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CD40 up-regulation on dendritic cells correlates with Th17/Treg imbalance in chronic periodontitis in young population

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Abstract

We aimed to discover the influence of age on the development of chronic periodontitis and illustrate the molecular mechanism in this process. Blood samples were collected from 63 chronic periodontitis patients and 30 healthy controls. Th17 cell/Foxp3$^+$ regulatory T cell (Treg) ratio and expression of costimulatory molecules in dendritic cells (DCs) were analyzed by flow cytometry. The serum levels of soluble CD40 ligand (CD40L) and IL-17 were examined by ELISA. In young chronic periodontitis patients, the Th17/Treg ratio was significantly higher than that in old patients. CD40 on DCs and serum levels of CD40L and IL-17 were all higher in young chronic periodontitis patients. Mature DCs with high CD40 expression level elevated the Th17/Treg ratio *in vitro*. During the pathogenesis of chronic periodontitis, young patients had higher Th17/Treg ratio than old patients and this phenomenon was in line with the differential expression levels of CD40 in DCs.

Keywords

Chronic periodontitis, Th17 cells, regulatory T cells, dendritic cells, CD40 up-regulation

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Introduction

As an inflammatory disease which originates from the dentition supporting structures, periodontitis is initiated by the infection of bacteria on the surface of teeth.[1] Periodontitis is considered a complex disease with a variety of contributory and etiologic factors.[2] The pathogenesis and development of periodontitis are triggered by a combination of both dysbiosis of microbiome on the surface of teeth and the dysregulated inflammation response of the host.[3] The infections in periodontitis are mediated by enhanced growth of commensal bacteria, while many other infectious diseases are initiated by other exogenous pathogens.[4] Understanding of the mechanism of inflammatory response initiation in periodontitis is crucial for the illustration of its pathogenesis. The interaction between immune regulation mechanism and the response of effector T cells plays an important role in innate and adaptive immunity.[5] During the pathogenesis and development of periodontitis, immature CD4+ T cells are activated and differentiate into distinct subsets to play either promoter or suppressor functions.[6] Foxp3$^+$ regulatory T cells (Treg) are cells which express the lineage-specific transcription factor Forkhead box P3 (Foxp3) and are crucial for immune induction.[7] Th17 cells participate in the regulation of inflammation and autoimmunity through the production of IL-17.[8] The activity of Th17 is inhibited by the migration of Treg into the inflammation site.[9] In recent years, the imbalance between the two CD4+ Th cell subsets, Th17 and Treg, is demonstrated to have an important function in the pathogenesis of periodontitis.[10] Dendritic cells (DCs) are considered to be one of the most effective Ag-presenting cells in the immune system and are crucial for the regulation of both innate and adaptive immunity.[11] It is reported that

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the imbalance between Th17 and Treg is influenced by the activation of DCs.[12]

The initiation of periodontitis may occur in both childhood and adolescence. Based on previous researches, periodontitis pathogenesis usually initiates in early adulthood and sometimes in later years.[13,14] Recently, the age of the periodontitis patients is demonstrated to be one of the factors influencing the clinical diagnosis of periodontitis and the choice of therapeutic strategy.[15] Several researches have reported that the incidence of periodontitis is higher in aging populations.[16,17] A recent published study focuses on the periodontitis in young populations and demonstrates that the prevalence of periodontitis ranges widely in young patients.[18]

In this research, we aimed to discover the influence of age on the development of chronic periodontitis and illustrate the molecular mechanism in this process.

Methods and materials

Patients and tissue samples

This research was approved by Fourth Affiliated Hospital of Harbin Medical University. All patients involved in this research were recruited at Fourth Affiliated Hospital of Harbin Medical University, who signed the corresponding informed consent. In the control group, a total of 30 healthy individuals (16 females) with the age of 39.67 ± 17.08 yr were recruited. Sixty-three patients with chronic periodontitis participated in this research and were divided into two different groups based on their ages (> 40 or ≤ 40 yr old). In the young group, 31 periodontitis patients (13 females) of 29.39 ± 6.49 yr old were enrolled. In the old group, 32 patients (17 females) of 58 ± 10.22 yr old were enrolled.

Blood samples from patients with chronic periodontitis and healthy individuals were collected for flow cytometry analysis. Plasma samples were collected after centrifugation and stored at $-80°C$ for cytokine measurement.

Flow cytometry

Peripheral blood mononuclear cells were treated with Cell Stimulation Cocktail and Protein Transport Inhibitor Cocktail (eBioscience, San Diego, CA, USA) and then stained with anti-CD4-FITC and anti-CD25-PE (eBioscience, San Diego, CA, USA) for surface Ags. Then the cells were permeabilized with permeabilization/fixation buffer (eBioscience, San Diego, CA, USA) and stained with anti-Foxp3-APC and anti-IL-17A-PE-Cy7 (eBioscience, San Diego, CA, USA). DCs were washed and incubated with anti-CD40-APC, anti-CD80-PE-Cy7, and anti-CD86-PE (eBioscience, San Diego, CA, USA). Cells were re-suspended for flow cytometric analysis. Flow cytometry was performed by FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was performed by FACS Diva software (BD Biosciences, San Jose, CA, USA).

ELISA

The IL-17 and soluble CD40 ligand (CD40L) concentrations in the serum were analyzed by corresponding commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) based on manufacturer's protocols.

Cell culture

Mouse bone marrow cells were isolated from the tibiae and femurs and treated with red blood cell lysing buffer. Cells were cultured with phenol red-free RPMI 1640 medium (HyClone, Shanghai, China) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 μg/ml penicillin-streptomycin (Sigma, St Louis, MO, USA), 10 ng/ml recombinant granulocyte-macrophage CSF (PeproTech, Rocky Hill, NJ, USA), and 1 ng/ml recombinant IL-4 (PeproTech, Rocky Hill, NJ, USA) at 37°C with 5% CO_2. Non-adherent and loosely adherent cells were harvested after cultured for 6 d. Cells with specific DC markers were isolated by fluorescence-activated cell sorting. Then mouse bone marrow-derived dendritic cells (BMDCs) were cultured for experiments. $CD4^+$ T lymphocytes were isolated by cell negative isolation kit (Invitrogen Dynal AS, Oslo, Norway) and cultured in RPMI-1640 medium (HyClone, Shanghai, China) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 μg/ml penicillin-streptomycin (Sigma, St Louis, MO, USA), 1% non-essential Aas, 4 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 2×10^{-5} M 2-β-mercaptoethanol at 37°C with 5% CO_2. BMDCs were stimulated by LPS (100 ng/ml; Sigma-Aldrich, Missouri, USA) for 24 h before co-culture with $CD4^+$ T lymphocytes. T lymphocytes were cultured with DCs at a ratio of 5:1 and the cells were analyzed after 3 d.

Statistical analysis

SPSS 17.0 software was used for the statistical analyses. Data are shown as mean ± SEM. One-way ANOVA with a Bonferroni post hoc test was used to calculate the differences between each group. Statistical analysis was significant when $P < 0.05$.

Results

Th17/Treg ratio was significantly increased in chronic periodontitis patients

To explore whether the age of patients played a role in the pathogenesis of chronic periodontitis, we divided all the participants in this research into three groups and the characteristics of the participants are shown in Table 1. Thirty healthy individuals (16 females) were enrolled in this research as the control group. Sixty-three chronic periodontitis patients were recruited in this research and were divided into two different groups, the young group ($n = 31$, age $\leq$ 40) and the old group ($n = 32$, age > 40).

We examined the proportion of circulating Treg cells ($CD4^+$, $CD25^+$, $Foxp3^+$) in the peripheral blood from three different groups. As shown in Figure 1a and b, in samples from both young group and old group, the proportions of Treg cells were significantly lower than those from the control group. Meanwhile, patients in the young group had lower Treg cell proportion than those in the old group (Figure 1a and b). In addition, the proportion of Th17 cells was significantly higher in patients with chronic periodontitis when compared with the healthy individuals (Figure 1c and d). Patients in the young group had higher Th17 cell proportion than those in the old group (Figure 1c and d). We also analyzed the ratio of Th17/Treg in different groups and the result was shown in Figure 1e. When compared to the control group, chronic periodontitis patients displayed significantly up-regulated ratio of Th17/Treg. Chronic periodontitis patients in the young group had a higher Th17/Treg ratio than patients in the old group. Based on these data, Th17/Treg ratio was significantly increased in chronic periodontitis patients and correlated with the age of the patients.

CD40 on DC was up-regulated in young chronic periodontitis patients

To investigate the function of CD11c+ DCs in the change of Th17/Treg ratio during the pathogenesis of chronic periodontitis, we examined the expression of costimulatory molecules CD80, CD86, and CD40 through flow cytometry. When compared to the control group, the expression of CD80 and CD86 were both up-regulated in chronic periodontitis patients in both the young and old groups (Figure 2a to d). However, there were no differences in the expression levels of CD80 and CD86 between patients in the young and old groups (Figure 2a to d). So, the expression levels of CD80 and CD86 in DCs were not altered by the age of the chronic periodontitis patients.

Table 1. Patients with chronic periodontitis were divided into the two groups by age.

		Chronic periodontitis	
	Healthy control	Young group	Old group
n	30	31	32
age	39.67 ± 17.08	29.39 ± 6.49	58 ± 10.22
age range	15–70	18–40	41–76
female	16 (53.33%)	13 (41.9%)	17 (53.1%)

The expression of CD40 in DCs was also enhanced by the pathogenesis of chronic periodontitis and significantly higher in the young group than in the old group (Figure 2e and f). These data indicated that the higher Th17/Treg ratio in young chronic periodontitis patients was correlated with the enhanced CD40 expression in DCs.

Serum level of CD40L and IL-17 were up-regulated in chronic periodontitis patients

The levels of CD40L and IL-17 in the serum of particpates in each group were examined through ELISA to further confirm the previous results in this research. As shown in Figure 3a, the serum level of IL-17 was significantly elevated by the pathogenesis of chronic periodontitis. Meanwhile, the CD40L level in the serum of chronic periodontitis patients was also higher than in healthy individuals (Figure 3b). In the serum of chronic periodontitis patients in the young group, the levels of CD40L and IL-17 were dramatically higher than those in the old group (Figure 3a and b). It was further confirmed that the higher Th17/Treg ratio in young chronic periodontitis patients was correlated with the enhanced CD40 expression in DCs.

Mature DCs with high CD40 could affect the Th17/Treg ratio in vitro

To assess whether CD40 expression in DCs affected Th17/Treg ratio *in vitro*, we examined the proportion of Th17 and Treg in co-culture of allogeneic CD4+ T lymphocytes with BMDCs that with or without LPS treatment to induce the CD40 expression. Based on Figure 4a, co-culture of LPS pre-treated BMDCs with CD4+ T lymphocytes caused a dramatic decline in the proportion of Treg. In contrast, LPS pre-treated BMDCs significantly elevated the proportion of Th17 (Figure 4b). So, the ratio of Th17/Treg was up-regulated by LPS pre-treated CD40hi BMDCs (Figure 4c). It was demonstrated that CD40 highly expressed DCs was correlated with the up-regulation of Th17/Treg ratio *in vitro*.

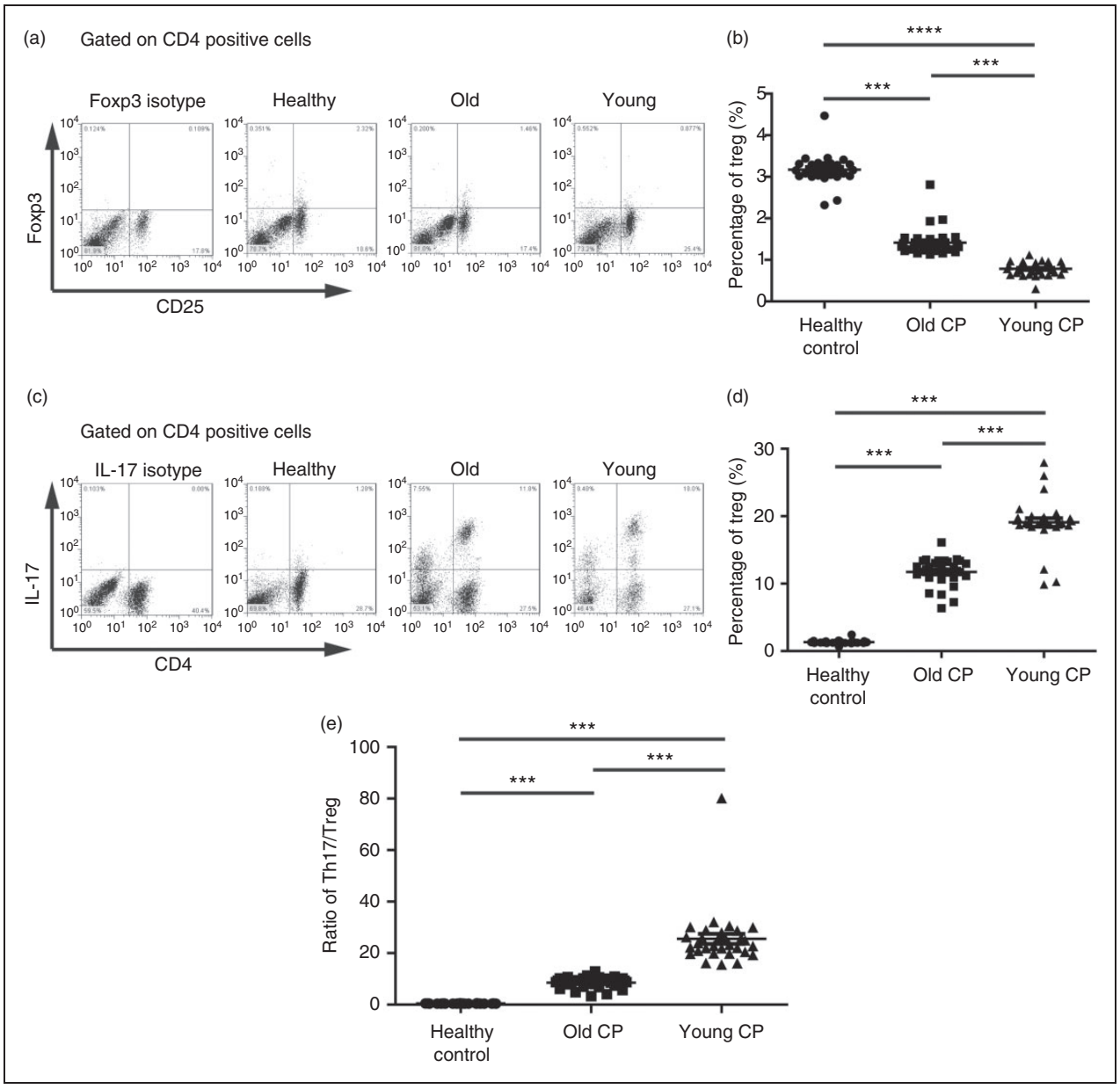

Figure 1. Th17/Treg ratio was significantly increased in chronic periodontitis patients. (a) Representative flow cytometry picture shown the percentage of Treg cells ($CD4^+CD25^+Foxp3^+$) in peripheral blood from each group. (b) Scatter plot shown the percentage of Treg cells ($CD4^+CD25^+Foxp3^+$) in peripheral blood of all the samples. (c) Representative flow cytometry picture shown the percentage of Th17 cells ($CD4^+IL17^+$) in peripheral blood from each group. (d) Scatter plot shown the percentage of Th17 cells ($CD4^+IL17^+$) in peripheral blood of all the samples. (e) The ratio of Th17/Treg in different groups. All the data are shown as mean $\pm$ SEM, *** indicates $P < 0.001$.

Discussion

Oral health is important to the individual's quality of life. Periodontitis is considered as one of the most universal oral diseases all over the world.[19] Based on the statistical data, more than 50% of the adult Chinese are affected by periodontitis.[20] In recent years, the age of the patients is considered to be another factor in the pathogenesis of periodontitis. But the differences in the pathogenesis of periodontitis between young and old patients are not reported. Although the infection of bacteria is necessary for the periodontitis initiation, the immune response in the host, especially the response mediated by $CD4^+$ T cells, is critical for the subsequent development of periodontitis and the destruction of tissues.[21] Keeping the balance between Th17 and Treg is thought to be important for the

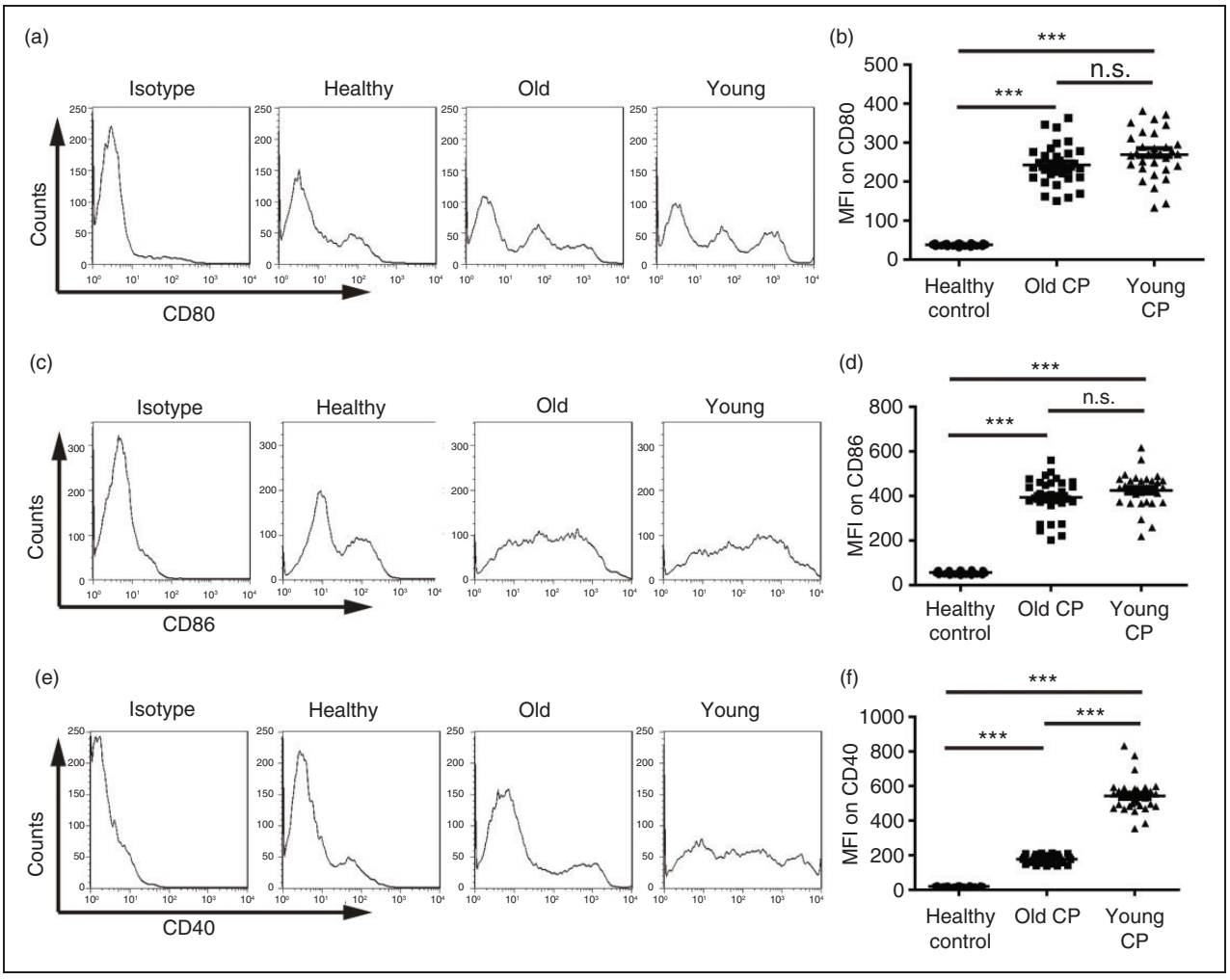

Figure 2. CD40 on DC was up-regulated only in young chronic periodontitis patients. (a) Representative flow cytometry picture shown expression level of CD80 on DCs in peripheral blood from each group. (b) Statistical analysis of CD80 expression on DCs in all the samples. (c) Representative flow cytometry picture shown expression level of CD86 on DCs in peripheral blood from each group. (d) Statistical analysis of CD86 expression on DCs in all the samples. (e) Representative flow cytometry picture shown expression level of CD40 on DCs in peripheral blood from each group. (f) Statistical analysis of CD40 expression on DCs in all the samples. All the cells were gated on CD11c, data are presented as mean $\pm$ SEM, *** indicates $P < 0.001$ and n.s. indicates $P > 0.05$.

maintenance of immune homeostasis. Several studies have shown that changes in the Th17/Treg balance are correlated with the pathogenesis of periodontitis.[22–24] In this research, we aimed to figure out the variation in the pathogenesis of periodontitis influenced by the age of patients and explore whether the imbalance between Th17 and Treg during the periodontitis pathogenesis is influenced by the age of patients.

In our research, we analyzed the percentages of Th17 and Treg in the peripheral blood from the participants in each group by flow cytometry. The pathogenesis of periodontitis significantly up-regulated the proportion of Th17 and down-regulated the proportion of Treg. These data confirmed that the balance between Th17 and Treg was disrupted in the patients with chronic periodontitis. In the group of young patients, the proportion of Th17 in the peripheral blood was significantly higher than in the group of old patients. In contrast, young chronic periodontitis patients had a lower proportion of Treg than old patients. The elevated ratio of Th17/Treg in chronic periodontitis was more pronounced in the young patients. These results indicated that the disruption of the balance between Th17 and Treg in chronic periodontitis pathogenesis was correlated with the age of patients.

DCs belong to the Ag-presenting cells and are thought to have a predominant function in the initiation and regulation of adaptive immunity.[25]

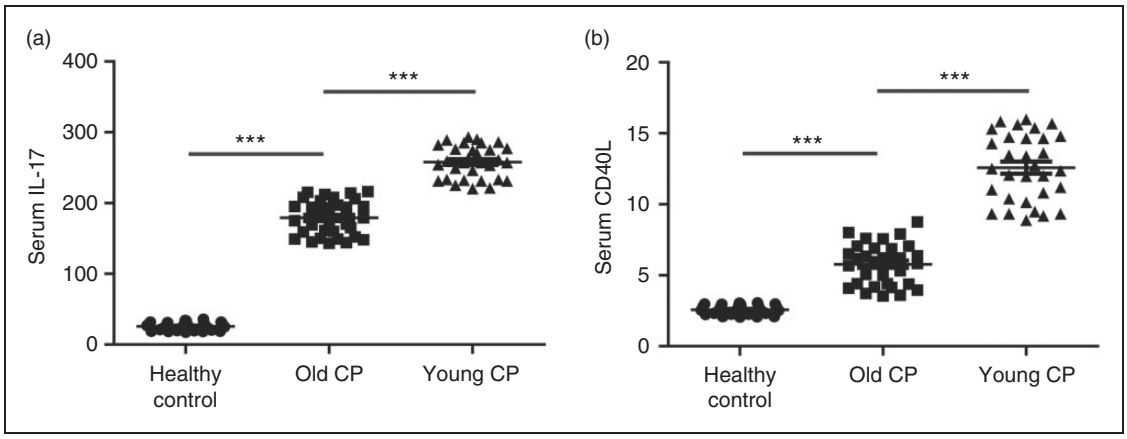

Figure 3. Serum level of CD40L and IL-17 were higher in the chronic periodontitis patients than in control. ELISA was used to examine the serum level of IL-17 (a) and CD40L (b). All the data are shown as mean ± SEM, *** indicates $P < 0.001$.

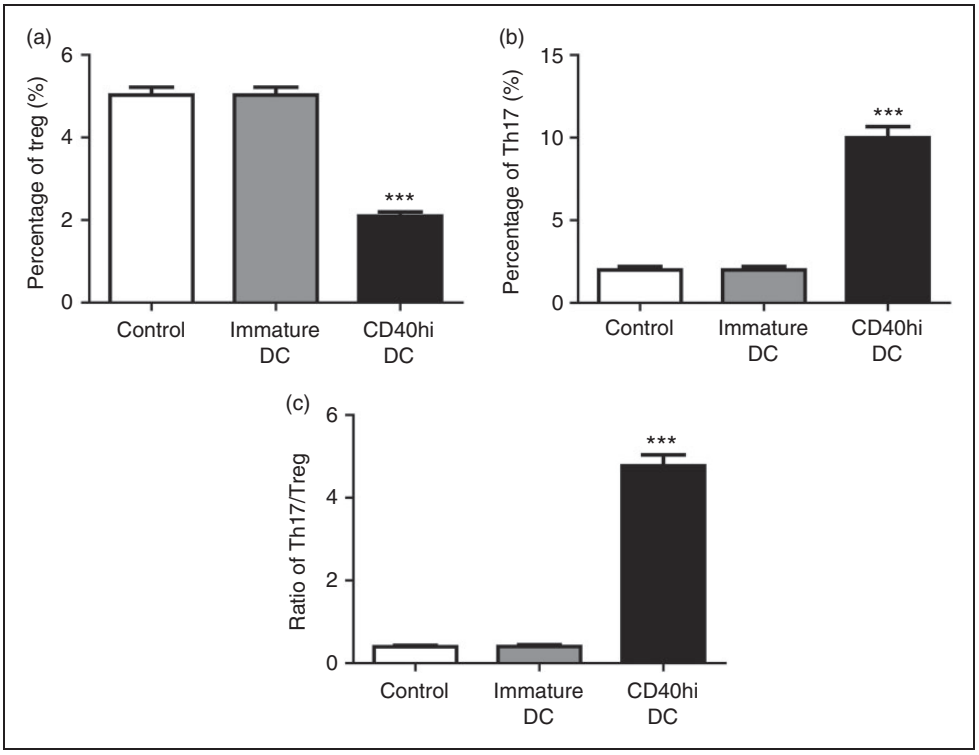

Figure 4. Mature DC with high CD40 could affect the Th17/Treg ratio *in vitro*. BMDCs were treated with 1 μg/ml LPS to induce the CD40 expression. CD40hi DCs or the immature DCs were co-cultured with the allogeneic $CD4^+$ T cells for 72 h. The percentage of Treg (a) and Th17 (b) and the ratio of Th17/Treg (c) were analyzed through flow cytometry. All the data are shown as mean ± SEM, ***indicates $P < 0.001$.

Since adaptive immunity is critical during the pathogenesis of periodontitis, DCs are demonstrated to participate in the development process of periodontitis.[26] According to their distinct mature states, DCs are divided into immature and mature DCs. During the migration towards lymphoid tissue, immature DCs turn into mature DCs and trigger the differentiation of T cells to effector T cells.[27] During the maturation process of DCs, the endocytic activity is reduced and the expression of costimulatory molecules (CD80, CD86, and CD40) and inflammatory cytokines are enhanced.[28] With the help of mature DCs, $CD4^+$ T cells differentiate into different subsets of Th cells, including Th1, Th2, Th17, and Treg.[29] Since DCs play an important function in both the differentiation of T cells and the pathogenesis of periodontitis, the

elevated ratio of Th17/Treg in chronic periodontitis may be influenced by DCs.

Through flow cytometry, we analyzed the expression of costimulatory molecules (CD80, CD86, and CD40) in DCs in peripheral blood from each group. The expression levels of CD80, CD86, and CD40 in DCs were all elevated during the pathogenesis of chronic periodontitis. This phenomenon demonstrated that the maturation process of DC was enhanced by chronic periodontitis. Then we compared the expression of these costimulatory molecules between young and old chronic periodontitis patients. There was no difference in the expression levels of CD80 and CD86 between young and old patients. However, in young patients, the DCs had a significantly higher CD40 expression level than those in old patients. To further confirm our results, the serum levels of CD40L and IL-17 were analyzed through ELISA. When compared to the old patients, young patients had higher serum levels of CD40L and IL-17. We also performed *in vitro* experiment to illustrate the function of DCs in the regulation of Th17/Treg ratio. Co-culture of $CD4^+$ T lymphocytes with immature DCs did not influence the balance between Th17 and Treg. In contrast, the ratio of Th17/Treg was significantly up-regulated when $CD4^+$ T lymphocytes were co-cultured with DCs with high CD40 expression. The highly expressed CD40 in DCs of the young chronic periodontitis patients contributed to the elevated ratio of Th17/Treg.

In conclusion, during the pathogenesis of chronic periodontitis, young patients had a higher Th17/Treg ratio than old patients, and this phenomenon was in line with the differential expression level of CD40 in DCs. The results in this research suggested that the mechanism of periodontitis pathogenesis in young and old people were likely to be different. The age of the patients should be considered as an important factor in the diagnosis and therapy of chronic periodontitis.

Declaration of conflicting interests

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