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Expression of CCL2 signaling pathway genes in patients with periodontitis and atherosclerosis

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Summary. Objective. Periodontitis and atherosclerosis are chronic inflammatory diseases characterized by leukocyte infiltration. We investigated the expression of CCL4, CCR5, c-Jun, c-Fos, NF-κB, and CCL2 as well as the possible mechanism involved in the regulation of CCL2 in human periodontitis tissues and atherosclerotic aorta based on previous research on the CCL4/CCR5/c-Jun and c-Fos/CCL2 pathway leading to CCL2 expression in collagen-induced arthritis (CIA) rat.

Methods. Sixty-five volunteers were recruited and the condition of their gingiva and coronary arteries were assessed. The subjects were divided into four groups: healthy control, chronic periodontitis (CP), coronary artery diseases (CAD), and noncoronary artery diseases (non-CAD). Total RNA was isolated from gingiva in periodontitis patients and control populations and from the aorta in patients with and without CAD. PCR was used to examine CCL4, CCR5, c-Jun, c-Fos, NF-κB, and CCL2 levels. The production of CCL2 in the gingiva and aorta was analyzed by immunostaining.

Results. PCR revealed that CCL4, CCR5, and CCL2 mRNA levels were increased in CP patients' gingivae and aortas from coronary artery bypass grafting (CABG) patients. Marked c-Jun, c-Fos, and NF-κB gene productions were detected in CP patients' gingivae but did not show statistical differences between the CAD and non-CAD groups. Stronger immunoreactivity against CCL2 was observed in periodontitis gingiva and aorta from CABG patients.

Conclusions. Our findings suggest that the CCL4/CCR5/c-Jun and c-Fos/CCL2 pathways may be involved in CCL2 expression in periodontitis. CCL4, CCR5, and CCL2 might act as possible nodes to link the presence of periodontitis and atherosclerosis.

Key words: CCL2, Periodontitis, Atherosclerosis, Aorta, Gingival tissue

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Introduction

Both periodontitis and atherosclerosis are chronic illnesses with inflammatory factors that lead to the amplification of the host's immunologic/inflammatory response. In atherosclerosis, inflammation leads to atheromatous plaque formation, while in periodontal disease (PD), it is responsible for local bony destruction. These changes result from the action of inflammatory mediators that are secreted both locally at sites of active periodontitis or atherosclerosis and systemically.

The pathogenesis of periodontitis seems to be related to the recruitment of leukocyte migration into the inflamed periodontal tissues promoting the progression of destruction (Garlet et al., 2005). Chemokines, which can recruit inflammatory cells expressing chemokine receptors, are found in chronic periodontitis (CP) at the site of infection and damage (Sugita et al., 1998). Since leukocyte recruitment is a dynamic biological process that involves several factors, the evaluation of the associations between chemokines and their receptor expression might define the network of inflammatory cell traffic and signaling events in periodontal tissues. Among the members of the CC chemokine family, CCL2 has been strongly implicated in CP. CCL2 is a chemoattractant for monocytes and a small subset of lymphocytes (Lee et al., 2003). Published studies have reported that peripheral blood monocytes elevate *CCL2* gene and protein expression after *P. gingivalis* infection (Mao et al., 2002). CCL4 is a potent chemotactic factor for neutrophils, monocytes, T lymphocytes, and osteoclasts (Ko and Lim, 2002). CCL4 also can induce the adhesion of T lymphocytes. The absence of CCR5

results in decreased inflammatory cell recruitment and bone loss (Ferreira et al., 2011). CCR5 is expressed on monocytes/macrophages.

The elevation in cytokine expression by various cells in CP lesions can theoretically enter the bloodstream where it can cause systemic effects (de Queiroz et al., 2008). CCL2 overexpression has been detected in many chronic systemic inflammatory disorders, such as osteoarthritis, atherosclerosis, tumors, rheumatoid arthritis (RA), and delayed-type hypersensitivity reactions (Gupta et al., 2013). The possibility that morbidity and mortality from chronic systemic inflammatory disorders may be reduced by maintaining periodontal health makes it essential to explore the impact of periodontitis on systemic disease.

Atherosclerosis is increasingly widely accepted as a chronic inflammatory disease of the cardio-cerebral vascular system. A previous study revealed significant leukocyte recruitment in atherosclerotic lesions. Monocyte cells play a pivotal role in orchestrating the inflammatory responses involved in atherosclerosis initiation and development (Aiello et al., 1999). CCL2 may have not only a central role as a chemotactic factor for monocytes but may increase monocyte adhesion as well as lead to the oxidative modification of lipids in the vascular wall. CCL2 signal transduction modulates transcriptional gene expression to influence the cell differentiation and death involved in cardiovascular diseases. CCL4 may have a chemoattractive effect on various cell types, such as monocytes, natural killer cells, macrophages, coronary endothelial cells (ECs), and immature dendritic cells (Chang and Chen, 2016). LPS-induced CCL4 secretion from human mononuclear cells was positively and strongly correlated with the total and LDL cholesterol concentration (Chang and Chen, 2016). Given the controversy in human reports, several investigations indicated the important role of CCR5 in plaque development in atherosclerosis animal models (Chang and Chen, 2016). The *in vivo* evidence indicating the significant contribution of CCL4 to vascular and myocardial alterations is still lacking.

The pathophysiological processes underlying chronic inflammation can lead to multiple diseases, such as atherosclerosis, CP, Rheumatoid arthritis (RA), and even cancer. Atherosclerosis is the leading cause of cardiovascular diseases (CVD) (Pizzo et al., 2010). Given that periodontitis, atherosclerosis, and RA are major chronic health problems worldwide, their associations are likely important. Several research groups have demonstrated a link between RA and atherosclerosis, and a link between RA and periodontitis (Abou-Raya et al., 2007). Therefore, we propose that the common inflammatory signaling pathways are implicated in the pathogenesis of periodontitis as well as atherosclerosis.

An earlier report from the author demonstrated that the CCL4/CCR5/c-Fos and c-Jun/CCL2 pathways were implicated in the regulation of CCL2 expression in collagen-induced arthritis (CIA) rat joint tissue (Zhang et al., 2011). Several common signaling mechanisms linking periodontitis to atherosclerosis have been hypothesized. Inflammatory chemokines and chemokine signaling pathways such as CCL4/CCR5/c-Fos and c-Jun/CCL2 might serve as possible network nodes to link the presence of periodontitis and atherosclerosis.

The present study was undertaken to examine gingival tissues from CP patients and the aorta of CVD patients for constitutive gene expression for CCL4, CCR5, c-Fos, c-Jun, NF-κB, and CCL2. Aorta and gingival tissue samples were analyzed. In particular, we investigated the role of the CCL4/CCR5/c-Fos and c-Jun/CCL2 pathways in mediating the CCL4-stimulated expression of CCL2 in gingiva and aorta, elucidating the relationship between CCL4 and CCL2 since they significantly contribute to the progression of periodontitis and atherosclerosis. Our results have important implications for understanding the role of CCL4, CCR5, c-Fos, c-Jun, CCL2, and NF-κB in the inflammatory processes of periodontitis and atherosclerosis.

Materials and methods

Patients and periodontal samples

A total of 38 patients, who reported to the outpatient Department of Periodontology, Hospital of Stomatology, Tianjin Medical University, Tianjin, from February to April 2021, were included in the study. Individuals with systemic disease or immunologic abnormalities, on any form of medication, and current and former cigarette smokers were not invited to participate. All individuals did present untreated CP, of whom 24 underwent tooth extraction due to advanced periodontitis and were enrolled as cases. Fourteen controls were selected from subjects who required tooth extraction for prosthetic or endodontic reasons other than PD, and who were periodontally healthy. The CP patients consisted of 14 male and 10 female patients with a mean age of 56.2 years. The median age of healthy control patients was 22.7 years, with 7 male and 7 female control subjects. Periodontitis was diagnosed based on the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. Diagnosis of CP and periodontal health were recorded according to a comprehensive periodontal examination, which included probing depth, clinical attachment level, and bleeding upon probing. Bone loss was routinely assessed from standardized radiographs. Patients presenting interdental CAL≥2 mm in ≥2 non-adjacent teeth or buccal or oral CAL≥3 mm and pockets >3 mm in ≥2 teeth were diagnosed with CP. Gingival tissues were obtained as remnants of discarded tissues during tooth extractions. Between 1 to 2 mm of the gingival sample was biopsied from the buccal and lingual/palatal side of the tooth to be extracted.

Patients with coronary artery disease

A total of 17 angiographically defined CAD patients

with clinical manifestations of atherosclerosis were included in the analysis. They had been symptomatic for two years or more. All had undergone elective surgery for CABG (Department of Cardiovascular Surgery, Tianjin Chest Hospital, Tianjin, and Department of Cardiovascular Surgery, General Hospital of Tianjin Medical University, Tianjin, from August 2021 to March 2022). Antianginal agents had been administered in all individuals for at least 3 years. Most patients were treated with antihyperlipidemic agents. Small ascending aorta specimens were collected during CABG surgery.

Patients without coronary artery disease

Ten angiographically defined non-CAD individuals without clinical signs of atherosclerosis consented to participate in this study. All had rheumatic aortic valve insufficiency and required open heart surgery for aortic valve replacement (AVR). They received regularly timed injections of benzathine penicillin in recent years. Some individuals were treated with antihyperlipidemic and antihypertensive agents. Small ascending aorta segments were obtained during AVR surgery. Clinical information of CAD and non-CAD patients is shown in Table 1.

Each sample was divided into two equal parts. A portion of each sample was stored frozen at -80°C and used for PCR, whereas the remainder of each sample was immediately transferred to 10% formalin solution and processed and blocked in paraffin wax for immunostaining and histopathological assessment.

Table 1. Clinical information of coronary artery disease (CAD) and non-coronary artery disease (non-CAD) subjects.

	CAD patients (n=17)	Non-CAD patients (n=10)
Age (Year)	66.5 (5.8)	56.9 (13.1)
Weight (kg)	72.9 (15.4)	71.4 (11.3)
BMI (kg/m2)	26.1 (4.7)	24.8 (3.1)
Sex (M/F)	10/7	8/2
Cigarette smoking[a]	35%	40%
Hypertension[b]	59%	50%
Dyslipidemia[c]	35%	10%
Diabetes mellitus[a]	24%	0%

[a]: at least 10 cigarettes per day; b: blood pressure more than 145/85; [c] total cholesterol more than 200 mg/dl.

This study was approved by the Human Ethics Review Committee of Tianjin Medical University, School of Dentistry (approval number: TMUhME-C20210715). All patients gave informed consent.

Real-time PCR analysis

Total RNA was isolated from the gingival tissue and aorta using TRIZOL (Invitrogen, Shanghai, China) according to the manufacturer's instructions. cDNAs were synthesized using a reverse transcription kit (Vazyme, Nanjing, China) and the isolated RNA samples. The relative quantity of gene expression changes in samples was performed by quantitative PCR and using the delta-delta CT method. *GAPDH* was used as an internal control gene to normalize the target genes. Control samples were used as the calibrator. The PCR conditions were as follows: one cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. The primer sequences used and the sizes of the PCR products are shown in Table 2. The PCR results were expressed as the amount of the *CCL4*, *CCR5*, *CCL2*, *c-Jun*, *c-Fos*, and *NF-κB* genes normalized to GAPDH and relative to the control samples.

Immunohistochemistry staining

Histopathologic evaluation tissue specimens were formalin-fixed and embedded in paraffin. The embedded sections (4 μm) were then prepared for H&E staining and immunostaining analysis. HE-stained sections were examined for atherosclerotic and periodontal inflammation. For immunostaining examination, specimens were subjected to antigen retrieval (30 min) and endogenous peroxidase blocking (20 min). Sections were saturated with 5% BSA in PBS, and incubated overnight at 4°C in a humidified chamber with anti-CCL2/MCP-1 mouse Monoclonal antibody (NBP2-22115, Novus Biologicals, Centennial, CO) at a 1:200 dilution. Subsequently, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Goat anti-mouse IgG, RS0001, Immunoway, Jiangsu, China) for 2 hours at 37°C. Color was developed using the DAB Immunohistochemistry Color Development kit (catalog no.: DA1010, Solarbio, China), and all sections were then routinely counterstained with Mayer's hematoxylin.

Table 2. Primers used for real time-PCR.

Gene name	Forward primer	Reverse primer	Size (bp)
CCL2	5′-CTTCTGTGCCTGCTGCTCAT- 3′	5′-CGGAGTTTGGGTTTGCTTGTC- 3′	273
CCL4	5′-CTGTGCTGATCCCAGTGAATC-3′	5′-TCAGTTCAGTTCCAGGTCATACA-3′	61
CCR5	5′-TTCTGGGCTCCCTACAACATT-3′	5′-TTGGTCCAACCTGTTAGAGCTA-3′	93
c-Jun	5′-CGCCCCTGTCCCCCATCG-3′	5′-TGTGCCACCTGTTCCCTG -3′	202
c-fos	5′-GGGGCAAGGTGGAACAGTTAT-3’	5′-CCGCTTGGAGTGTATCAGTCA-3′	126
NF-κB	5′-GCAGCACTACTTCTTGACCACC-3′	5′-TCTGCTCCTGAGCATTGACGTC -3′	130
GAPDH	5′-ATTGCCCTCAACGACCACT-3′	5′-ATGAGGTCCACCACCCTGT -3′	83

The H-score was utilized to evaluate the grade of CCL2 immunoreactivity. The results were calculated according to the H-score method applied by Ilgin et al. (Ilgin et al., 2020) (Table 3).

Statistical analysis

The Mann-Whitney *U* test was performed for pairwise comparisons between groups. Potential differences in mean H-scores between the CP and control group were assessed using the student's t-test. The SPSS statistics software was used for statistical analyses (SPSS version 12.0, Software Package Programme, Chicago, Illinois, USA). A *P* value less than 0.05 was taken as statistically significant.

Results

Quantification of CCL4, CCR5, CCL2, c-Jun, c-Fos, and NF-κB gene expression with real-time PCR

To measure the rates of increased expression for these six genes, we used real-time PCR. After PCR result conversion to mRNA, the fold changes were calculated. As shown in Figs. 1, 2, there was a significant increase in the levels of the *CCL4*, *CCR5*, and *CCL2* genes in gingival tissues of CP patients and the aorta of CAD patients. The marked *c-Jun*, *c-Fos*, and *NF-κB* gene expressions were detected in gingival tissues from CP patients but did not show any statistical differences between the CAD and non-CAD groups.

Immunohistochemistry

CCL2 is produced by fibroblasts, leucocytes, and ECs, primarily on the basal layer of the epithelium. CCL2 is present in gingivae of all CP groups, whereas control samples showed little CCL2 expression, this protein level was higher in CP gingiva (Fig. 3).

CCL2 production was increased in the aortas of CABG patients (Fig. 4). In the CAD groups, H&E staining showed that partial ECs were detached, neointima was abnormally thickened, and smooth muscle cells were irregularly arranged. As shown in Figures 4J,M,P, CCL2 was expressed in aortic specimens from CAD patients who underwent CABG surgery. CCL2 proteins were dramatically expressed in vascular endothelial and smooth muscle cells. CCL2-positive expression was shown as a chocolate brown or clay-bank product, which was granular, striped, or clustered. Control samples had CCL2 weak or no expression according to immunostaining as indicated by areas of light brown or blue in the aorta of non-CAD patients who underwent AVR surgery. The expression levels of CCL2 differed significantly in the aorta between individuals with and without CAD.

We used negative control sections from study samples without primary antibodies. All sections were evaluated at 100x, while some sections were photographed at 400x using an Olympus microscope.

The assessment of the H score revealed that CCL2 was more intense in tunica intima and tunica media in the CAD group compared with the non-CAD group ($P<0.001$). CCL2 immunostaining was significantly different between the CP and control groups (($P<0.001$). All the CCL2 H scores are summarized in Table 3.

Table 3. The statistical values for the CCL2 H-Score in the study groups.

Groups	CCL2 H-Score
Chronic periodontitis group CP (n:7)	16±29.9*
Healthy control group (n:6)	44±24.5
Coronary artery diseases group CAD (n:12)	69±11.2**
Non-coronary artery diseases group (non-CAD) (n:9)	14±4.6

(Mean ± SD). *CP vs. healthy control group $P<0.001$, student's t-test; **CAD vs. non-CAD $P<0.001$, Mann-Whitney *U* test.

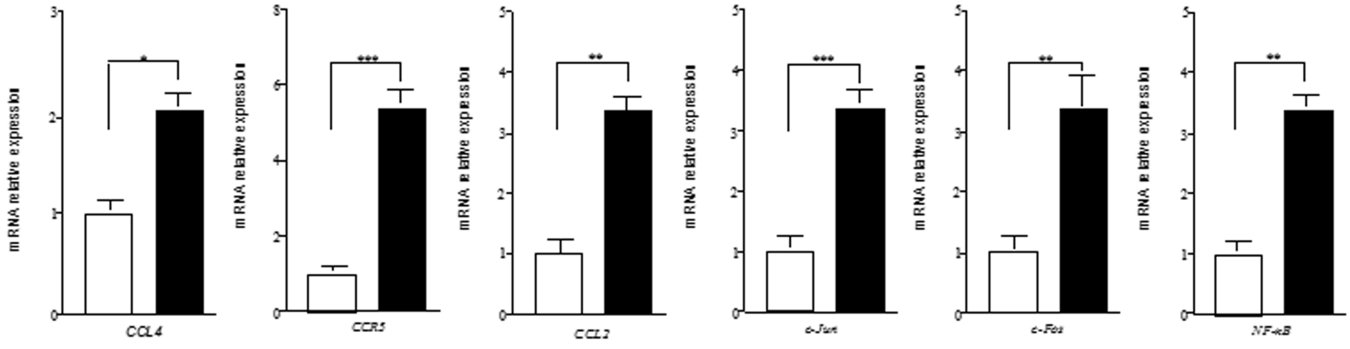

Fig. 1. Real-time PCR analysis of *CCL4*, *CCR5*, c-Jun, *c-Fos*, *NF-κB*, and *CCL2* mRNA levels in gingival tissue. Results are expressed as the relative amount of targets normalized to *GAPDH* and relative to the control samples. Control samples always had a relative quantity of 1 in this figure. The significance of differences was determined using the Mann-Whitney *U* test. *$P<0.05$, **$P<0.01$, ***$P<0.001$, n=3 for each group, representative of three independent experiments (open bars healthy controls, solid bars CP patients)

Discussion

Assessing the patterns of chemokine gene expression may be a more fruitful approach to understanding the immunopathology of periodontitis, particularly concerning inflammatory cell infiltration. Our study demonstrates the expression of CCL2 in gingivae from all CP patients, with no expression in those from healthy individuals. It is secreted in diseased gingivae by ECs, keratinocytes, fibroblasts, and monocytes of CP patients (Pradeep et al., 2009). The local production of CCL2 at inflamed periodontal sites may recruit circulating mononuclear cells to the periodontally diseased tissue, where they differentiate into macrophages upon exposure to different types of stimuli, including chemokines. Thus, the secretion of CCL2 provides a molecular mechanism accounting for the migration of monocytes observed in bacterially induced inflammatory reactions in human gingiva. Monocytes are the predominant cell type expressing CCL2 (Graves, 1999) and vascular ECs tend to express CCL2 in diseased gingiva. CCL2 expressed by endothelial cells may promote the migration of monocytes into the peripheral blood. We have shown that CCL2 expression is markedly increased in mildly and highly inflamed gingival tissue, suggesting a possible leakage to the serum. These observations strongly suggest that periodontitis may promote a systemic low-grade inflammatory response primarily through the expression of CCL2 to recruit and activate adjacent macrophages and other immune cells. These recruited and activated cells then release massive proinflammatory factors to initiate the inflammatory immune response. CP apparently and strongly modulates MIP-1 systemically. Our results also show that the expression of CCL4, and CCR5 was more intense in CP patients than in healthy control subjects. The expression of CCL4 may recruit systemic monocytes expressing CCR5 to the local periodontitis lesions, where they differentiate into macrophages following responses to various stimuli (Gemmell et al., 2001). CCL4 levels were also directly correlated with periodontal disease severity (Haytural et al., 2015). CCL4 enhances chemotaxis of CD3-stimulated lymphocytes (Graves, 1999).

Increased CCL2 has been found in atherosclerotic lesions but not in normal vascular walls, suggesting its critical role in monocyte infiltration and the progression of atherosclerosis (Aiello et al., 1999). CCL2 plays an important role in the chemotaxis of monocytes in the vascular walls and foam cell formation and is proposed to contribute to atherosclerosis progression (Aiello et al., 1999). The major cell types in the vasculature are macrophages, smooth muscle cells, and ECs, all contribute to the upregulation of CCL2 in atherosclerotic vessels. The ability of CCL2 to enhance monocyte recruitment depends on CCL2 being produced at the site of inflammations. Thus, factors that decrease CCL2 production may have therapeutic effects in treating atherosclerosis (Jones et al., 2011). CCR5 immunoreactivity was observed in atherosclerotic plaque lesions with the upregulation of CCR5 expression detected in unstable carotid atherosclerotic plaque compared with stable plaque. CCR5 was related to the progression of atherosclerosis (Wei et al., 2019). CCR5 is expressed in various types of inflammatory cells, such as monocytes and neutrophils, which promotes the development and vulnerability of atherosclerotic plaques (Luehmann et al., 2014). CCR5 might be important in late atherosclerosis, rather than in the early stages of atherosclerotic plaque development (Chang et al., 2020). Animal experiments revealed that inhibition of CCR5 can reduce atherosclerotic plaque progression. CCL4 seems likely to only be an agonist to CCR5 (Jones et al., 2011). Secreted by multiple types of vascular and blood cells, including leucocytes, vascular ECs, and vascular smooth muscle cells, CCL4 is particularly chemotactic for CD4+ T cells and memory T cells and enhances the ability of T cells to bind to vascular ECs (Chang et al., 2020). T-cell immunity may be related to atherosclerotic inflammation, as atherosclerosis can be caused by

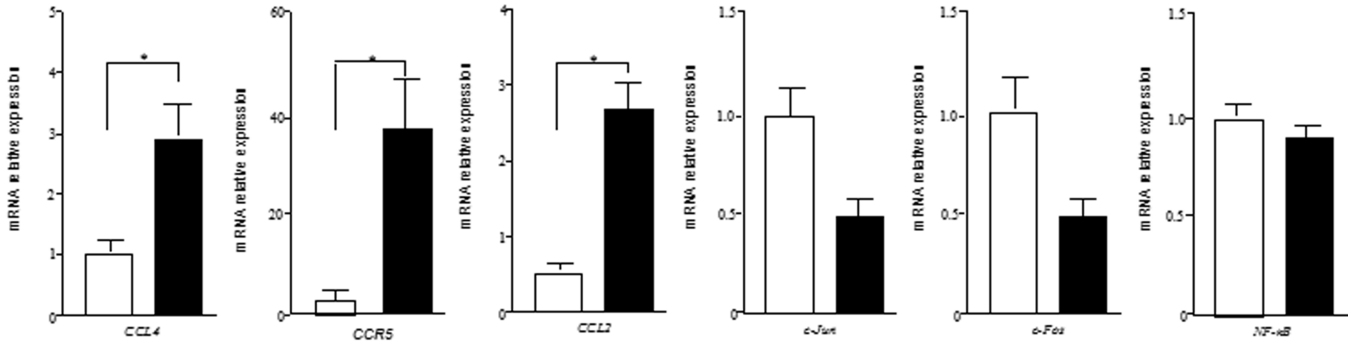

Fig. 2. Real-time PCR analysis of *CCL4*, *CCR5*, *c-Jun*, *c-Fos*, *NF-κB*, and *CCL2* mRNA levels in the aorta. Results are expressed as the relative amount of targets normalized to *GAPDH* and relative to the aortic samples of non-CAD patients. The aortic specimens from non-CAD patients always had a relative quantity of 1 in this figure. The significance of differences was determined using the Mann-Whitney U test. **P*<0.05, n=3 for each group, representative of three independent experiments (open bars non-CAD patients, solid bars CAD patients)

immune responses to plaque antigens such as ox-LDL (Chang et al., 2020). Previous studies and ours showed the possible effects of CCL4 in the progression of atheromas and atherosclerosis, which may be linked to its role in the activation of macrophages and ECs. The circulating level of the CCL4 is higher in atherosclerotic patients. Nevertheless, mononuclear cells in peripheral blood from CAD patients have enhanced CCL4 production, which is decreased by statin therapy (Jones et al., 2011). The results we report support the suggested role of CCL4 in atherosclerosis and present a rationale for a new anti-atherosclerosis strategy targeting CCL4.

AP-1 proteins and activity in endothelial cells can be induced by diverse proatherogenic stimuli, including inflammatory cytokines, physical forces, high levels of LDL, and oxidant stress (Wang et al., 1999). AP-1 overexpression is itself sufficient to modulate chemokine genes in ECs through a mechanism independent of NF-

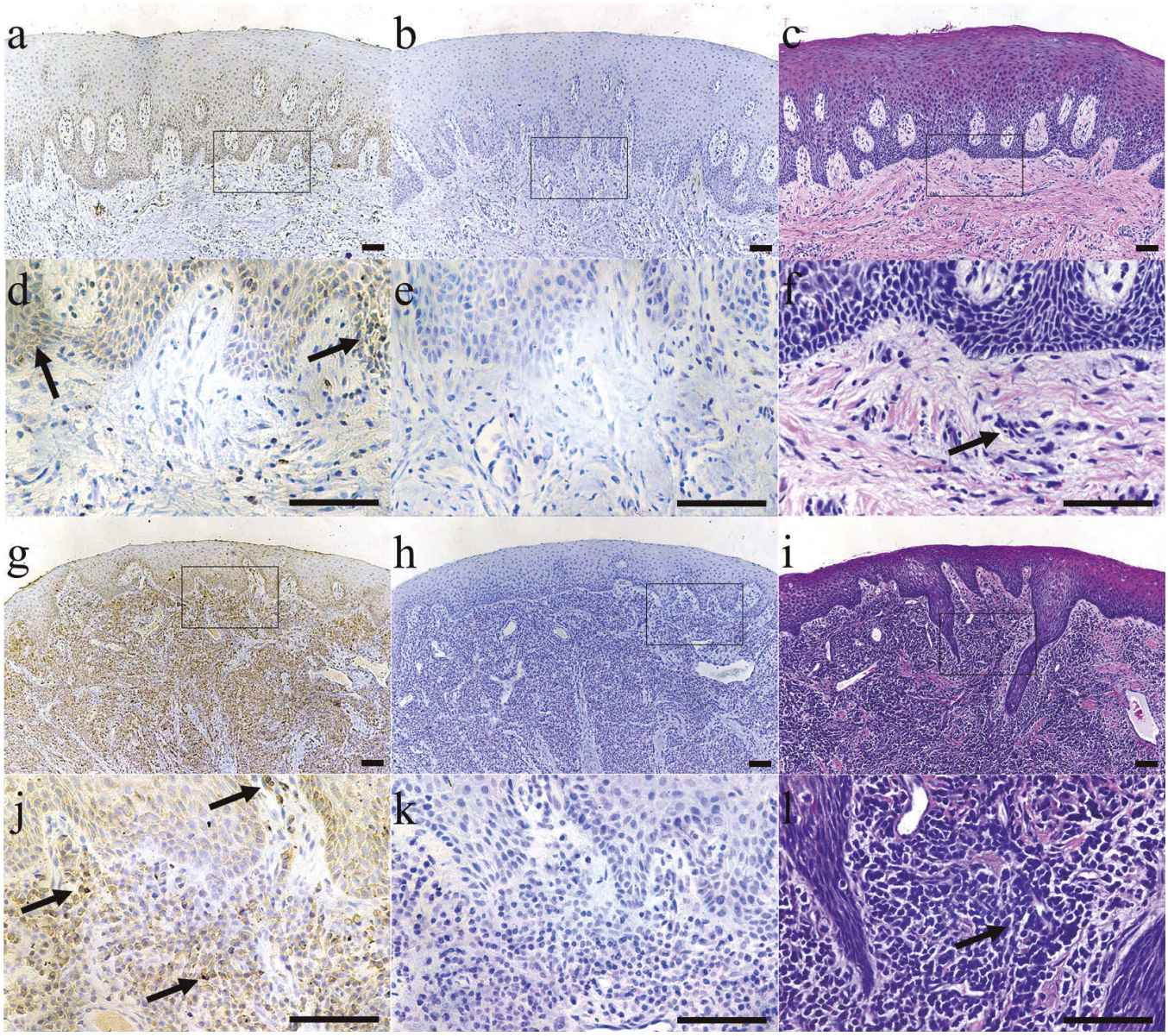

Fig. 3. Immunohistochemical study of CCL2 expression in gingival biopsies of CP patients. Photomicrographs were taken from healthy controls **(a-f)** and CP patients **(g-l)**. **b, e, h, k.** CCL2 antibody omitted. **d, e, f, j, k, l,** area of interest of gingival tissue in **a, b, c, g, h, l** at a high magnification. **g, j,** CCL2 with high intensity and high number of positive cells, strong immunoreactivity (black arrow). **a, d,** CCL2 with low intensity and low number of positive cells, mild immunoreactivity (black arrow). CCL2 expression in the CP group was higher than in the healthy control group. Hematoxylin and eosin staining in **i, l,** showing maximal inflammatory cell infiltration (black arrow). **c, f,** the density of infiltrating inflammatory cells (black arrow) is lower than in CP samples. Bar: 100 μm.

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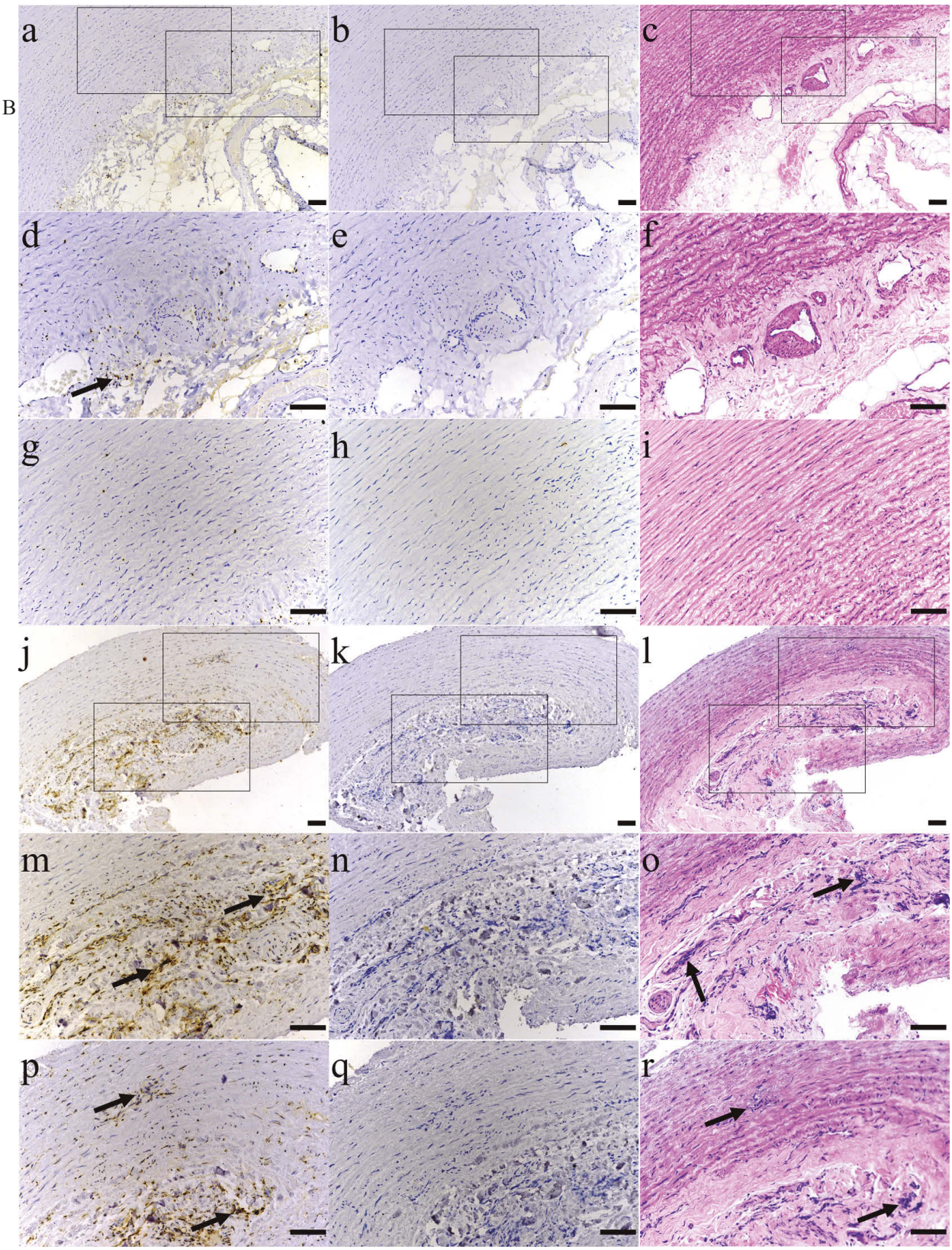

Fig. 4. Immunohistochemical study of CCL2 expression in the aorta of patients with and without CAD. Photomicrographs were taken from CAD patients **(J-R)** and non-CAD patients **(a-i)**. **b, e, h, k, n, q** CCL2 antibody omitted. **d, e, f, g, h, i, m, n, o, p, q, r,** area of interest of the aorta in **a, b, c, j, k, l** at a high magnification. **j, m,** (intima), **p** (tunica intima), CCL2 with high intensity and high number of positive cells, strong immunoreactivity (black arrow). **a, d** (tunica intima), **g** (tunica media), CCL2 with low intensity and low number of positive cells, mild immunoreactivity (black arrow). CCL2 expression in the CAD group was higher than in the non-CAD group. Hematoxylin and eosin staining in **l, o** (intima), and **r** (tunica intima), showing maximal inflammatory cells (black arrow). **c, f** (tunica intima), and **i** (tunica media), the density of infiltrating inflammatory cells is lower than in the CAD samples. Bar: 100 μm.

(nuclear factor-kappa B). AP-1 may also mediate its regulatory effects through direct interaction with several other regulatory proteins, including NF-κB (Wang et al., 1999). AP-1 plays a critical regulatory role, whereby diverse stimuli activate ECs in a particular pattern of gene expression and subsequently modulate the pathological processes of both cardiovascular and cerebrovascular disease. Activation of NF-κB (p50/p65) is significant in periodontitis tissues, suggesting the potential of NF-κB regulators in PD management. The target genes of NF-κB in inflammatory reactions include 27 of its downstream-regulated inflammatory factors, including CCL2. Many of these cytokines and chemokines regulated by NF-κB affect PD progression (Ambili et al., 2005). Many systemic diseases linked with periodontitis are also associated with NF-κB activation (Ambili et al., 2005). AP-1 and NF-κB activation may play critical roles in the development of atherogenesis, inflammation, and angiogenesis. In addition, high levels of AP-1 and NF-κB in the circulation, and the presence of periodontopathogens at systemic locations, link PD to various systemic disorders with chronic inflammatory backgrounds including cardio-cerebral vascular disease, RA, and kidney disease.

There are several binding sites for different transcription factors, including NF-κB and AP-1, in the promoter of the CCL2 gene. The AP-1 and NF-κB binding sites seem to be the key site for CCL2 production. PD activates both NF-κB and AP-1 in periodontopathogen-infected human umbilical vein ECs and the cooperative action of the two transcription factors results in increased transcription of the CCL2 gene. Periodontopathogen-activated tyrosine kinase in human gingival fibroblasts leads to CCL2 gene expression through NF-κB and AP-1 (Wang and Ohura, 2002). In gingival CP tissues, AP-1 and NF-κB expression were increased compared with the control group. AP-1 can bind to AP-1-binding sites in target gene promoters and may initiate the transcription of multiple target genes in response to different cytokines and chemokines; while AP-1 functions are regulated through alterations in the expression of Jun and Fos (Kida et al., 2005). The co-expression of both c-jun and c-fos was more efficient in the regulation of CCL2 expression. Significant overexpression of cytoplasmic NF-κB was also found in periodontally diseased tissue. CCL2 contains AP-1 and NF-κB transcription factor binding motifs, which strongly implicates AP-1 and NF-κB in the production of CCL2. For these reasons, we cannot rule out the possibility that the AP-1 and NF-κB pathways are involved in CCL2 production. From our findings, it is reasonable to speculate that CCL2 expression is upregulated by the activation of the NF-κB and AP-1 pathway in periodontitis, though it remains unclear whether these two pathways work together or in parallel.

Previous studies indicated that AP-1 and NF-κB were highly expressed as atherosclerotic lesions progressed. Both the AP-1 and NF-κB pathways might be implicated in the modulation of CCL2 expression. In contrast to these results, our study found no statistical difference in AP-1 and NF-κB levels in the aorta between the CAD and non-CAD groups. The specimens in both studies were prepared differently, previous studies used arterial tissues without atherosclerotic lesions collected from subjects who donated kidneys or autopsy cases as the control group while the present study employed the aorta from non-CAD patients who underwent AVR surgery since the aortic wall can rarely be ethically obtained from a healthy individual. The intensity of AP-1 and NF-κB expression was relatively strong in late-stage atherosclerotic lesions compared with the lesion-free parts, whereas our study was designed to evaluate AP-1 and NF-κB expression in the aorta between two groups of individuals with various degrees of atherosclerosis, which may partly explain the contradictory results of the two experiments. The different methods of specimen processing may not have produced statistically significant amounts of AP-1 and NF-κB.

Previous work from the author using the comprehensive microarray-based pathway analysis identified candidate genes and the CCL4/CCR5/c-Fos and c-Jun/CCL2 pathways involved in the knee joint synovium of CIA rats. However, the mechanism of interaction of these genes in PD and atherosclerosis is not understood. RA, atherosclerosis, and adult PD are common, chronic inflammatory disorders that share pathologic similarities. Common pathways may explain the connections between RA, atherosclerosis, and PD. These early findings prompted us to hypothesize that CCL4 induces CCL2 expression through the CCL4/CCR5/c-Fos and c-Jun/CCL2 pathways in PD and atherosclerosis. Our data showed that the levels of CCL4, CCR5, c-Jun, c-Fos, and CCL2 mRNA in periodontally diseased tissue were significantly increased compared with healthy subjects. Therefore, it is possible that the CCL4/CCR5/c-Fos and c-Jun/CCL2 pathways leading to CCL2 production may be activated upon CCL4 chemokine expression in PD.

CCL2 production has been detected in a spectrum of disease processes characterized by a chronic low-grade inflammatory condition with leukocyte recruitment, including atherosclerosis, RA (Graves, 1999), chronic obstructive pulmonary disease, tumor, diabetes mellitus, and renal ischemia. Some spillover of such higher GCF CCL2 levels from inflamed periodontal tissues may be present in the serum. High levels of CCL2 in the systemic bloodstream due to local periodontitis are a potent risk factor for atherosclerosis and other above-mentioned diseases. The raised serum levels of CCL2 might increase the predisposition to develop cardiovascular diseases in systemically healthy subjects and may accelerate atherosclerosis development in CAD patients. More than 50% of cardio-cerebral vascular disease cases are not associated with traditional risk factors, and a chronic inflammatory disease like

periodontitis may also play an important complementary role in this causal chain (Gupta et al., 2013). These studies reinforce the hypothesis that periodontitis due to its chronic low-level inflammatory response affects systemic vascular physiology, which induces thrombogenesis and/or arteriosclerosis (Gupta et al., 2013). Analysis of the mechanisms of monocyte infiltration into diseased periodontal tissues is an essential step toward elucidating CP pathogenesis. To be able to assess and regulate the expression of CCL2 in PD and atherosclerosis, knowledge of the corresponding intracellular molecular pathway is needed. We considered the possibility that CCL4-induced production of CCL2 in PD and atherosclerosis uses the same chemokine signaling pathway. By analyzing local levels of various pro-inflammatory factors, we reinforced the hypothesis that periodontitis is an inflammatory condition that triggers the immune response with a cascade of inflammatory events that are not limited to diseased sites. More recently, this concept has been extended to other systemic diseases, including adverse pregnancy outcomes, Alzheimer's disease, HIV type-1-associated dementia, malignant neoplasia, chronic kidney disease, and multiple sclerosis (Sabharwal et al., 2018). The cytokine storm induced by COVID-19 is similar to that caused by CP, atherosclerosis, and RA. The nature of COVID-19 pathogenesis is the cytokine storm with increased levels of CCL2 and CCL4, which have been implicated in COVID-19, periodontal disease, atherosclerosis, and RA (Mancini et al., 2022). Recent studies suggested that the common inflammatory pathways point towards a potential link between COVID-19, periodontitis, and other above-mentioned diseases. Those pathways might serve as ideal targets for the development of more efficient and effective means to prevent and treat RA, PD, atherosclerosis, and COVID-19. There are common biomarkers of severity for these diseases, and the clinical efficacy of various treatment options for RA also work well in periodontitis, atherosclerosis, and other chronic systemic inflammatory diseases. Besides, it might help to deepen our understanding of the underlying mechanisms of some of the drugs commonly used to control COVID-19 infection. Exploring the use of CCL2 and CCL4/CCR5/c-Fos and c-Jun/CCL2 signaling pathways as inflammatory biomarkers and novel therapeutic targets in periodontitis and other above-mentioned systemic diseases can be active fields of study in the near future.

The main advantage of this study is that it examined fresh surgical specimens, while autopsy specimens may be deteriorated by postmortem processes. In this study, we investigated the CCL2 pathway associated with CCL4, CCR5, c-Fos, c-Jun, NF-κB, and CCL2 in PD and atherosclerosis, both at the mRNA and protein level. Based on the present and other previous findings, we considered the possibility that the ligand for CCR5, CCL4, may be secreted from inflamed gingival tissue to modulate CCL2 expression via the CCL4/CCR5/c-Fos and c-Jun/CCL2 signaling pathways, which may be related to the chronic inflammation associated with PD. This pathway may be more general in other chronic systemic inflammatory diseases. CCL4, CCR5, CCL2, and even this pathway and NF-κB might act as possible nodes to link the presence of periodontitis and atherosclerosis.

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References

Abou-Raya S., Abou-Raya A., Naim A. and Abuelkheir H. (2007). Chronic inflammatory autoimmune disorders and atherosclerosis. Ann. NY Acad. Sci. 1107, 56-67.

Aiello R.J., Bourassa P.A., Lindsey, S., Weng W., Natoli E., Rollins B. J. and Milos P.M. (1999). Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler. Thromb. Vasc. Biol. 19, 1518-1525.

Ambili R., Santhi W.S., Janam P., Nandakumar K. and Pillai M.R. (2005). Expression of activated transcription factor nuclear factor-kappaB in periodontally diseased tissues. J. Periodontol. 76, 1148-1153.

Chang T.T. and Chen J.W. (2016). Emerging role of chemokine CC motif ligand 4 related mechanisms in diabetes mellitus and cardiovascular disease: friends or foes? Cardiovasc. Diabetol. 15, 117.

Chang T.T., Yang H.Y., Chen C. and Chen J.W. (2020). CCL4 inhibition in atherosclerosis: Effects on plaque stability, endothelial cell adhesiveness, and macrophages activation. Int. J. Mol. Sci. 21, 6567.

de Queiroz A.C., Taba M., O'Connell P.A. Jr, da Nóbrega P.B., Costa P.P., Kawata V.K., Trevisan G.L., Novaes A.B., de Souza S.L. Jr, Palioto D.B. and Grisi M.F. (2008). Inflammation markers in healthy and periodontitis patients: a preliminary data screening. Braz. Dental J. 19, 3-8.

Ferreira S.B., Repeke C.E. Jr, Raimundo F.M., Nunes I.S., Avila-Campos M.J., Ferreira B.R., Santana da Silva J., Campanelli A.P. and Garlet G.P. (2011). CCR5 mediates pro-osteoclastic and osteoclastogenic leukocyte chemoattraction. J. Dental Res. 90, 632-637.

Garlet G.P., Avila-Campos M.J., Milanezi C.M., Ferreira B.R. and Silva J.S. (2005). Actinobacillus actinomycetemcomitans-induced

periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration. Microbes Infect. 7, 738-747.

Gemmell E., Carter C.L. and Seymour G.J. (2001). Chemokines in human periodontal disease tissues. Clin. Exp. Immunol. 125, 134-141.

Graves D.T. (1999). The potential role of chemokines and inflammatory cytokines in periodontal disease progression. Clin. Infect. Dis. 28, 482-490.

Gupta M., Chaturvedi R. and Jain A. (2013). Role of monocyte chemoattractant protein-1 (MCP-1) as an immune-diagnostic biomarker in the pathogenesis of chronic periodontal disease. Cytokine 61, 892-897.

Haytural O., Yaman D., Ural E. C., Kantarci A. and Demirel K. (2015). Impact of periodontitis on chemokines in smokers. Clin. Oral Investig. 19, 979-986.

Ilgin C., Çomut E., Sarıgül Ç., Korkmaz S., Vardar E. and Müftüoğlu S.F. (2020). The evaluation of the distribution of CD133, CXCR1 and the tumor associated macrophages in different molecular subtypes of breast cancer. Histol. Histopathol. 35, 83-96.

Jones K.L., Maguire J.J. and Davenport A.P. (2011). Chemokine receptor CCR5: from AIDS to atherosclerosis. Br. J. Pharmacol. 162, 1453-1469.

Kida Y., Kobayashi M., Suzuki T., Takeshita A., Okamatsu Y., Hanazawa S., Yasui T. and Hasegawa K. (2005). Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. Cytokine 29, 159-168.

Ko H.J. and Lim S.S. (2002). Production of macrophage inflammatory protein (MIP)-1alpha and MIP-1beta by human polymorphonuclear neutrophils stimulated with Porphyromonas endodontalis lipopolysaccharide. J. Endod. 28, 754-757.

Lee E., Yang Y.H., Ho Y.P., Ho K.Y. and Tsai C.C. (2003). Potential role of vascular endothelial growth factor, interleukin-8 and monocyte chemoattractant protein-1 in periodontal diseases. Kaohsiung J. Med. Sci. 19, 406-415.

Luehmann H. P., Pressly E. D., Detering L., Wang C., Pierce R., Woodard P. K., Gropler R. J., Hawker C. J. and Liu Y. (2014). PET/CT imaging of chemokine receptor CCR5 in vascular injury model using targeted nanoparticle. J. Nucl. Med. 55, 629-634.

Mancini L., Americo L.M., Pizzolante T., Donati R. and Marchetti E. (2022). Impact of COVID-19 on periodontitis and peri-Implantitis: A narrative review. Front. Oral Health 3, 822824.

Mao S., Maeno N., Yoshiie K., Matayoshi S., Fujimura T. and Oda H. (2002). CD14-mediated induction of interleukin-8 and monocyte chemoattractant protein-1 by a heat-resistant constituent of Porphyromonas gingivalis in endothelial cells. Scand. J. Immunol. 56, 484-491.

Pizzo G., Guiglia R., Lo Russo L. and Campisi G. (2010). Dentistry and internal medicine: from the focal infection theory to the periodontal medicine concept. Eur. J. Intern. med. 21, 496-502.

Pradeep A.R., Daisy H. and Hadge P. (2009). Gingival crevicular fluid levels of monocyte chemoattractant protein-1 in periodontal health and disease. Arch. Oral Biol. 54, 503-509.

Sabharwal A., Gomes-Filho I. S., Stellrecht E. and Scannapieco F. A. (2018). Role of periodontal therapy in management of common complex systemic diseases and conditions: An update. Periodontol. 2000. 78, 212-226.

Sugita N., Kimura A., Matsuki Y., Yamamoto T., Yoshie H. and Hara K. (1998). Activation of transcription factors and IL-8 expression in neutrophils stimulated with lipopolysaccharide from *Porphyromonas gingivalis*. Inflammation. 22, 253-267.

Wang N., Verna L., Hardy S., Forsayeth J., Zhu Y. and Stemerman M.B. (1999). Adenovirus-mediated overexpression of c-Jun and c-Fos induces intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in human endothelial cells. Arterioscler. Thromb. Vasc. Biol. 19, 2078-2084.

Wang P.L. and Ohura K. (2002). *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. Crit. Rev. Oral Biol. Med. 13, 132-142.

Wei L., Petryk J., Gaudet C., Kamkar M., Gan W., Duan Y. and Ruddy T.D. (2019). Development of an inflammation imaging tracer, 111In-DOTA-DAPTA, targeting chemokine receptor CCR5 and preliminary evaluation in an ApoE-/- atherosclerosis mouse model. J. Nucl. Cardiol. 26, 1169-1178.

Zhang L., Zhao J., Kuboyama N. and Abiko Y. (2011). Low-level laser irradiation treatment reduces CCL2 expression in rat rheumatoid synovia via a chemokine signaling pathway. Lasers Med. Sci. 26, 707-717.

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