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Down-regulated Treg cells in exacerbated periodontal disease during pregnancy

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ABSTRACT

Pregnancy is a special period marked with complicated changes in various immune responses. Although pregnant women are prone to developing gingival inflammation, its immunological mechanism remains to be clarified. In a modified ligature-induced periodontal disease murine model, pregnant mice developed more severe alveolar bone loss. Using this model, we investigated the Treg responses during exacerbated periodontal disease in pregnant mice. We tested Treg-associated molecules in gingival tissues by quantitative real-time PCR and found decreased gingival expression of Foxp3, TGFβ, CTLA-4, and CD28 in pregnant mice after periodontal disease induction. We further confirmed that lower number of Treg cells were present in the cervical lymph nodes of pregnant periodontitis mice. Treg cells from the cervical lymph nodes of ligated pregnant mice and non-pregnant mice were tested for their suppressive function in vitro. We manifested that Treg suppressive function was also down-regulated in the pregnant mice. Additionally, we demonstrated that more inflammatory Th17 cells were present in the cervical lymph nodes of ligated pregnant mice. Therefore, impaired Treg development and function, together with upregulated Th17 response, may contribute to the exacerbated periodontal disease during pregnancy.

1. Introduction

Pregnant women are more prone to gingival inflammation and periodontal disease [1–14], which can be detrimental to pregnant women's health and dangerous to their fetuses [15–20].

It is critical to understand the immunological mechanisms of more severe periodontal disease during pregnancy. Various immune cells are responsible for the aggravated periodontal conditions during pregnancy [21]. As one of the most important components of host immunity, T cells are involved in almost all immune interactions. Regulatory T cells (Treg) are a subset of T cells that emerge as critical immune regulators and maintain immune homeostasis. Transcription factor Foxp3 has been found to program Treg cell development as well as function [22,23]. Treg cells suppress T cell proliferation and cytokine production [24–26] as well as the innate immune responses [27]. Treg cells are enriched in the periodontal lesions and play critical roles in periodontal diseases [28–30]. In certain infectious diseases, Treg cells can be exploited by pathogens to down-regulate host immune responses and facilitate the survival of the pathogens [31]. On the other hand, Treg cells can down-regulate excessive host immune responses and control the diseases. The balance between Treg and other immune cells satisfies the survival strategy of both pathogens and the hosts [31,32].

Treg is believed to play a protective role in periodontal disease by suppressing excessive inflammation and collateral tissue damages [33,34]. During pregnancy, Treg cells in uterus-draining lymph nodes and systemic Treg are up-regulated [35,36]. The upregulation of Treg cells seems contradictory to the symptom of elevated periodontal inflammation during pregnancy. To this date there is no research on the Treg change responding to oral infection and its role in periodontal disease during pregnancy. It is possible that the Treg response at the fetal-maternal interface is different from that in the oral cavity during pregnancy.

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Periodontal pathogens are reported to be critical in exacerbated periodontal diseases during pregnancy [10,37]. *Porphyromonas gingivalis*, one of the most widely investigated oral pathogens, has been found in pregnant women with aggravated gingival inflammation [1,5,38,39]. *P. gingivalis* is well known for its function to regulate host immunity and induce oral microbiota dysbiosis, thus causing inflammation and collateral tissue damage. Interestingly, *P. gingivalis* infection induced Treg change in vitro and in vivo [40]. *P. gingivalis* can also promote Th17 development [41]. Th17 is one of the newly discovered inflammatory T cell subsets, which is involved in various inflammatory diseases including periodontitis [41–43]. RORγt has been regarded as specific for Th17 and responsible for the production of pro-inflammatory cytokines in Th17 cells [44]. Th17 development is closely related to Treg development in that TGFß is critical for the development of both cell subsets [45,46]. It is also intriguing to assess the Th17 response in periodontal disease during pregnancy.

In our report, by the placement of silk ligature around 2nd molar and *P. gingivalis* infection, we induced more severe inflammatory bone loss in the pregnant mice, accompanied by the down-regulated gingival expression of Treg-related molecules. We illustrated that there were fewer Treg cells in the cervical lymph nodes from the pregnant mice with periodontal disease, accompanied by an increased presence of Th17 cells. Additionally, the Treg cells from the pregnant mice exhibited compromised suppressive function. These finding demonstrated that the mechanism of more severe periodontal disease might be attributed to fewer Treg cells and impaired anti-inflammatory regulation by Treg cells.

2. Materials and methods

2.1. Mice

C57BL/6 mice (8–10 wk of age) were purchased from Jackson Lab and kept in animal facilities at University of Louisville, in appliance with the established Federal and State policies. All handling and processing were approved by Institutional Animal Care and Use Committee.

2.2. Ligature-induced periodontal disease model

Female C57BL/6 mice were paired with male mice and checked for vaginal plugs, which indicates successful copulation. As soon as vaginal plugs were found, females were separated from males and counted as day 0 of gestation, upon confirmation of pregnancy at a later date. The pregnant mice were ligated around the maxillary 2nd molar with 6-0 silk suture at day 8 of gestation; the suture resided within the gingival sulcus for the remainder of the experiment. During the experiments, the mice were infected with 10^9 CFU *P. gingivalis* ATCC 33277 in 2% carboxymethylcellulose every other day. Ten days after ligature placement, the experiments were terminated and jawbones were harvested. The distance from the cemento-enamel junction to the alveolar bone crest (CEJ-ABC) was measured on the ligated second molar (three sites corresponding to mesial cusp, palatal groove, and distal cusp) and the affected adjacent regions (sites corresponding to distal cusp and distal groove of the first molar, and palatal cusp of the third molar). To calculate pro-inflammatory periodontal bone loss, the mean CEJ-ABC distance from the group of sham-ligated mice was subtracted from the CEJ-ABC distance for each mouse. The results were presented in mm; the values indicated bone loss relative to controls. Gingival tissues or cervical lymph nodes were harvested for the later assays, depending on the purpose of the experiments.

2.3. Gingival mRNA expression

Gingival tissue was excised from around the maxillary molars for mRNA harvest. The expression of the interested molecules was determined by quantitative real-time PCR. Briefly, mRNA was extracted from gingival tissue, using the PerfectPure RNA kit (5 Prime; Fisher, Waltham, MA, USA). High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe mRNA, and quantitative real-time PCR (qPCR) was performed using the ABI 7500 System, according to the manufacturer's protocol (Applied Biosystems). TaqMan probes, sense primers, and antisense primers for genes of interest and a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) were purchased from Applied Biosystems.

2.4. Lymphocyte isolation and detection

Cervical lymph nodes were removed from mice and homogenized through a nylon mash (70 μM). The cells were then stained with fluorescence-conjugated anti-CD3, anti-CD4, anti-RORγt, and anti-Foxp3 (eBioscience). Transcription factor staining buffer set (eBioscience) was used for RORγt and Foxp3 intracellular staining. Stained cells were then acquired and analyzed on FACSCalibur using CellQuest (BD Biosciences) on FACSCelesta using Flowjo.

2.5. T cell in vitro proliferation

Foxp3-EGFP transgenic mice (B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J) [47], in which EGFP and the Treg-specific transcription factor Foxp3 are co-expressed, were induced for gingival inflammation during pregnancy as mentioned before. Treg cells were sorted by fluorescence flow cytometry based on EGFP expression from gingival tissue of B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J mice. As previously reported as a standard method to test Treg suppressive function, we determined the ability of these Tregs to suppress effector T cell proliferation [48,49]. Briefly, CD4 + CD25- naïve T cells were harvested from spleens and lymph nodes of naïve WT C57BL/6 mice using magnetic column and anti-CD4 and anti-CD25 beads (Miltenyi Biotec, Auburn, CA). Irradiated spleen cells were used as antigen presenting cells. Treg cells (GFP + cells) were sorted from infected pregnant or non-pregnant B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J mice. Treg cells were then co-cultured with 25,000 CD4 + CD25 − effector cells at different ratios in the presence of antigen presenting cells (100,000 cells) and 10 μg/ml anti-CD3 antibody. Cells were cultured in complete media (RPMI 1640, 10% heat-inactivated FCS, 2 mM glutamine, 10 mM Hepes, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 10^{-5} M 2-mercaptoethanol) at 37 °C and 5% CO_2 for 3 days, after which the CCK-8 solution was added to each well to a final concentration of 100 μl/ml. The cells were incubated for an additional 2 h, and the absorbance was then measured at 450 nm with a microplate reader.

2.6. Statistics

Data were evaluated by two-way ANOVA (GraphPad Prism7). Two-tailed *t*-tests were also performed where appropriate (comparison of two groups only). Differences were considered statistically significant at the $p < 0.05$ level.

3. Results

3.1. Exacerbated periodontal conditions in pregnant mice

A ligature induced periodontal disease animal model, in which periodontal disease was induced by molar ligation and *P. gingivalis* infection, was modified as described in our previous publication [50]. Our results validated the model by showing that ligation and *P. gingivalis* infection caused significantly higher alveolar bone loss in pregnant mice (Fig. 1).

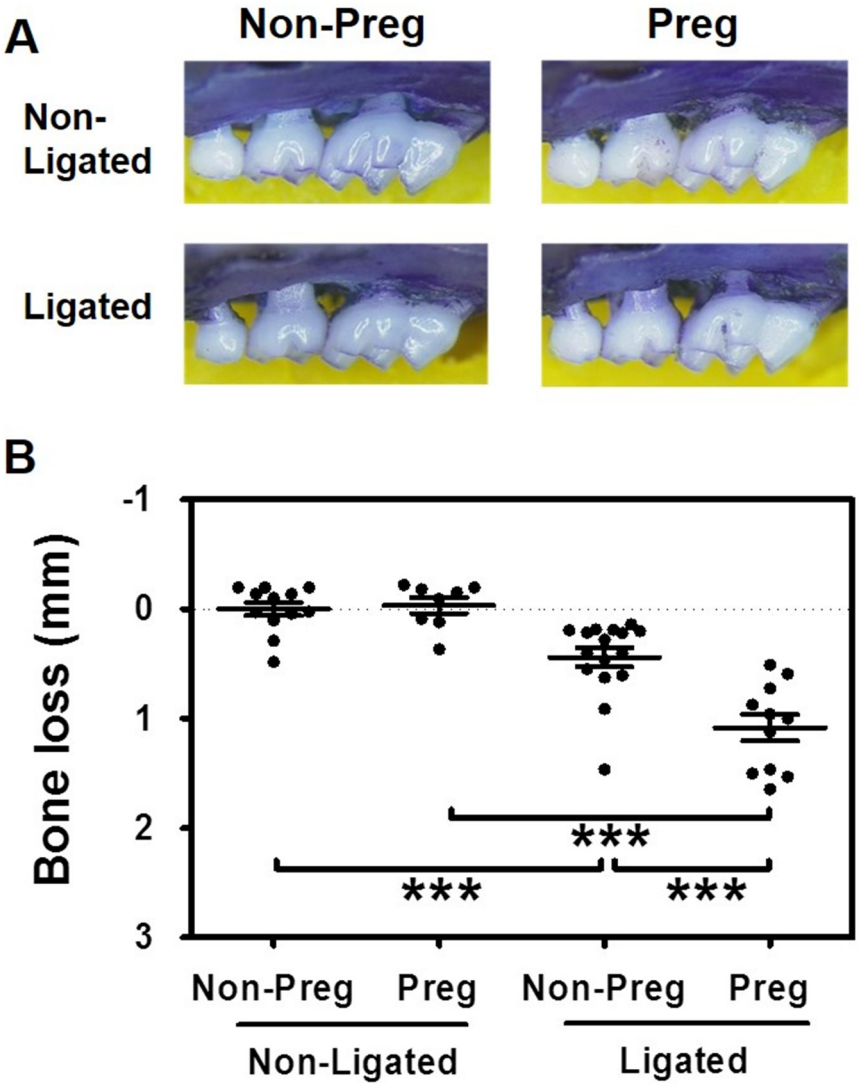

Fig. 1. Periodontal bone loss in ligated pregnant and non-pregnant mice. (A) Representative images from the maxillae of ligated (lower panels) or non-ligated (upper panels) non-pregnant (Non-preg; left panels) and pregnant (Preg; right panels) mice. (B) The mm distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 most affected maxillary palatal sites and the readings were totaled for each mouse. The CEJ-ABC reading of each mouse was represented by each dot. The data from *CEJ-ABC readings* were transformed to indicate bone loss, as outlined in Materials and methods section. Asterisks indicate statistically significant (***, $p < 0.001$) differences between different groups.

3.2. Reduced gingival expression of Treg-related molecules in pregnant mice

As one of the most important cell subsets in regulating host immune responses, Treg can curb both innate immunity and adaptive immunity. Foxp3 is a transcription factor that is specific to Treg cells and is critical in Treg development and function [22,23]. TGFβ [48,51,52], CTLA-4 [53,54], and CD28 [55–58] are important for Treg cell development and function. We tested the gingival expression of these Treg-related molecules. In non-pregnant mice, periodontal disease induction led to higher Foxp3 expression. While in pregnant mice, Foxp3 expression is significantly lower than that in ligated non-pregnant mice (Fig. 2A). After ligation, the expression of TGFβ (Fig. 2B) in pregnant mice was also significantly lower than non-pregnant mice. Similarly, both CTLA-4 and CD28 were expressed at lower level in the pregnant mice with periodontal disease (Fig. 2C and D).

3.3. Lower number of Treg cells in cervical lymph nodes from pregnant periodontal disease mice

In order to confirm the Treg change during exacerbated periodontal disease in pregnant mice, the lymphocytes from cervical lymph nodes were tested by flow cytometry. The percentage of CD3 + CD4 + Foxp3 + cells (Treg) in CD3 + CD4 + cells (Th) was determined. Consistent with the expression of Treg related molecules in gingival tissues, the frequency of Treg cells in ligated pregnant mice was significantly lower than ligated non-pregnant mice (Fig. 3).

3.4. Impaired suppressive function of Treg cells from pregnant mice

Besides the ratio/number of Tregs, the regulatory/suppressive function of these cells is also critical in affecting the immune response and disease progression. To determine the suppressive function of Treg cells, GFP + Treg cells were sorted by fluorescence flow cytometry from cervical lymph nodes and co-cultured with effector T cells at different dosages. Treg cell itself did not proliferate in this experiment [59] (Fig. 4). At lower dosages (1:16 and 1:8), Treg cells from ligated pregnant mice were less potent than the ones from non-pregnant mice in suppressing effector cell proliferation (Fig. 4), indicating that the Treg cells from ligated pregnant mice exhibited an impaired regulatory function.

3.5. Higher number of Th17 cells in cervical lymph nodes from pregnant periodontal disease mice

We further tested the presence of Th17 cells in cervical lymph nodes. The lymphocytes from cervical lymph nodes were stained with fluorescence-conjugated anti-CD3, CD4, and Th17 specific transcription factor RORγt before tested by flow cytometry. The percentage of CD3 + CD4 + RORγt + cells (Th17) in CD3 + CD4 + cells (Th) was determined. We found that the frequency of Th17 cells in ligated pregnant

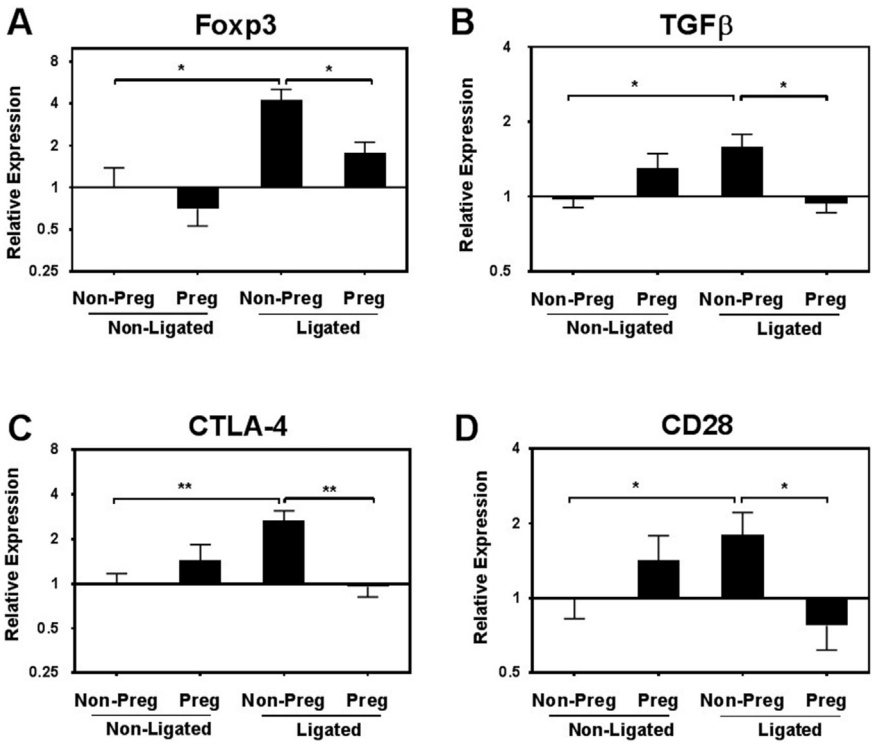

Fig. 2. Relative expression of Treg-related cytokines and molecules in the gingivae of ligated pregnant and non-pregnant mice. Quantitative real-time PCR (qPCR) was used to determine gingival mRNA expression levels for the indicated molecules (normalized against GAPDH mRNA levels). The gingivae used were excised from pregnant (Preg) and non-pregnant (Non-Preg) C57/BL6 mice. Results are shown as fold change relative to non-pregnant sham-ligated mice. Each group represents the mean ± SD of at least 5 separate expression values, corresponding to qPCR analysis of individual mice. Asterisks indicate statistically significant (*, $p < 0.05$; **, $p < 0.01$) differences between different groups.

mice was significantly higher than ligated non-pregnant mice (Fig. 5).

4. Discussion

While the inflammation during pregnancy can be detrimental to pregnant women's health, negatively impact their quality of life, and may even cause adverse pregnancy outcomes, the mechanism of the more severe oral inflammation is illusive. We modified a widely applied ligature model and utilized it to understand the pathogenesis of the disease in pregnant mice, as described in our previous publication [50]. This model induced inflammation in a shorter time period, which enabled us to fit the experiment into the time frame of pregnancy. We ligated the maxillary 2nd molar of the pregnant mice with sutures and infected the mice with *P. gingivalis* which induced significantly more severe alveolar bone loss in ligated pregnant mice than ligated non-pregnant mice (Fig. 1), confirming that periodontal deterioration could be accelerated by pregnancy [10,14].

P. gingivalis is one of the most important periodontal pathogens and readily found in the pregnant women with gingival inflammation [38]. A series of recent research of periodontal disease pathogenesis indicated that *P. gingivalis* infection caused disruption of immune homeostasis and normal oral microflora, subsequently leading to periodontitis [60,61]. *P. gingivalis* might exploit similar pathogenic mechanisms to induce more severe periodontal disease during pregnancy. In this paper, our research investigated the complicated yet seldom investigated T cell responses to periodontal pathogens in the oral cavity during pregnancy; whereas previous research focused on the immune responses at the fetal-maternal interface.

Treg cells have manifested their critical roles in down-regulating the host immunity and inflammation [24–27]. Indeed, we found that after periodontal disease induction, IL-10 expression was lower in the pregnant mice than non-pregnant mice [50], which led us to further investigate Treg cell responses in the exacerbated periodontal disease during pregnancy. We tested the gingival mRNA expression of Treg-related molecules, including Foxp3, TGFβ, CTLA4, and CD28. We found that after ligation and *P. gingivalis* infection, the gingival expressions of the Treg-specific transcription factor Foxp3 was significantly lower in pregnant mice than non-pregnant mice (Fig. 2A). TGFβ, which is famous for its anti-inflammatory function, has been well manifested to be critical in Treg development and function [48,51,52]. Gingival expression of TGFβ was also lower in the pregnant mice with periodontal diseases (Fig. 2B). CTLA-4, which is highly expressed in Treg cells and important for Treg development [51,53,54], is expressed in T cells from patients with periodontitis [62]. CD28, which shares ligands CD80/CD86 on the antigen presenting cells with CTLA-4, is also critical for Treg development and maintenance [55–58]. Lower gingival expression of these molecules in pregnant mice indicated that Treg cell development was impaired in the pathogenic environment (Fig. 2C and D).

To confirm that the periodontal pathogen-induced Treg cell development is down-regulated during pregnancy, we harvested lymphocytes from cervical lymph nodes and tested the frequency of Treg cells through flow cytometry. Our results in Figs. 2A and 3 showed that Treg cells were up-regulated in non-pregnant mice after periodontal disease induction. This observation is consistent with previous reports, which have shown that Treg cells were enriched in periodontal lesions [28,29], and fulfilled protective function in inflammation-induced bone loss [30]. This protective mechanism from Treg cells might be impaired in the pregnant mice. Indeed, in the pregnant mice that had been induced for periodontal disease, we found lower frequency of Treg cells in helper T cells than the non-pregnant mice with periodontal disease (Fig. 3). Pregnancy status reversed the periodontal disease-induced Treg development. No significant difference in Foxp3 expression (Fig. 2A) or Treg cell (Fig. 3) was found between non-pregnant and non-ligated mice versus pregnant and ligated mice. These results imply that the periodontal pathogenesis during pregnancy could be attributed, at least partially, to the compromised Treg development.

Besides their roles in Treg development and maintenance, Foxp3, TGFβ, and IL-10 are also important for Treg suppressive function [51,54,63–65]. While CTLA-4 also plays a critical role in Treg development and maintenance, its role in Treg function is controversial [66,67]. Since Treg-related molecules were down-regulated in the pregnant mice with periodontal disease, it is possible that Treg cells from these mice exhibit impaired regulatory function and are less effective in suppressing effector T cell proliferation. Therefore, we co-

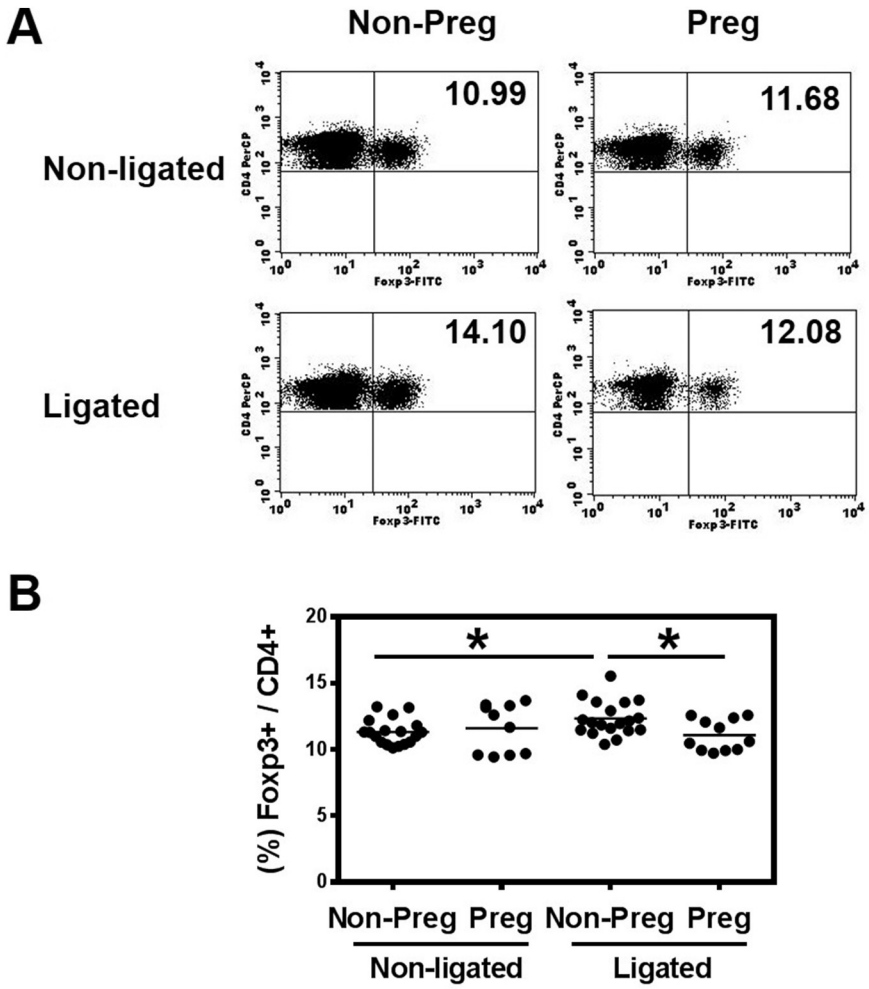

Fig. 3. Treg cells in the cervical lymph nodes of ligated pregnant and non-pregnant mice. (A) Representative flow cytometry images of the gated CD3 + CD4 + cells from non-ligated (upper panels) or ligated (lower panels), non-pregnant (left panels) and pregnant (right panels) mice; (B) the frequency of Treg cells were shown as percentage of CD3 + CD4 + Foxp3 + cells in CD3 + CD4 + cells. Each mouse was represented by each dot. Asterisks indicate statistically significant (*, $p < 0.05$) differences between different groups.

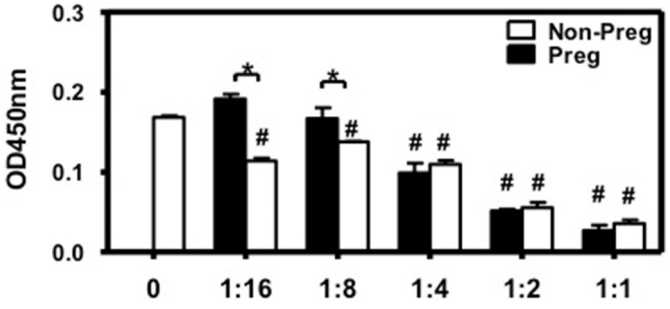

Fig. 4. Treg cells from ligated pregnant mice exhibited less potent regulatory function in vitro than those from ligated non-pregnant mice. After periodontal disease induction in pregnant mice and non-pregnant mice, GFP^+ cells (Treg cells) were sorted from cervical lymphocytes by fluorescence-activated cell sorter and tested for regulatory function in vitro. For this assay, 25,000 freshly harvested CD4 + CD25 − cells (effector cells) were purified and cocultured at varying (Treg/effector cells) ratios with GFP^+ cells from pregnant mice (Preg) or non-pregnant mice (Non-preg) in the presence of irradiated spleen cells (APC) and anti-CD3 for 3 days. Asterisks indicate statistically significant (*, $p < 0.05$) differences between different groups. #, indicate statistically significant difference from the group without Treg (group 0).

cultured Treg cells with effector T cells and tested the ability of Treg cells to suppress effector T cell proliferation in a commonly accepted method [48,49]. Indeed, we showed that after periodontal disease induction, the suppressive function of Treg cells from pregnant mice were not as potent as the ones from non-pregnant ones (Fig. 4). Our results showed that both Treg number and Treg function were down-regulated in the pregnant mice with periodontal disease.

Th17 cells are a subset of inflammatory T cells and are involved in various inflammatory diseases [41–43]. Th17 development is closely related to Treg development. While TGFβ promotes Treg development, it directs Th17 differentiation with the presence of IL-6 [45]. Th17 can be induced in periodontal disease or in response to periodontal pathogen stimulation; however, its effect on periodontal disease progression is still unclear. It is intriguing to reveal whether the Th17 response is up-regulated in periodontal disease during pregnancy. Transcription factor RORγt is specifically expressed in Th17 and was labeled to determine the presence of Th17 by flow cytometry. Indeed, we detected higher number of Th17 cells in the cervical lymph nodes from pregnant periodontal disease mice (Fig. 5). Recent publication showed that Treg cells converted into inflammatory Th17 cell in periphery in the inflammatory environment [68,69], which may be the reason that fewer Treg cells and more Th17 cells were detected in pregnant mice. Treg/Th17 plasticity will be our future research interest.

In summary, pregnant mice showed exacerbated inflammatory alveolar bone loss after periodontal disease induction in our animal

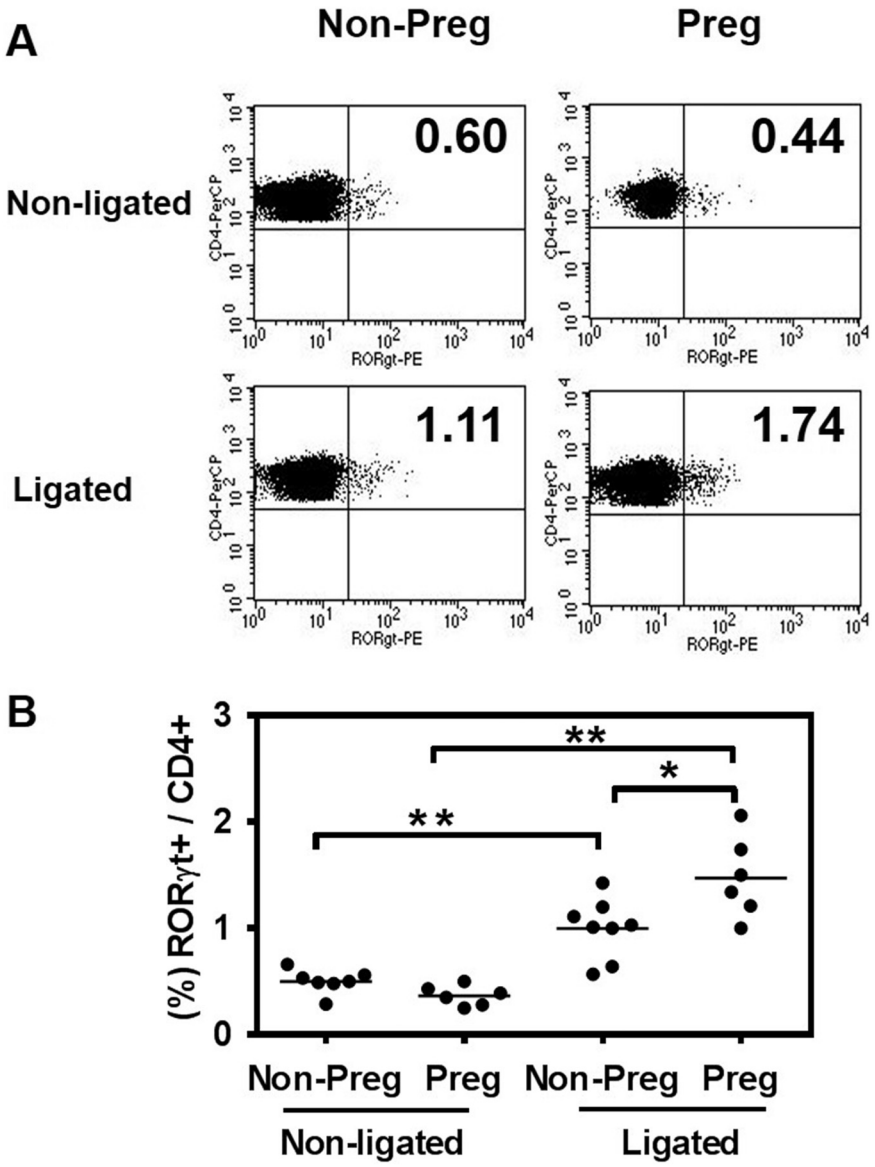

Fig. 5. Th17 cells in the cervical lymph nodes of ligated pregnant and non-pregnant mice. (A) Representative flow cytometry images of the gated CD3 + CD4 + cells from non-ligated (upper panels) or ligated (lower panels), non-pregnant (left panels) and pregnant (right panels) mice; (B) the frequency of Th17 cells were shown as percentage of CD3 + CD4 + RORγt + cells in CD3 + CD4 + cells. Each mouse was represented by each dot. Asterisks indicate statistically significant (*, $p < 0.05$; **, $p < 0.01$) differences between different groups.

model. Lower gingival expression of Treg-related molecules implied that down-regulation of Treg is involved the pathogenesis of the disease. Pregnant mice with periodontal disease showed decreased Treg cells in their cervical lymph nodes. Besides, these Treg cells exhibited less potent regulatory function than the cells from non-pregnant periodontal disease mice. Furthermore, Th17 cell increased in the pregnant mice with periodontal disease, which implies that an imbalance in Th17/Treg might contribute to the more severe periodontal disease during pregnancy. Our finding has therefore helped us to better understand the mechanisms underlying periodontal disease susceptibility and/or progression during pregnancy.

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