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Original Article

The expression of PD-1 and LAG-3 in periapical lesions

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Abstract: Periapical lesions are the distinct result of chronic root canal infection and could generate severe bone resorption surrounding apical regions. Despite the local cytokine and cell-mediated immune responses, periapical lesions are also characterized by its auto-restrict inflammation. However, the detailed mechanism related to its auto-restriction of immune response is still unclear. Co-inhibitory immune checkpoints are important molecules which could negatively modulate immune response especially in T cell function. In this study we detected the expressional pattern of PD-1/LAG-3 in periapical lesions. Immunohistochemical staining showed that the inflammatory response including up-regulation of TNF-α and the infiltration of T cells, was severe in granuloma and restricted in periapical cyst. PD-1 and LAG-3 both could be detected in granuloma and cyst, while scarcely observed in control group. Exhausted T cells, characterized by PD-1 or LAG-3 positive, accumulated within granuloma and reduced in cyst. Our study revealed that in periapical lesions, T cell exhaustion characterized by PD-1 or LAG-3 positive, might contribute to the auto-restriction of inflammatory response in periapical lesions.

Keywords: Periapical lesions, inflammation, programmed cell death protein 1 (PD-1), lymphocyte activating gene 3 (LAG-3)

Introduction

Periapical lesions formed as a result of continuous root canal infection are characterized by host inflammatory response and periapical bone destruction. Granulomas are histologically identified by fibrous and granular tissues. Radicular cysts contain cavities completely enclosed by epithelial lining, and present remitted inflammatory response than that in granuloma [1-5]. Studies have shown periapical lesions infiltrated by different inflammatory cells such as lymphocytes [6]. The presence of mass immune cells indicates that local cytokine and cell-mediated immune responses are taking place [7-9]. However, the modulation of the immune cell activation and inactivation in periapical lesions is still unclear.

Multiple subpopulations of immune cells are found in periapical lesions, while $CD8^+$ and $CD4^+$ T cells seem to be the dominant lymphocytes in periapical granuloma [1]. Appropriate activation of antigen-specific T cells leads to their proliferation and acquisition of effector function [10]. In some situation such as chronic infection, T cell dysfunction occurs as a result of prolonged antigen exposure: the T cell loses the ability to proliferate in the presence of the antigen and progressively fails to produce cytokines to lyse target cells. Several studies have shown that the expression of certain inhibitory receptors is associated with compromised function of T cells in patients with chronic infection.

Co-inhibitory or immune checkpoint receptors have a critical role in the maintenance of immune homeostasis: their expression on effector T cells ensures the proper contraction of effector T cell responses and their expression on regulatory T (Treg) cells guarantees the proper function of Treg cells to control effector T cells [11]. Programmed cell death protein 1 (PD-1) is a negative immune checkpoint molecule [9]. It was firstly reported as a type I transmembrane protein and has two reported ligands,

programmed cell death ligand 1 and ligand 2 (PD-L1 and PD-L2) [12]. In healthy people, PD-1 is generally expressed on activated T cells, natural killer (NK) cells, B cells and some myeloid cells [13]. Lymphocyte activation gene-3 (LAG-3) was discovered as a molecule that is upregulated on activated $CD4^+$ and $CD8^+$ T cells and a subset of natural killer (NK) cells [14]. The expression of PD-1 and LAG-3 indicated the dysfunctional or exhausted T cells [13].

The objective of the present study was to determine the expression of PD-1 and LAG-3 in chronic infection in periapical lesions, and identify the possible role of exhausted $PD\text{-}1^+$ or $LAG\text{-}3^+$ T cells in the inflammatory auto-restriction in periapical lesions.

Materials and methods

Tissue samples

All the samples were collected from the School and Hospital of Stomatology, Wuhan University with informed consent. 13 cases of human radicular cysts and 14 cases of human periapical granulomas were studied and all the cases were collected during last 6 months. The patients (ranging from 21-56 yrs) had radiographic evidence of periapical lesions and excluded systemic diseases. They had not been treated with antibiotics for at least 1 month before surgery. No distinctions between specimens were made regarding etiology or tooth type. The periapical lesions were diagnosed on the basis of clinical and histopathologic criterions. Radicular cysts were diagnosed as below: (a) a lesion situated on the periapical region of a non-vital tooth, (b) a cavity with fluid or semisolid content, (c) histologic observation of stratified nonkeratinizing squamous epithelium lining a cystic cavity [4, 15]. Dental granulomas were diagnosed by the observation of a chronic inflammatory reaction, with a large number of infiltrating inflammatory cells (macrophages, lymphocytes and plasma cells) [1]. The control group was healthy oral mucosa tissues (n=6), they were obtained from residual mucosa after third molar extraction which did not show any inflammation.

Immunohistochemical staining

Human tissue samples, including healthy oral mucosa (n=6), periapical granuloma (n=14), and radicular cyst (n=13) respectively were fixed in 4% buffered paraformaldehyde, then dehydrated and embedded in paraffin. The paraffin block-embedded tissue was cut into 4 μm sections, deparaffinized, and rehydrated. Antigen retrieval was performed by treatment with 10 mM citrate buffer (pH 6.0) for 5 min. The immunohistochemical staining was performed as previously described. Briefly, sections were incubated with first antibodies overnight at 4°C. The antibodies included: anti-CD4 (Abcam, Cambridge, MA, USA), anti-CD8 (Abcam, Cambridge, MA, USA), anti-CD3 (Abcam, Cambridge, MA, USA), anti-LAG-3 (Cell Signaling Technology, Danvers, MA, USA), anti-tumor necrosis factor (TNF)-α (Cell Signaling Technology), anti-PD-1 (Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibody was used to detect antibody binding using a diaminobenzidine substrate kit according to the manufacturer's protocol. Sections of human squamous-cell carcinoma were used as positive control. As negative control, samples were treated as described, except that the primary antibody was replaced by a solution of bovine serum albumin in PBS.

Immunostaining assessment

After the immunohistochemical treatment, the tissue sections were examined by three observers under a light microscope. Semi-quantitative analysis of immunohistochemistry was considering both the staining intensity and the positive proportion. The positive reactions were defined as brown staining within cells, and the counts were calculated by positive cells/total number. At least five areas at 400× magnifications were calculated by three different experimenters. The intensity was scored as followed: 0, negative; 1, weak; 2, moderate; 3, strong. The proportion was scored as followed: 0, less than 5%; 1, 5%-25%; 2, 25%-50%; 3, 50%-75%; 4, more than 75%. The immunohistochemical evaluation index was calculating by multiplying the intensity and proportion.

Double immunofluorescence staining

Double immunofluorescence staining was performed as previously describe [11]. Tissue sections were disposed to deparaffinized, rehydrated, and antigen retrieval. After blocked with 2.5% BSA for 1 h at 37°C, tissues were incubated with primary antibody PD-1/CD4, PD-1/

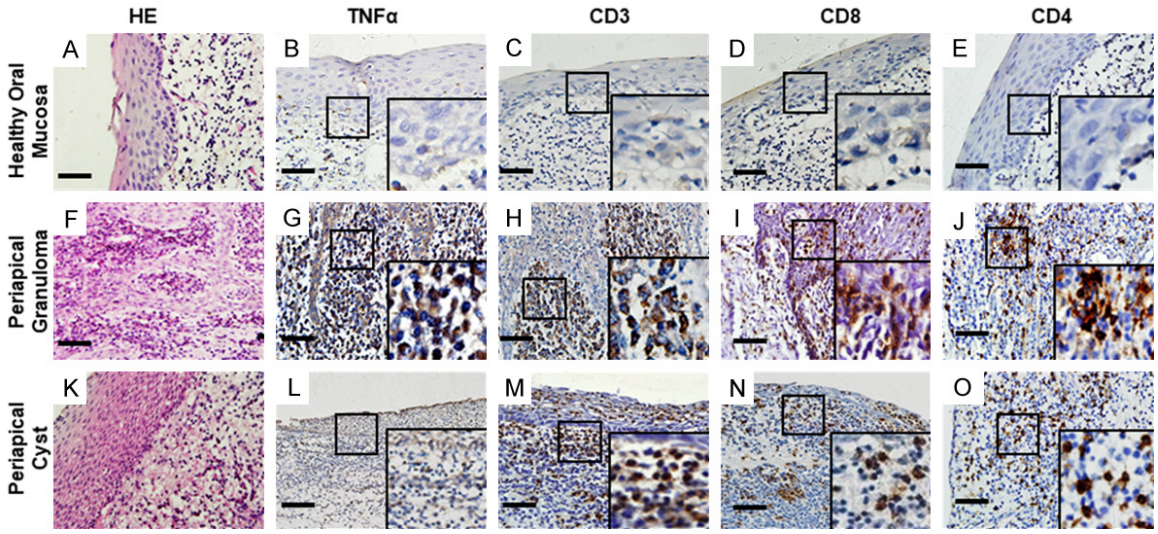

Figure 1. The expression of TNF-α, CD3, CD4 and CD8 in periapical lesions. Inflammatory response of healthy oral mucosa (n=3), periapical granuloma (n=14) and radicular cyst (n=13) was detected by immunohistochemical staining. A, F, K. HE staining of healthy oral mucosa, periapical granuloma and radicular cyst. B, G, L. TNF-α significantly overexpressed in periapical granuloma, and mildly decreased in radicular cyst. In the healthy oral mucosa, TNF-α were scarcely detected. C, H, M. The infiltration of total T cells was detected by CD3 immunostaining. Similar to the expression of TNF-α, $CD3^+$ T cells accumulated in granuloma and reduced in radicular cyst, while it is seldom observed in healthy oral mucosa. D, I, N, E, J, O. CD8 and CD4 were stained, the positive cells accumulated within periapical granuloma and radicular cyst, while $CD8^+$ and $CD4^+$ cells in healthy mucosa were barely detected. Scale bar = 50 μm.

CD8, LAG-3/CD4 and LAG-3/CD8 respectively overnight at 4°C. Then they were incubated with Alexa Fluor 488- and 549- conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA). Following counterstained with 4',6-diamidino-2-phenylindole (DAPI). Tissues sections were observed and photographed using a fluorescence microscope (Leica, Germany).

Statistical analyses

All the data presented as Mean ± SEM. Percentage of positive cells in immunohistochemical staining and the ratio of co-localization were calculated by Image Pro Plus 6.0. Data were analyzed and visualized using Graph Pad Prism 5.0. One-way analysis of variance followed by post-Tukey test. All experiments were independently repeated in triplicate. $P<0.05$ was regarded as statistically significant.

Results

Expression of TNF-α and the infiltration of T cells in periapical lesions

To characterize the inflammatory condition of periapical lesions, we performed immunohistochemical staining of TNF-α, CD4, CD8, CD3 in human samples including periapical granuloma and radicular cyst as compared with oral mucosa. The expression of TNF-α and the accumulation of $CD4^+$, $CD8^+$, $CD3^+$ T cells were found to be significantly increased in periapical granuloma samples (**Figure 1G-J**). Radicular cyst samples were mildly stained for TNF-α and the number of $CD4^+/CD3^+/CD8^+$ T cells were less than that in periapical granuloma (**Figure 1L-O**). TNF-α and T cell markers were barely expressed in healthy oral mucosa (**Figure 1B-E**).

Expression of PD-1 and LAG-3 in periapical lesions

Immune checkpoint molecules PD-1 and LAG-3 were stained among healthy oral mucosa and periapical lesions (**Figure 2A**). The percentage of positive cells in the lesions was determined and quantified (**Figure 2B** and **2C**). Both PD-1 and LAG-3 were scarcely detected in healthy oral mucosa. The adaptive increase of LAG-3 was found within the epithelial fraction of periapical granuloma, while in radicular cyst, it was mainly located beneath epithelium layer. The expression of PD-1 was statistically higher in radicular cyst ($P<0.05$) and periapical granulo-

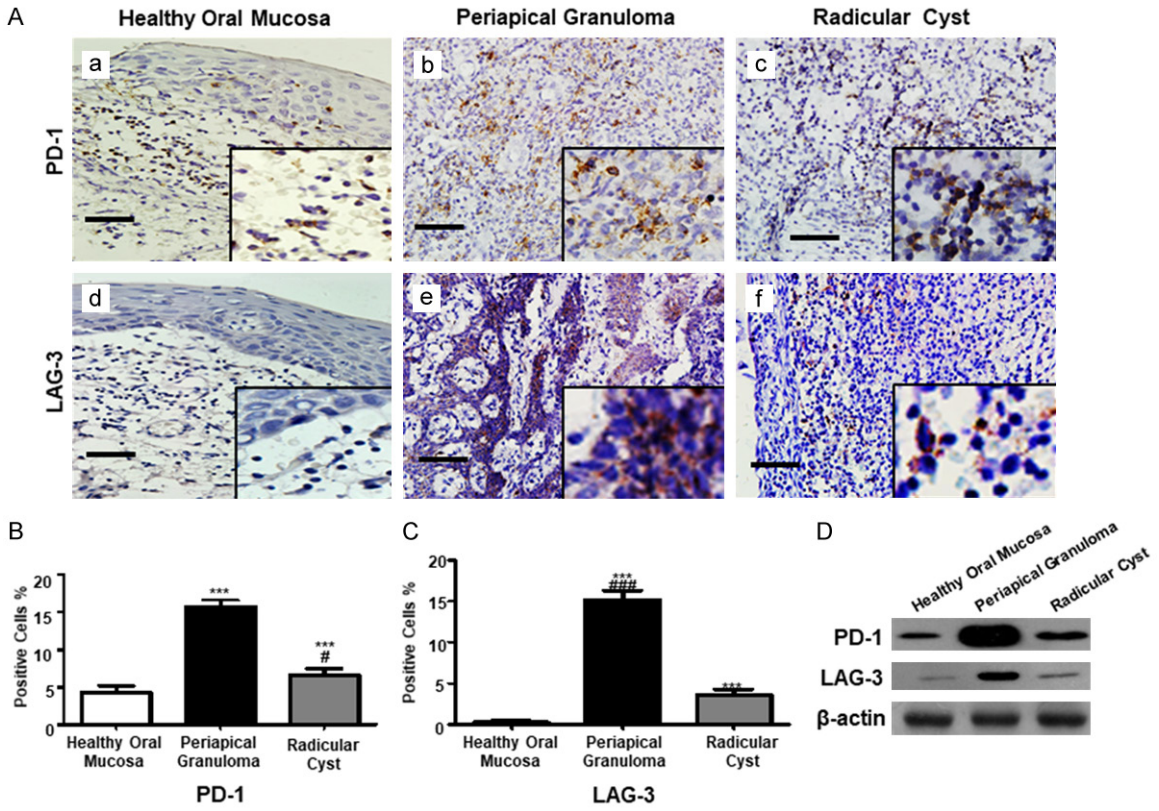

Figure 2. The expression of PD-1 and LAG-3 in periapical lesions. A. We stained both PD-1 and LAG-3 in periapical lesions also in healthy oral mucosa. B and C. PD-1$^+$ and LAG-3$^+$ cells in each group were calculated. Comparing to the healthy oral mucosa, both PD-1 and LAG-3 were significantly upregulated in periapical granuloma and radicular cyst. Both PD-1 and LAG-3 mostly expressed in periapical granuloma. D. Western blotting analysis showed similar result, PD-1 and LAG-3 mainly expressed in periapical granuloma and decreased in radicular cyst. Experiments were repeated in triplicate. Mean ± SEM; **, P<0.05; **, P<0.01; ***, P<0.001* versus healthy oral mucosa group. *#, P<0.05; ##, P<0.01*; versus periapical granuloma group. Scale bar = 50 μm.

ma (*P<0.001*) as compared with oral mucosa. Remarkably, the immunoreactivity of PD-1 was significantly increased in periapical granuloma as compared with radicular cyst (*P<0.05*). Similar expressional pattern of LAG-3 could be detected within periapical lesions. The immunostaining of LAG-3 was statistically higher in granuloma than that in the radicular cyst (*P<0.001*) and oral mucosa (*P<0.001*).

Western blot analysis indicated same results that the expression of TNF-α, LAG-3, and PD-1 peaked in granuloma and decreased in radicular cyst, but barely expressed in oral mucosa (**Figure 2D**).

Co-localization of CD8 with PD-1 or LAG-3 in periapical lesions

To determine the dysfunction or exhaustion of CD8$^+$ T cells in periapical lesions, we double labeled CD8 with PD-1 or LAG-3 (**Figure 3A**), and statistically analyzed the ratio of co-localization (**Figure 3B** and **3C**). Both CD8$^+$PD-1$^+$ and CD8$^+$LAG-3$^+$ T cells mostly accumulated within granuloma, and barely could be detected in healthy mucosa or periapical cysts. Statistical analysis showed that the differences between granuloma and radicular cyst in the ratio of co-localization is significant, as well as comparing with oral mucosa (**Figure 3B** and **3C**, *P<0.001*).

Co-localization of CD4 with PD-1 or LAG-3 in periapical lesions

In order to better illustrate the dysfunctional or exhaustion condition of CD4 positive T cells, we also double labeled CD4 with PD-1 or LAG-3 (**Figure 4A**). The ratio of CD4 and PD-1 co-localization were calculated in column graph (**Figure 4B**, *P<0.001* respectively as compared with mucosa). The CD4 and LAG-3 double positive T

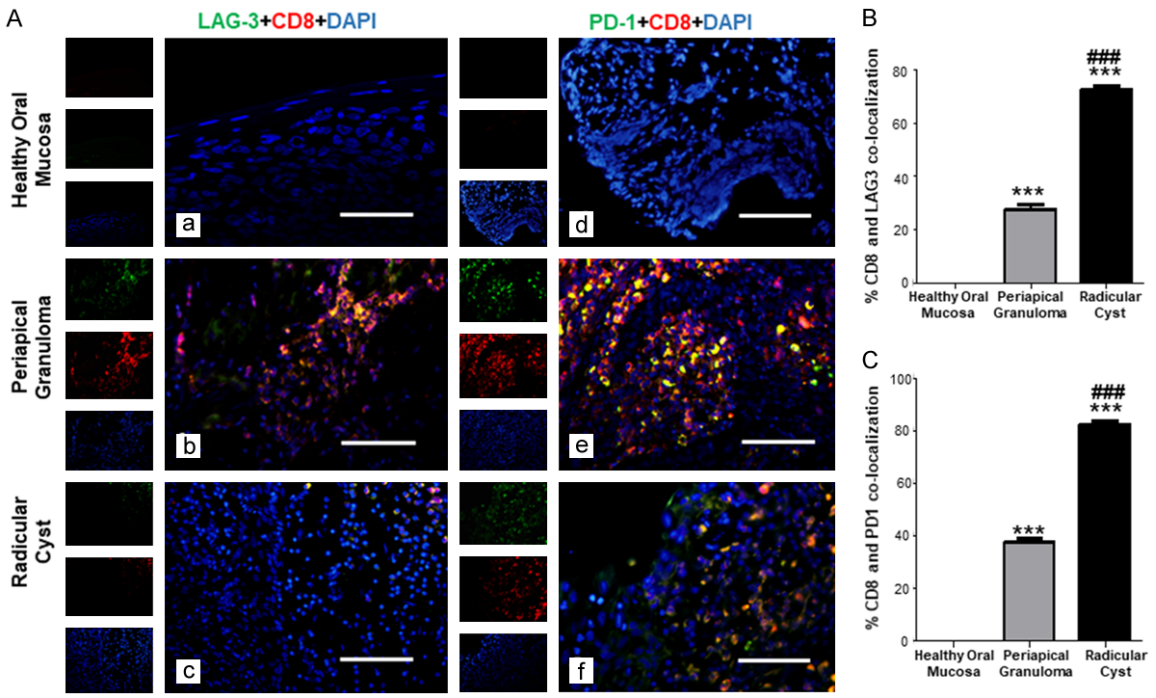

Figure 3. Co-localize of $CD8^+$ T cells with PD-1 and LAG-3 in periapical lesions. A. CD8 was co-localized with LAG-3 and PD-1 respectively in (a, d) healthy oral mucosa, (b, e) periapical granuloma, and (c, f) radicular cyst. B, C. Rate of co-localization of CD8 with LAG3 and CD8 with PD-1 were calculated, both of the rate significantly increased in periapical granuloma and even higher in radicular cyst. Experiments were repeated in triplicate. Mean ± SEM; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus healthy oral mucosa group. #, $P<0.05$; ##, $P<0.01$; versus periapical granuloma group. Scale bar = 50 μm.

cells also significantly decreased in radicular cyst than that in periapical granuloma (*P<0.001* as compared with periapical granuloma). Likewise, consistent with former results, neither PD-1, LAG-3, CD4 expressed in healthy oral mucosa.

Discussion

In periapical inflammation, the balance between persistent infection and continuously activated immune response leads to the differences in immune response between periapical granuloma and radicular cyst [4, 6, 7]. Our study showed expression of the putative immune checkpoint molecules PD-1 and LAG-3 in periapical lesions.

T cells were reported as crucial immune cells participated in the transition of periapical lesions [4]. The function of T cells had been wildly explored in recent years, especially the dysfunction and exhaustion of T cells and their roles in but the inflammation differs between exact way it modulates the inflammatory response of periapical lesions is still unclear. In our study, we detected the severity of immune response in periapical lesions. Both the expression of TNF-α and the infiltration of $CD4^+/CD8^+$ T cells were significantly increased in periapical granuloma and remitted in radicular cyst, these results are conformity with other studies [3, 4, 7]. As shown in our data, $CD4^+/CD8^+$ T cells infiltrated both in granuloma and cyst, despite its progressive role in inflammation progression, vast studies had reported a dysfunctional differentiation state of T cells in chronic infections. This phenomenon could negatively regulate the immune response and contribute to inflammatory anesis [14, 16, 17]. Thus, we hypothesized that the deactivated T cells during the inflammatory progression might contribute to the inflammatory auto-restriction of periapical lesions.

Co-expression of LAG-3 and PD-1 within T cells indicated the exhaustion of T cell function[14]. We stained LAG-3 in human periapical granuloma and radicular cyst. According to the statistical analysis, LAG-3 positive cells significantly accumulated in granuloma. Previous study showed that LAG-3 impacts the function of

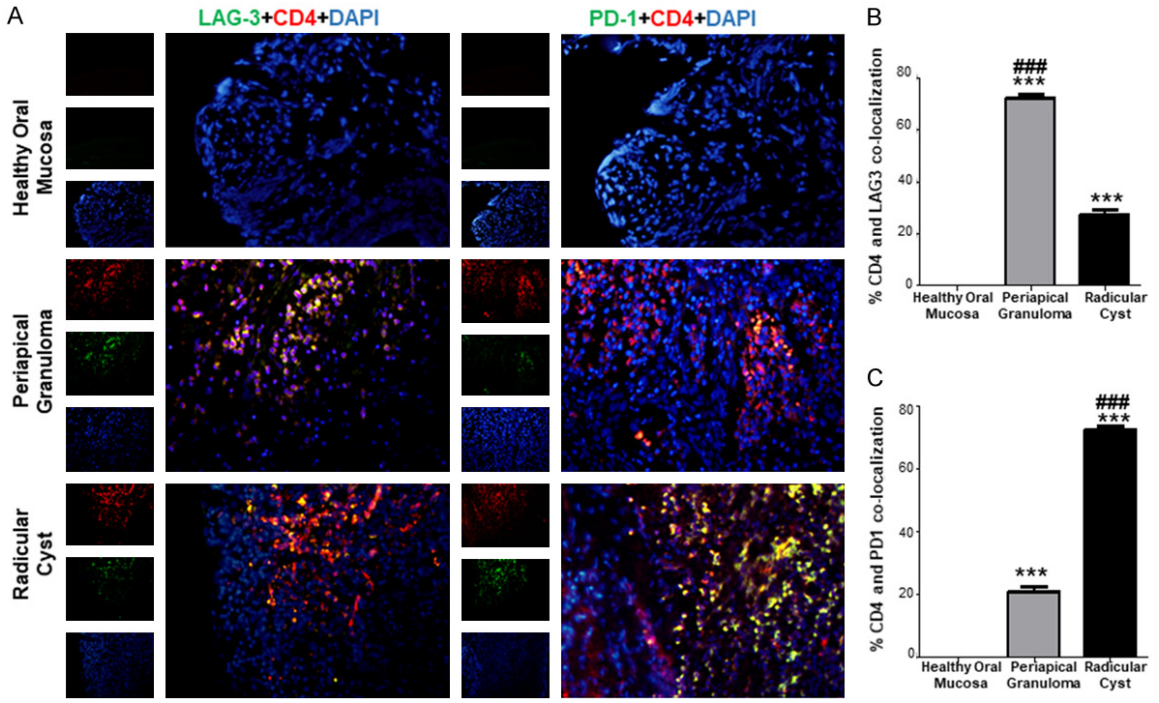

Figure 4. Co-localize of $CD4^+$ T cells with PD-1 and LAG-3 in periapical lesions. A. The $CD4^+PD\text{-}1^+$ and $CD4^+LAG\text{-}3^+$ T cells were double labeled in periapical lesions. B. CD4 and LAG-3 scarcely observed in healthy oral mucosa, and mainly co-localized in periapical granuloma. C. Co-localization of CD4 and PD-1 was detected in periapical granuloma, while $CD4^+PD\text{-}1^+$ cells mostly gathered in periapical granuloma and decreased in radicular cyst. ***, $P<0.001$ versus healthy oral mucosa group. ###, $P<0.001$ versus radicular cyst group. Scale bar = 50 μm.

$CD8^+$ T cells and NK cells by binding to LSECtin, a member of the DC-SIGN family of molecules, is another ligand for LAG-3 (Xu et al., 2014). While in $CD4^+$ T cells, LAG-3 is expressed on both activated natural Treg (nTreg) and induced $CD4^+FoxP3^+$ Treg (iTreg) cells, where expression levels are higher than that observed on activated effector $CD4^+$ T cells (Huang et al., 2004). In our study, we double labeled LAG-3 with CD4 or CD8, the results showed that in granuloma both CD4 and CD8 T cells highly expressed LAG-3, which indicated that large percentage of T cells in granuloma were exhausted. This phenomenon revealed that the progression of inflammation might accompanied by suppression of immune response.

T cell exhaustion might contribute to the self-restriction of the inflammation in periapical lesions. In viral infection, immune modulation, tumor immune escape and T cell exhaustion are wildly explored [12, 18-20]. DCs genetically modified to overexpress PD-L1 to diminish macrophage, $CD4^+PD\text{-}1^{high}$ and $CD8^+PD\text{-}1^{high}$ T cell infiltration thus inhibits T cell function [21, 22]. In our study, $CD4^+PD\text{-}1^+/CD8^+PD\text{-}1^+$ T significantly accumulated in granuloma, which revealed that the inflammatory response in granuloma starts to restrict by increasing deactivated $CD4^+/CD8^+$ T cells. In the study of head and neck squamous cell carcinoma, PD-1/PD-L1 signaling axis, whose expression is upregulated in human and mouse HNSCC, inhibits the antitumor immunity response (Yu et al., 2015; Bu et al., 2017). Thus, in granuloma, the increased expression of PD-1 on CD4 and CD8 positive T cells indicated that immune negatively modulation also activated during infection and inflammation.

In conclusion, our results showed that the T cells could express PD-1 and LAG-3 due to progressive inflammation in periapical lesions. These T cells are characterized as dysfunctional or exhausted T cells, and might serve as a potentially novel way to hinder the progressive inflammation, thus restrict the severe bone destruction caused by periapical granuloma. However, further *in vivo* investigation by mouse model is needed to understand the function and precise mechanism of T cell dysfunction and exhaustion in the modulation of inflammatory response.

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Disclosure of conflict of interest

None.

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