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Original Article

Elevated interleukin (IL)-35-related sCD14 but not IL-23 is associated with the severity of chronic periodontitis

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Abstract: Chronic periodontitis (CP) was a chronic inflammatory disease, mediated by complex cytokine network released from neutrophil, macrophages, T and B lymphocytes. A potent immunosuppressive cytokine, interleukin-35 (IL-35), predominantly secreted by regulatory B (i35-Bregs) and T (iTR35) cells, had been reported to be related with the severity of periodontitis. However, the role of IL-35 on CP were not be elaborated. Herein, we wondered whether the elevated IL-35 affected cytokine production from host innate immune cells in CP patients. For this purpose, the relationships among the aberrant expression of IL-35, IL-23 and sCD14 in gingival crevicular fluid (GCF) and serum from 30 CP patients after non-surgical periodontal therapy matched with 30 healthy volunteers were evaluated. As a result, we found that the levels of IL-35, IL-23 and sCD14 were elevated in CP patients. IL-35, IL-23 and sCD14 from GCF positive correlation with those from blood before periodontal therapy on CP patients. Importantly, elevated IL-35-related sCD14 but not IL-23 is associated with the severity of CP. Together, for the first time, our data suggested that IL-35 may facilitate sCD14 to induce innate immune cells mediated inflammatory response to involve in CP pathogenesis. However, further research should be carried out to understand the mechanisms of them.

Keywords: Chronic periodontitis, interleukin-35, interleukin-23, gingival crevicular fluid, soluble cluster differentiation 14, serum, periodontal inflammation

Introduction

Chronic periodontitis (CP) is a chronic inflammatory disease characterized by destruction of periodontal attachment apparatus including periodontal ligament, gingiva, alveolar bone and cementum with microbial infection. Neutrophil [1-4], macrophages [5-8], T and B lymphocytes [9-12] had been described to mediate the periodontal inflammation. The complex cytokine network released from these inflammatory cells played an important role in the initiation, progression and host modulation of periodontal disease.

Our previously study showed interleukin-35 (IL-35) was significantly elevated in gingival crevicular fluid (GCF) and reduced after periodontal therapy on CP patients [13]. This study suggested IL-35 may play an important role in the progression of periodontitis. However, the role of IL-35 expression on CP patients were not be elaborated. IL-35 was a potent immunosuppressive cytokine, predominantly secreted by regulatory B (i35-Bregs) and T (iTR35) cells [14-17]. Recently, both Mitani and colleagues [13], and our previous report [18] speculated that elevated IL-35 in GCF was likely to facilitate bacterial dissemination on CP patients. Therefore, we proposed a question that whether the elevated IL-35 affected cytokine production from host innate immune cell or not. To this end, soluble cluster differentiation 14 (sCD14) and IL-23, mainly produced by host innate immune cells were evaluated. sCD14 had been showed as a marker for prediction of the severity of periodontitis induced by *P. gingivalis* [19]. And our previously date also demonstrated that

Table 1. Summary of clinical data

Group	HV (n = 30)	CP patients	*P*
Female/Male, n	13/17	12/18	0.7934
Age (Year), Mean ± SEM	43.5 ± 1.2	44.0 ± 1.5	0.7500
Severe/Moderate, n	-	13/17	-
Smoking (smokers)	0/30	0/30	1
WBC (× 10^9/L), Mean ± SEM	6.4 ± 1.1	7.8 ± 1.9	0.5262
Anti-Porphyromonas gingivalis IgG (OD_{450})	0.6 ± 0.08	1.2 ± 0.09	0.0013

circulating IL-35 associated with IL-23 to mediate breast cancer progression and prognosis [20]. Inhere, the purpose of this study was to determine the interrelationships among the aberrant expression of IL-35, IL-23 and sCD14 in GCF and blood serum from 30 cases followed-up CP patients after non-surgical periodontal therapy matched with 30 cases gingiva healthy volunteers.

Material and methods

Subjects

30 cases CP patients and 30 cases gingiva normal healthy volunteers (HV) at the Department of Stomatology of Dongguan Hospital Affiliated to Medical College of Jinan University, between July 2014 and December 2015 were selected by periodontal faculty according to the classification of 1999 [21]. For CP patients, all subjects received a comprehensive periodontal evaluation (CPE) including gingival index (GI), periodontal probing depth (PPD) and clinical attachment loss (CAL) measurements. The teeth with periodontal disease were identified. Prior to non-surgical periodontal therapy (NSPT), gingival crevicular fluid (GCF) from the deepest pockets and peripheral blood were collected from CP patients. Following the NSPT and oral health education process, CPE, GCF from the same sites and peripheral blood were obtained again after 1 month. For HV individuals, after CPE checklist health, peripheral blood were collected. The inclusion criteria included individuals general health, no systemic disease, no smoking, women no pregnant, at the same time, no antibiotics, no immunosuppressive agents, no periodontal medical or no surgical treatment were used within the previous 3 months. The demographic and clinical characteristics of the selected subjects were summarized in **Table 1**. The study was approved by the Internal Review and the Ethics Board of Wuhan University, Dongguan Hospital Affiliated to Medical College of Jinan University, Guangdong Medical University, and Guangdong Medical University Affiliated Longhua Central Hospital, and informed consent was obtained from all study subjects.

Clinical CPE examination

The clinical CPE examinations including GI, PPD and CAL measurements were performed by the periodontists. The GI was scored as 0 (normal gingival), 1 (mild inflammation), 2 (moderate inflammation) or 3 (severe inflammation), as previously reported by Offenbacher and colleagues [22]. PPD, defined as the distance from the free gingival margin to the bottom of the sulcus, and CAL, defined as the distance from the cementoenamel junction to the bottom of the sulcus were measured by a manual periodontal probe (Kangqiao, Shanghai, China). Patients with PPD ≤ 4 mm and CAL 1~2 mm were defined as mild periodontitis, patients with PPD ≤ 6 mm and CAL 1~2 mm were defined as moderate periodontitis, patients with PPD ≥ 6 mm and CAL ≥ 5 mm were defined as severe periodontitis.

GCF sampling

Two sites per patient and per visit with maximum PPD and CAL were selected as the GCF sampling sites. Prior to collecting GCF, the sampling sites were cleaned with cotton rolls and dried gently with air spray. A filter paper (Whatman International Ltd., Maidstone, England) strip was inserted into the gingival sulcus until slight resistance was encountered and left there for 1 minute. During GCF collection, samples contaminated with blood and/or saliva were discarded. The strips were placed in sterilized Eppendorf tubes and stored in -80°C freezer until use. At the day of the assays, 300 μL of PBS (pH 7.2) and 2 μL of phenylmethanesulfonyl fluoride (20 mM) were added to the tubes containing the strips and incubated for 30 min at 4°C, then the tubes were centrifuged at 3000 g for 5 min and the supernatant was used for protein level detection.

Serum sampling

Blood samples were collected in the morning at the day of clinical CPE examination. 5 mL

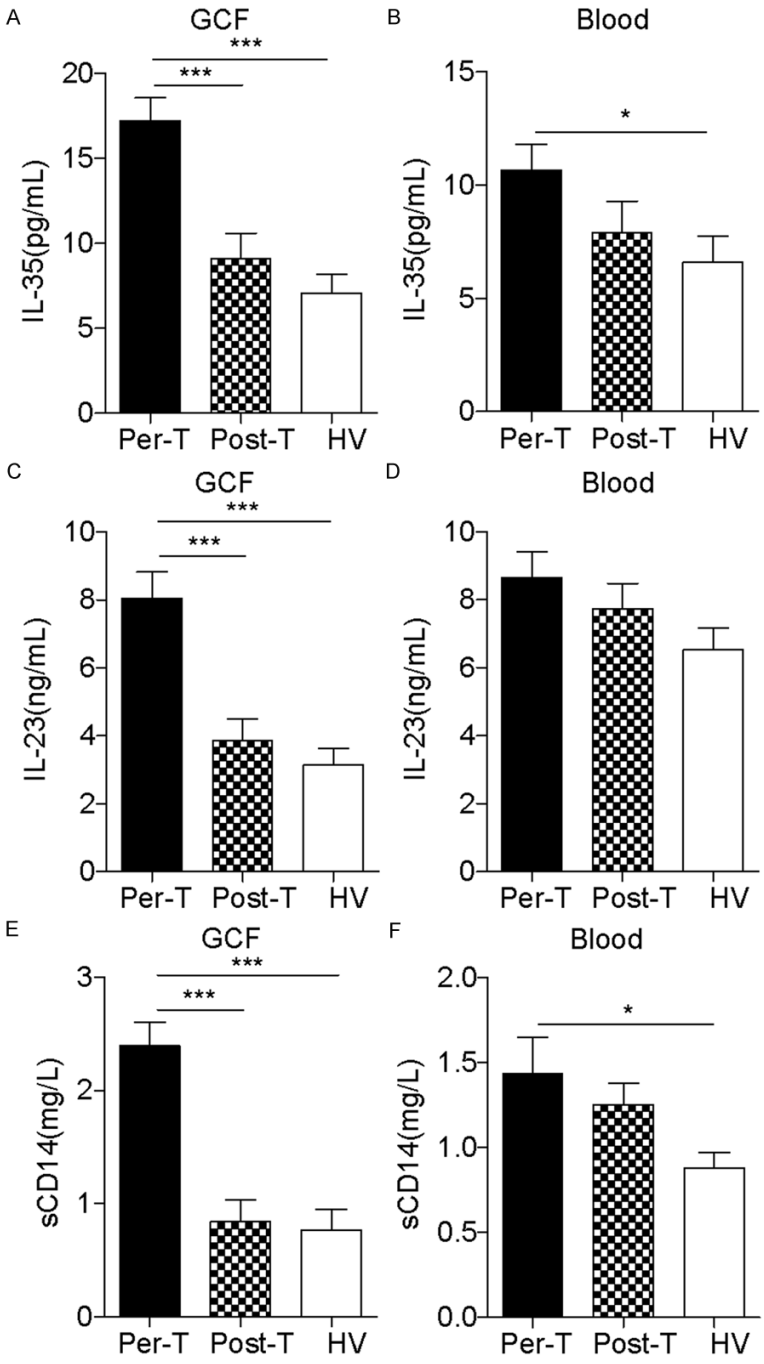

Figure 1. The levels of IL-35, IL-23 and sCD14 on CP patients The levels of IL-35 (A and B), IL-23 (C and D) and sCD14 (E and F) on GCF and blood serum were detected by ELISA from 30 cases pre-therapy (Per-T) and post-therapy (Post-T) CP patients, and 30 cases healthy volunteers (HV). Values are expressed as means ± SEM. *, P < 0.05; ***, P < 0.001.

venous blood was drawn from every subject into a free anticoagulant vacuum tube at room temperature. Serum was obtained after immediate centrifugation at 3000 g for 10 min and stored in -80°C freezer until use.

Enzyme-linked immunosorbent assay (ELISA)

The serum and GCF levels of the sCD14, IL-23 and IL-35 were determined by ELISA, according to manufacturer's instruction. sCD14 levels were measured using the Human sCD14 ELISA Kit (Quantikine; R&D Systems, Minneapolis, MN, USA), IL-23 levels were measured using the Human IL-23 ELISA Kit (BioLegend, San Diego, CA, USA), IL-35 levels were measured using the Human IL-35 ELISA Kit (BioLegend, San Diego, CA, USA).

Statistical analysis

Statistic data analysis were performed using the GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Comparisons were carried out using Student's *t*-tests, chi-square (χ^2) tests for 2-group comparisons when appropriate. Correlations were evaluated using Spearman's rank correlation coefficients following we previously described methods [23-25]. A *P*-value of 0.05 was considered significant.

Results

IL-35, IL-23 and sCD14 high expressed in CP patients

Our previously study showed that IL-35 levels on GCF were significantly reduced after periodontal therapy, and IL-35 may play an important role in the progression of periodontitis [13]. However, the role of IL-35 expression on CP were not be elaborated. IL-23 has also been reported to decrease after nonsurgical periodontal therapy on GCF of periodontitis patients [26]. Our previously date also suggested that circulating IL-35 associated with IL-23 to mediate

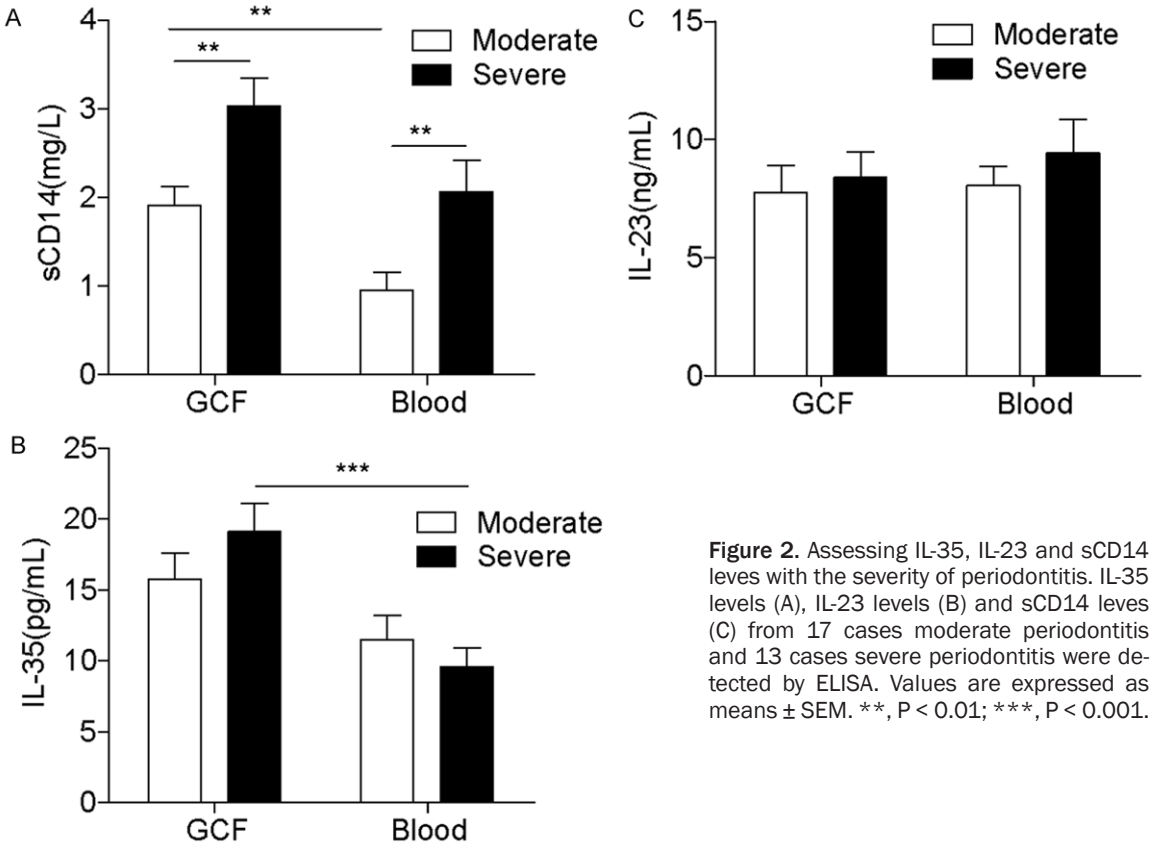

Figure 2. Assessing IL-35, IL-23 and sCD14 leves with the severity of periodontitis. IL-35 levels (A), IL-23 levels (B) and sCD14 leves (C) from 17 cases moderate periodontitis and 13 cases severe periodontitis were detected by ELISA. Values are expressed as means ± SEM. **, $P < 0.01$; ***, $P < 0.001$.

tumor resection, and circulating IL-23: IL-35 ratio may be an important indicator association with breast cancer progression and prognosis [20]. Therefore, we further evaluated IL-35 and IL-23 expression on GCF and blood serum of CP patients matched healthy volunteers (HV) by ELISA. Consistent with our previous report, IL-35 levels were higher in CP patients than that of HV control both on GCF (**Figure 1A**) and blood serum (**Figure 1B**) before periodontal therapy. GCF IL-35 levels was significantly reduced (**Figure 1A**), and a tendency towards decreased IL-35 levels was observed on blood of CP patients (**Figure 1B**) after periodontal therapy. In line with the IL-35 expression, IL-23 levels in GCF (**Figure 1C**) but not in blood (**Figure 1D**) were higher on CP patients than that on HV control, and GCF IL-23 levels was significantly reduced after periodontal therapy as shown in **Figure 1C**. Periodontitis is associated with elevated levels of sCD14 [27-29]. Inhere, we also observed sCD14 was high expression both on GCF (**Figure 1E**) and blood (**Figure 1F**) of CP patients before treatment. And GCF sCD14 levels was significantly decreased after periodontal therapy. Of noted, sCD14 levels both on GCF and blood from severe CP patients were significantly higher than those from moderate patients as shown in **Figure 2A** before periodontal therapy. Additionally, IL-35 levels on GCF was significantly higher than blood on severe but not moderate CP patients as shown in **Figure 2B**. However, we didn't find any significantly different of IL-23 levels between moderate or severe CP patients whether on GCF (or blood) as shown in **Figure 2C**. Altogether, those data suggested that IL-35, IL-23 and sCD14 especially on GCF maybe associated with the progression of periodontitis.

IL-35, IL-23 and sCD14 from GCF positive correlation with those from blood before periodontal therapy in CP patients

For further uncovering the relationship of IL-35, IL-23 or sCD14 between GCF and blood, the correlation of them were analyzed by *Pearson* correlation analysis. Results showed that IL-35 levels on GCF was noted to be positively correlated to those on blood on CP patient before periodontal therapy, as shown in **Figure 3A**.

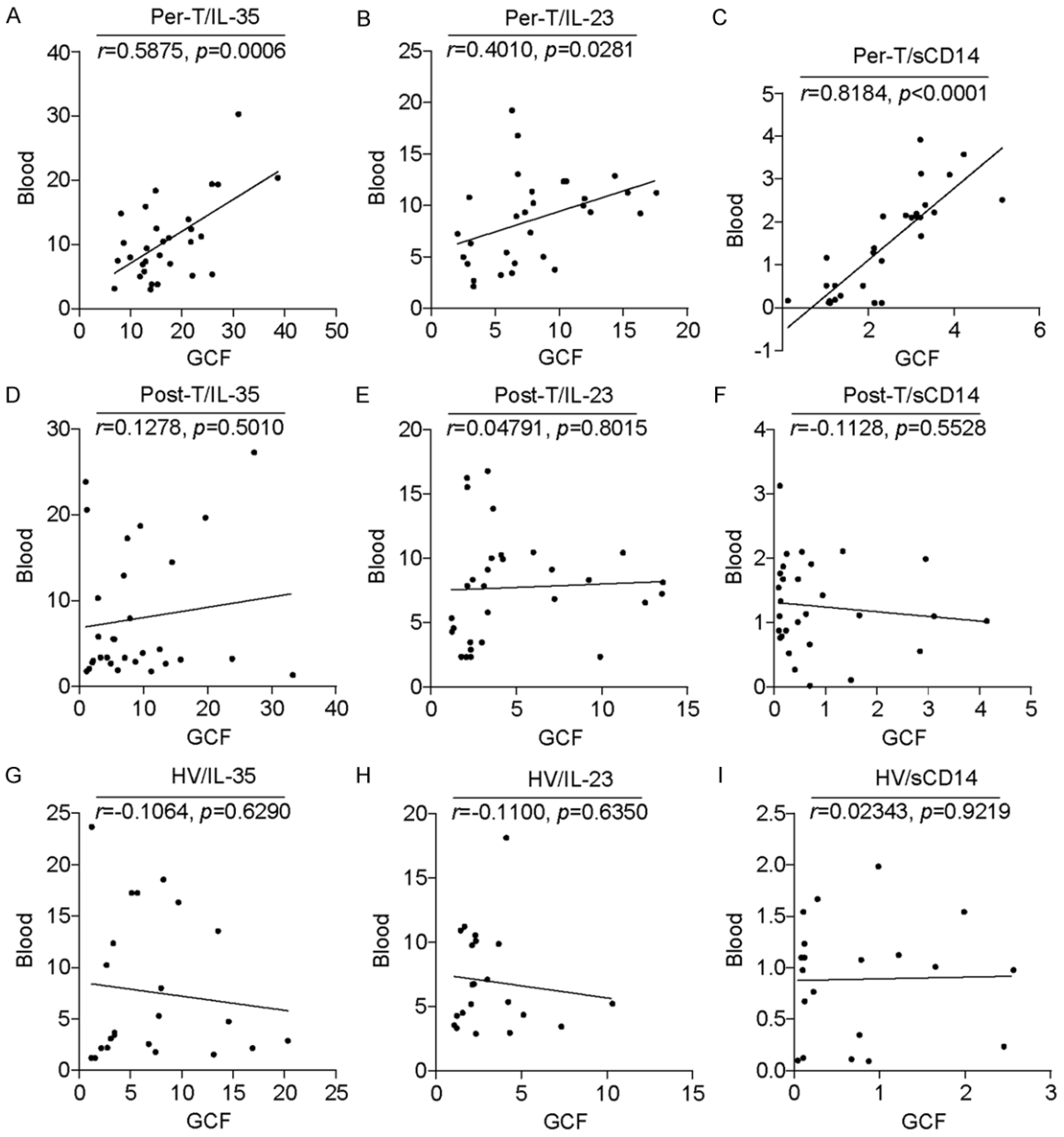

Figure 3. Correlation of IL-35, IL-23 or sCD14 levels between GCF and serum. The correlation of IL-35, IL-23, and sCD14 levels between GCF and blood serum were evaluated using Spearman correlation fromPer-T CP patients (A-C), Post-TCP patients (D-F), and HVhealthy group (G-I), respectively.

Similarly, IL-23 or sCD14 levels on GCF were also positively correlated to those on blood (**Figure 3B**, **3C**) before periodontal therapy. However, this relationship was broken after periodontal therapy, as shown in **Figure 3D-F**. There was no significantly correlation of IL-35, IL-23 or sCD14 between GCF and blood on HV group, as shown in **Figure 3G-I**. This results suggested that IL-35, IL-23 and sCD14 expression in GCF may relate to the expression of them on blood. Periodontal inflammation maydrive IL-35, IL-23, sCD14 abnormal expression on GCF and blood of CP patients.

Elevated IL-35-related sCD14 but not IL-23 is associated with the severity of periodontitis

There was a marked correlation between IL-35 and sCD14 levels on GCF of CP patient before periodontal therapy (Spearman's r = 0.5323, P

= 0.0025) but not on patient after periodontal therapy, as shown in **Figure 4A-C**. There was no significantly correlation between IL-35 and IL-23, IL-23 and sCD14 levels on GCF of CP patients (regardless of whether the periodontal therapy), or HV group, as shown in **Figure 4D-I**. At the same time, we didn't find any significantly correlation between IL-35 and sCD14, IL-35 and IL-23, IL-23 and sCD14 on blood of CP patient or HV group, as shown in Figure S1. These results suggested that aberrant expression of IL-35 may facilitate with sCD14 on GCF to mediate disease process in CP patients.

Discussion

In the present study we confirmed the presence of high levels of IL-35, IL-23 and sCD14 on GCF (or blood) of CP patients, and for the first time, we described high concentrations of IL-35, IL-23 and sCD14 on GCF may relate to the levels of them on blood. More importantly, we showed elevated IL-35-related sCD14 but not IL-23 is associated with the severity of periodontitis.

Periodontal inflammation including neutrophil [1-4], macrophages [5-8], T and B lymphocytes [9-12] mediated had been described as a cause of clinical manifestations on pathogenesis of periodontitis.

IL-35 was the most recently identified member of the IL-12 family cytokines to suppress immune response through regulatory T cells expansion and Th17 cell development suppression [14, 17]. IL-35 also induced the conversion of human B cells into Breg cells to inhibit antimicrobial immunity through production of IL-35 [15, 16]. Elevated IL-35 likely facilitated bacterial dissemination on patients with clinical and experimental sepsis [30], active tuberculosis [31], and CP as Mitani and colleagues, and we previously reported [13, 18]. However, the role of IL-35 during CP pathogenesis remains largely to be defined.

CP was initiated by sequential colonization with a broad array of bacteria and was perpetuated by the periodontal inflammatory response to the changing biofilm. Host recognition of microbes was largely mediated by toll-like receptors (TLRs). CD14 was a prototypical co-receptor for several TLRs to created an important link between the pathogenic microbes and host innate immune system by facilitating macrophages responses or activating circulating neutrophils for the development of lipopolysaccharide (LPS)-induced systemic inflammation [32-34]. The CD14 involvement in host defense against bacterial infection had been investigated in several infection disease. Echchannaoui and colleagues demonstrated CD14 played a protective role in pneumococcal meningitis by slowing polymorphonuclear (PMN) migration via MIP-2 and CXCR2 modulation [34]. CD14 polymorphisms were related to the predisposition to severe invasive infection caused by *S. pneumoniae* and *N. meningitidis* [35]. Recently, a meta-analysis perfomed by Han and colleagues also showed CD14 polymorphism was involved in the development of periodontitis [36]. And Wilensky and colleagues found porphyromonas gingivalis gingipains selectively reduce CD14 expression to induce macrophage hyporesponsiveness to bacterial infection [19].

Membrane-bound CD14 (mCD14) was mainly expressed on mature monocytes, macrophages and activated neutrophils [28, 32]. sCD14 can be the result of protease-mediated shedding/cleavage of mCD14 [28]. Recently, sCD14 aslo been showed as a marker for prediction of serious bacterial infection in bacterial meningitis or ventriculitis [37], chemotherapy induced severe neutropenia [38], early death in pneumococcal infection [34], and severity of periodontitis induced by *P. gingivalis* [19]. Inhere, we showed sCD14 was high expressed both on GCF and blood of CP patients, and GCF sCD14 levels strong correlation to blood sCD14 levels before periodontal therapy. Consistent with previous studies by Nicuand colleagues [28]. AndJinand colleagues [29], we also showed elevated levels of sCD14 associated with severity of periodontitis, and we observed strong correlations of sCD14 between GCF and blood. Interestingly, we found IL-35 positively correlated to sCD14 on GCF but not blood before periodontal therapy, and this correlation was broken after periodontal therapy. This results suggested that GCF IL-35 may related sCD14 to mediate pathogenesis of CP.

In line with CD14, IL-23 was mainly produced by host innate immune cells like macrophages and DCs to promote neutrophil recruitment or involve IL-23/Th17 axis-mediated adaptive im-

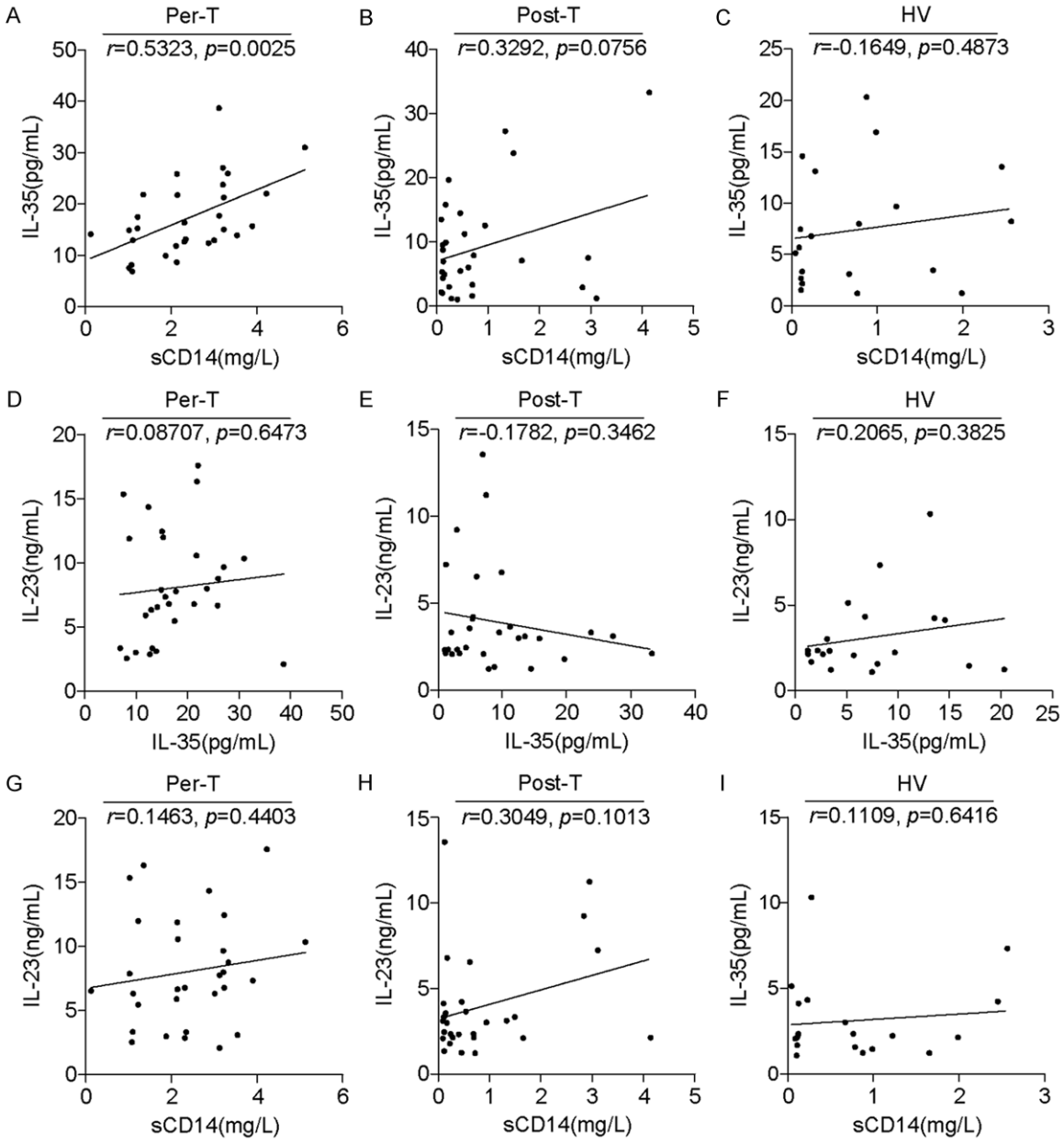

Figure 4. Correlation among IL-35, IL-23, and sCD14 levels in GCF of CP patients after periodontal therapy. The correlation between IL-35 and sCD14 (A-C), between IL-35 and IL-23 (D-F), and between IL-23 and sCD14 (G-I) were evaluated by Spearman correlationon GCF of CP patients after periodontal therapy, respectively.

mune response, in response to *C. difficile* infection [39], *Bacillus anthracis* infection [40], *Mycobacterium tuberculosis* infection [41] and *Arcobacter butzleri* infection [42]. Of noted, Luo et al. found recombinant human IL-23 also regulated IL-17A and/or IL-17F positive Th17 cells to involve in pathogenesis of periodontitis [43]. Consistent with Cifcibasiand colleagues reported on patients with generalized aggressive periodontitis [26], Himaniand colleagues reported on CP patients [44], we also found GCF IL-23 levels increased on CP patients, and GCF IL-23 levels decreased after periodontal therapy. In addition, different from Qi and colleagues reported [45], we found that blood IL-23 levels had no significantly different between CP patients and healthy group. And, we also didn't find any significantly correlation between IL-23

and IL-35, IL-23 and sCD14 on GCF (or blood) both on pre-therapy and pos-therapy CP patients or healthy volunteers.

Together, for the first time, our data suggested that IL-35 may facilitate sCD14 to induce innate immune cells mediated inflammatory response to involve in CP pathogenesis. However, further research should be carried out to understand the mechanisms of them.

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Disclosure of conflict of interest

None.

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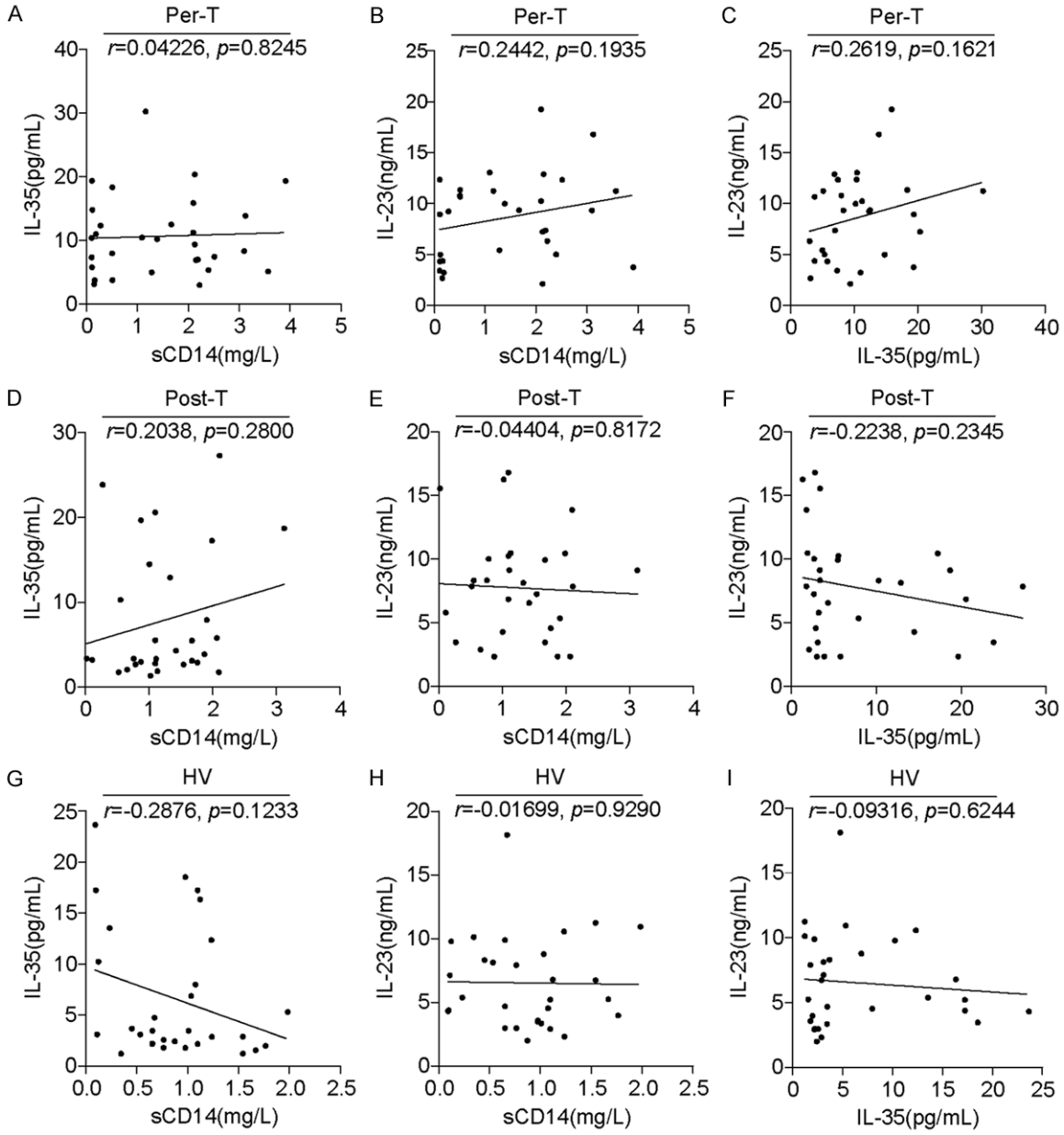

Figure S1. Correlation among IL-35, IL-23, and sCD14 levels in blood of CP patients after periodontal therapy. The correlation between IL-35 and sCD14, between IL-23 and sCD14, and between IL-35 and IL-23 were evaluated by Spearman correlation on blood serum from Per-T CP patients (A-C), Post-TCP patients (D-F), and HV healthy group (G-I), respectively.