeratosis, epithelial atrophy and acanthosis, vasodilatation, and elastosis (Fig. 2A). In the dermis was observed the presence of inflammatory infiltrate of intensity varying. To determine the lymphocytes profiles in AC lesions, isolation of leukocytes and flow cytometric experiments was performed. As shown in the insert in Fig. 2B, a great number of leukocytes (4.8 \pm 0.5 $\times 10^5$ cells) were isolated from AC lesions. AC samples exhibited accumulation of the CD3⁺ T cells (2.3 \pm 0.2 $\times 10^5$), CD4⁺ T cells (2 $\times 10^4$ to 2.4 $\times 10^5$ cells/biopsy), CD8⁺ T cells (0.5 $\times 10^4$ to 4.4 $\times 10^5$ cells/biopsy), CD19⁺ B cells (0.2 $\times 10^4$ to 1.4 $\times 10^5$ cells/biopsy), CD4⁺CD25⁺ T cells (0.5 $\times 10^4$ to 1.2 $\times 10^5$ cells/biopsy), and CD8⁺CD25⁺ T cells (0.5 $\times 10^4$ to 1.2 $\times 10^5$ cells/biopsy). As expected, AC lip-derived Treg cells express GITR (74.4 \pm 7.4%), CTLA-4 (67 \pm 13%), CCR4 (75.5 \pm 17.5%), Foxp3 (76.3 \pm 5.3%), and IL-10 (82.1 \pm 15.1%) (Fig. 2A) More intriguingly, a lower accumulation was observed of CD45RO⁺ (23.5 \pm 20%), LAP⁺ (3.3 \pm 0.2%), and CD103⁺ (1.4 \pm 0.2%) Treg cells in AC lesions (Fig. 2A).

In addition, lower levels of IFN- γ and higher levels of IL-10 and TGF- β into AC lesions were observed as compared to healthy gingival tissue (Fig. 2C). To verify the suppressor activity of AC lesion-derived CD4⁺CD25⁺ T cells, proliferative experiments were performed. CD4⁺CD25⁺ T cells isolated from AC samples inhibited the allogeneic T-cells proliferation induced by PHA (SI = 29.8 \pm 2.8%) (Fig. 2D). These results confirmed that functional Treg cells accumulate in AC lesions.

Discussion

Regulatory T cells are known to control the intensity of efficient responses through a large number of mechanisms, including the production of TGF- β , CTLA-4 expression on their cell membrane, and intracellular Foxp3 (11, 20–26). Growing evidences show that Tregs dampen T-cell immunity to tumor-associated antigens being one of the most important barrier damaging successful immune response and active vaccination (11, 25, 26).

In spite of studies concerning Tregs and cancer, there are limited data relating these cells and potentially malignant lesion. As we have previously demonstrated that Tregs are abundant in OSCC lesions (11, 27) and AC is described as a lesion with potential to develop in OSCC (2), we decided to evaluate the presence and function of these cells in blood and lesions from AC patients. We hypothesize that Treg cells in a malignant environment impairs T-cells-mediated immune response, thereby leaving the development of SCC. Because Tregs on AC lesion are originated from systemic circulation, first, we analyzed the phenotypic difference of circulating CD4⁺CD25⁺ T cells from AC individuals and controls subjects. No difference was found related to the proportion of CD4⁺CD25⁺T cells in PBMC from healthy individuals and AC patients. Circulating CD4⁺CD25⁺T cells from AC patients expressed high levels of IL-10 and significantly suppresses IFN- γ production. However, these cells presented similar Foxp3 expression and suppressor function compared with those cells from healthy control individuals. Although our data demonstrated that Tregs existed at the same frequency in both groups, the frequencies of IL-10-positive Tregs were higher in AC patients. This variability observed in the groups could be indicating a direct association between high frequencies of circulating IL-10-positive Treg cells which a presence of the anti-inflammatory response in AC patients (28). For example, in diseases with an IL-10 over-production, undesired immunosuppressive effects of IL-10 and the growth of some tumors can be observed (28).

It is possible that circulating IL-10-positive Tregs migrate to the potentially malignant lesion where they impair anticancer Th1 immunity. In fact, our results show that CD4⁺CD25⁺T cells were present in AC lesions and around 80% of them were Foxp3⁺. Tregs may be subdivided based on Foxp3 expression and cytokine profile (29). The main task of Foxp3⁺Treg cells is to migrate to inflammation sites and suppress various effector lymphocytes, especially helper T (Th) cell subsets: Th1, Th2, Th17, and follicular Th (Tfh) cells (30). Besides, Tregs have a core module of suppression driven by Foxp3 expression, where they are also able to adapt to changes in their environment and

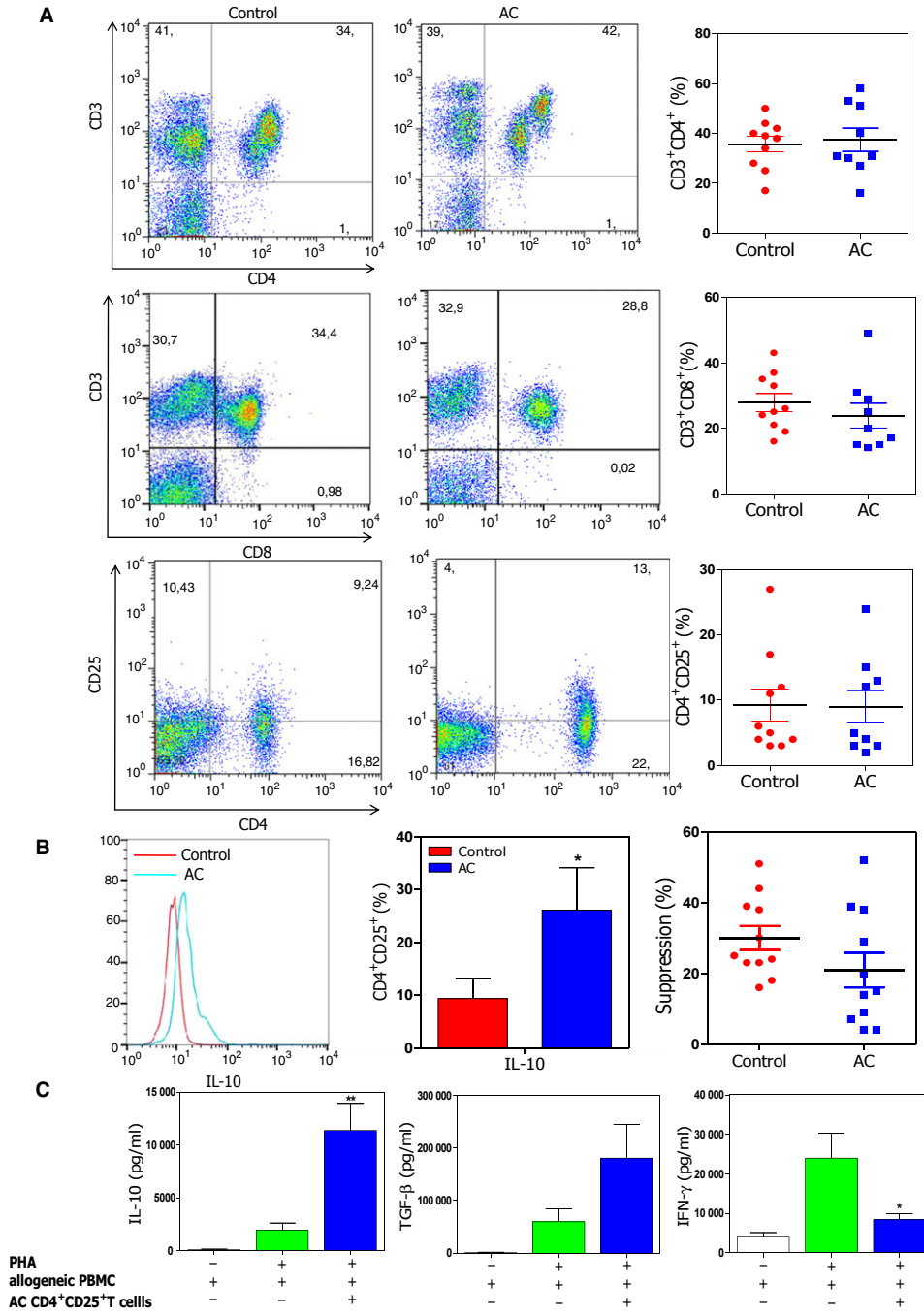


Figure 1 Characterization of blood lymphocytes from control subjects and actinic cheilitis patients. Peripheral blood mononuclear cells (PBMC) isolated from control subjects (red circle) and patients with actinic cheilitis (AC) (blue square) were analyzed by flow cytometry. (A) The subpopulations of lymphocytes in PBMC were represented as percentage of gated lymphocytes. Each dot plot in the right panel represents the percentages of CD3⁺, CD4⁺, CD8⁺, CD25⁺, and CD19⁺ cells. (B) Blood CD4⁺CD25⁺ T cells from controls and patients were analyzed for IL-10 positivity. Representative histograms of the percentage of IL-10⁺ Tregs in total T cells are shown. In the left panel is represented the suppressor activity of magnetic bead-sorted CD4⁺CD25⁺ T cells. (C) IL-10, transforming growth factor-β (TGF-β), and IFN-γ production in supernatants from in vitro suppression assays. The error bar indicate ± SEM. **P* < 0.05 compared with controls.

harness additional modules by the expression of other transcription factors normally associated with other T cell subtypes in order to better control immunopathology (30). Foxp3 regulates expression of a large number of genes including those responsible for key features of Tregs, such as high expression of cytotoxic T lymphocyte antigen-4

(CTLA-4), a critical molecule involved with the suppression function of Tregs (30). In fact, we found more than 70% of CD4⁺CD25⁺T cells expressing CTLA-4 in AC lesions but not in the blood from patients. Besides, high percentages of CD4⁺CD25⁺T cells from AC lesions expressed GITR, CCR4, and CTLA-4. Importantly, IL-10 and TGF-β were

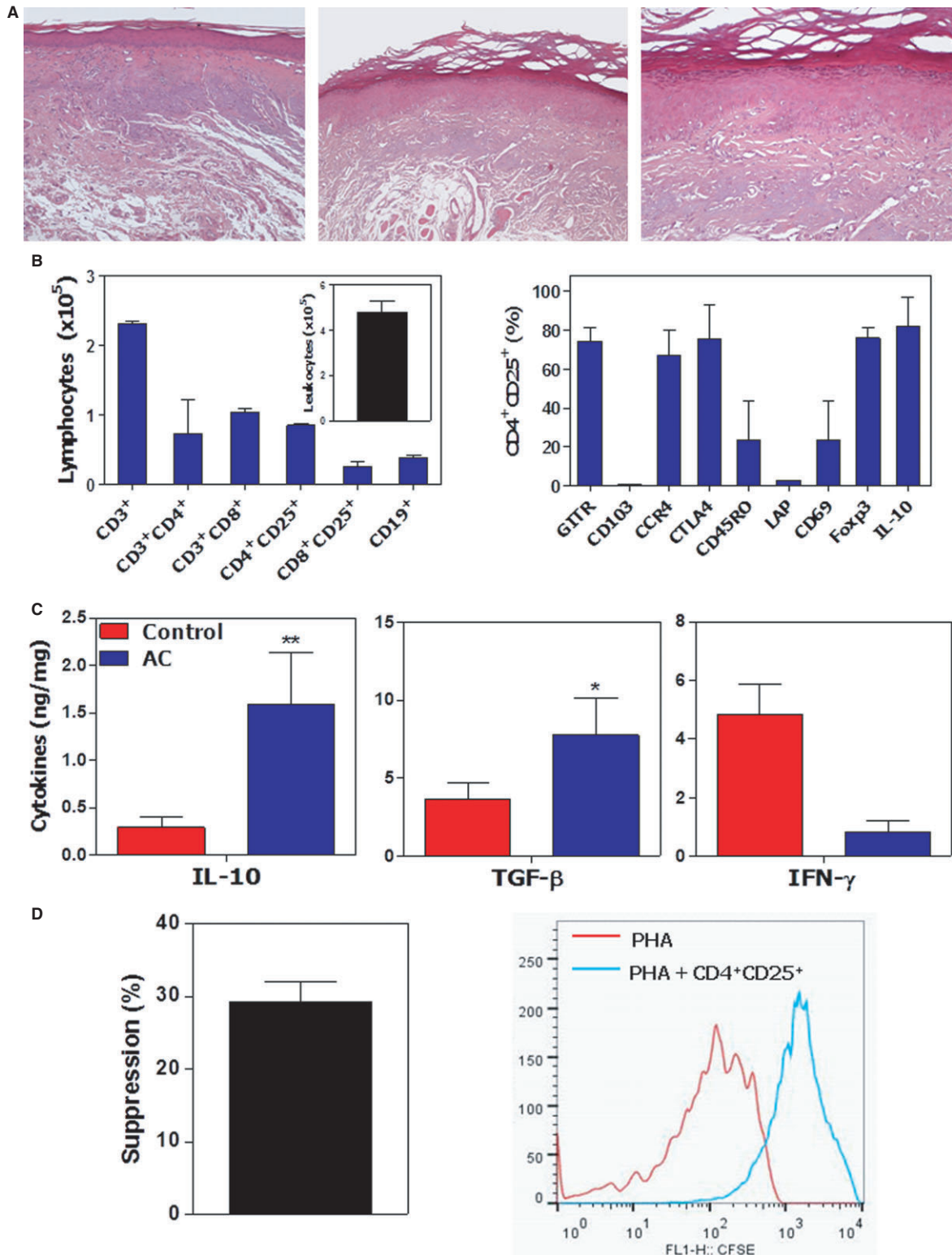


Figure 2 Phenotypic characterization of leukocytes derived from actinic cheilitis lesions (AC) and cytokine profiles of AC lesions and control tissue. (A) Representative of H&E-stained sections of lesions from AC patients. Magnification 200× (left side) 400× (center and right side). (B) Data represent the total number of CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD25⁺, CD8⁺CD25⁺, and CD19⁺ cells in lip actinic cheilitis lesions. Insert shows total number of lymphocytes infiltrating AC lesions. Data represent the percentage of CD4⁺CD25⁺ express CCR4, CTLA-4, GITR, CD103, CD45RO, CD69, LAP, Foxp3, and IL-10.(C) L-10, transforming growth factor-β (TGF-β), and IFN-γ protein levels were measured in AC lesions and control tissue by ELISA. (D) CD4⁺CD25⁺ T cells (1 × 10⁴ cells/well) isolated from AC lesions of patients were expanded with 0.5 μg/ml anti-CD3, 1 μg/ml anti-CD28, 1 μg/ml PHA, and exogenous 10 ng/ml rhIL-2 and tested for their ability to suppress the proliferation of allogeneic peripheral blood mononuclear cells (PBMC). Representative histograms of CFSE labeled allogeneic PBMC cultivated with PHA (red line) or PHA plus CD4⁺CD25⁺ T cells (blue line). The error bar indicates ± SEM. **P* < 0.05 and ***P* < 0.01 when compared with controls.

strongly detected in the AC microenvironment. High levels of IL-10 and TGF- β are a strong indicative the presence of Tregs in the AC microenvironment (29). Our data demonstrated that 75% of CD4⁺CD25⁺T cells expressed IL-10. Although many cell types might be producing them (26, 27), our results indicated that the Tregs might be the major source of IL-10 in AC microenvironment. IL-10-producing cells have been suggested to contribute to an immune suppressive tumor microenvironment (31, 32). It is possible to speculate that circulating IL-10⁺ Tregs migrate to the potentially malignant lesion and impair anticancer Th1 immunity. In fact, recently, it has been shown that tumor-associated IL-10 was produced by an activated Treg population (31). IL-10⁺ Tregs in the tumor required type I IFN signaling pathway and that, together, IL-10 and type I IFN act in a network that is required to limit Th17-type inflammation specifically in tumor microenvironment (31, 33). Although in this study, we not have focused on the role of Th17 cells on antitumor response, others have observed that Th17 cells and Th17-associated cytokines have been shown to have both antitumorigenic and pro-tumorigenic functions (34). Tregs and Th17 cells shared immunosuppressive mechanisms and have their function associated to TGF- β production (34). TGF- β contributes to the inhibition of anticancer immunity (15). During tumor progression, excess TGF- β suppresses immune surveillance by attenuating the antitumor functions of CD8⁺ T cells, CD4⁺ T cells, and dendritic cells (35). In this way, the presence of TGF- β Tregs could be impair anticancer Th1 immunity (19, 21, 35–37). The expression of Tregs-associated markers IL-10, TGF- β , Foxp3, and CTLA-4, as well as the influx of Tregs into the AC microenvironment could determine a worse prognosis to the host, as observed in others types of tumor (38, 39).

Actinic cheilitis lesions consists in morphologically altered tissue in which external factor is responsible for the etiology and malignant transformation; it carries the risk of oral OSCC (40). From this point, the presence of Foxp3⁺ Tregs accumulated in AC lesions would be able to inhibit T-cell proliferation in situ, generating a microenvironment poor in cytokines with known antitumor activity, high levels of suppressor cytokines (IL-10 and TGF- β) (20, 26, 27, 29, 30, 36–39, 41). In fact, our data demonstrated low levels of IFN- γ in AC lesions. Evidence has suggested a critical role of IFN- γ on tumor immunity, and this cytokine plays a role important in the antitumor effector mechanisms (42–44). Blockage of IFN- γ has been shown to inhibit the tumor regression as it plays two distinct roles in expressing the antitumor efficacy of IL-12: one is to support the T-cell acceptability of tumor masses, and the other is to mediate the antitumor effects of migrated T cells (43, 45). Thus, as the effective T cell antitumor response depends on the IFN- γ , its low levels detected in the lesions might facilitate the AC persistence, recurrence, progression, or malignization.

The specific inhibitor/regulatory role of CD25⁺Foxp3⁺ T cells in AC lesions had not been previously investigated. We observed that AC microenvironment exhibited infiltration of Tregs cells presenting phenotype and function consistent with natural Tregs (45). Treg-mediated immunosuppression may characterize one of the immune evasion mechanisms facilitating the relapse of this disease or even

its malignization to OSCC (2, 46, 47). According to our results, the presence of Tregs in AC lesions may be, in part, responsible for downregulation of immune responses impairs T-cell proliferation and cytokines production. As AC is a potentially malignant lesion, the presence of Treg cells could be one important factor addressing the tumor onset (2, 47). Further studies are necessary to establish exact influence of Tregs on activated T cells and their role in the regulation of AC lesion. Understanding the role of Tregs infiltrating AC lesion might contribute with novel therapeutic interventions. The presence of these cells might be responsible for impaired cellular immunity against this potentially malignant lesion and, consequently, be involved in its malignant transformation.

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