J Periodont Res 2008; 43: 471–477

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01080.x

CC Chemokine ligand 17 in periodontal diseases: expression in diseased tissues and production by human gingival fibroblasts

Y. Hosokawa[1], I. Hosokawa[1], K. Ozaki[2], H. Nakae[1], T. Matsuo[1]

[1]Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan and [2]Faculty of Dentistry, School of Oral Health and Welfare, Oral Health Care Promotion, The University of Tokushima, Tokushima, Japan

Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. CC Chemokine ligand 17 in periodontal diseases: expression in diseased tissues and production by human gingival fibroblasts. J Periodont Res 2008; 43: 471–477. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: It has been reported that T helper 2 (Th2) cells are related to exacerbation of periodontal disease. However, it is uncertain how the migration of Th2 cells is controlled. In this study, we examined the expression of CC chemokine ligand 17 (CCL17), which is a Th2 chemokine, in periodontal tissues. Moreover, we investigated the effects of cytokines and toll-like receptor (TLR) ligands on the production of CCL17 by human gingival fibroblasts (HGFs).

Material and Methods: We used immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) to detect CCL17 in periodontal tissues. HGFs were exposed to cytokines and TLR ligands. Expression of CCL17 was examined by RT-PCR and enzyme-linked immunosorbent assay. We used signal transduction inhibitors in some experiments.

Results: Both CCL17 and its receptor, CC chemokine receptor 4 (CCR4), were expressed in diseased periodontal tissues. A combination of tumour necrosis factor α (TNF-α) and interleukin (IL)-4/IL-13 increased CCL17 expression. Moreover, treatment of HGFs with a low dose of interferon-γ (IFN-γ) in combination with TNF-α and IL-4 or IL-13 had synergistic effects on the production of CCL17, whereas a high dose of IFN-γ inhibited CCL17 production. Furthermore, *Escherichia coli* (E. coli) lipopolysaccharide (TLR4 ligand) and Pam3CSK4 (TLR2 ligand) inhibited CCL17 production by TNF-α + IL-4-stimulated HGFs, while CpG DNA (TLR9 ligand) enhanced TNF-α + IL-4 induced-CCL17 production by HGFs. Furthermore, a c-Jun NH_2 terminal kinase (JNK) inhibitor, a phosphatidylinositol-3-kinase (PI3K) inhibitor and a nuclear factor κB (NF-κB) inhibitor inhibited CCL17 production by HGFs.

Conclusion: These results suggest that the CCL17 produced by HGFs may be involved in the migration of Th2 cells into inflamed tissues, and provide evidence that CCL17 production is controlled by cytokines and TLR ligands in periodontal disease.

Yoshitaka Hosokawa, Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan
Tel: 011 81 886 33 7340
Fax: 011 81 886 33 7340
e-mail: hosokawa@dent.tokushima-u.ac.jp

Key words: CC chemokine ligand 17; CC chemokine receptor 4; human gingival fibroblast; periodontal disease

Accepted for publication December 16, 2007

Periodontal disease is characterized by chronic inflammation associated with the presence of gram-negative bacteria in the oral cavity (1,2), resulting in the destruction of soft tissue and resorption of periodontal bone. The host immune response to these bacteria has been suggested to be associated with pathological changes or disease progression (3,4). Diseased periodontal tissue shows an accumulation of T cells, B cells, macrophages and dendritic cells (5,6). T cells are reportedly involved in the pathogenesis of periodontal disease (7). It has been reported that both T helper (Th)1 cells and Th2 cells infiltrate the inflamed tissues, producing Th1 [interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α)] and Th2 cytokines (IL-4 and IL-13) (7). However, it is unclear which cell types are most related to the pathogenesis of periodontal disease. Seymour *et al*. (8) reported that Th1 cells are associated with stable areas in periodontal tissues and Th2 cells with regions where the disease is progressing. In contrast, Taubman *et al*. (9) reported that Th1 cells are involved in periodontal destruction and Th2 cells abrogate periodontal disease symptoms. The migration of T cells into inflamed tissue is thought to contribute to the interaction between chemokines and chemokine receptors, and the roles of chemokines in periodontal disease have been analysed (7,10–15). However, most studies reported Th1-type chemokine expression in periodontally diseased tissues. Thus, the mechanism of Th2 cell migration into inflamed periodontal tissues is still uncertain.

The receptor for thymus and activation-regulated chemokine (TARC/CCL17), CC chemokine receptor 4 (CCR4) is most highly expressed on differentiated Th2 lymphocytes (16,17). Thus, CCL17 is implicated in the recruitment of Th2 lymphocytes and the maintenance of the Th2 immune response (18), as well as in the suppression of classically activated macrophages (19). Expression levels of CCL17 correlate with the severity of disease in some chronic allergic pathologies, including asthma (20), atopic dermatitis (21) and cutaneous lupus erythematosus (22). In addition, *in vivo* neutralization of CCL17 can limit Th2 lymphocyte recruitment and inflammation (23,24), underlining the significance of this chemokine to allergic pathologies and the importance of understanding how its expression is regulated in cells of the innate immune system. The CCL17 is produced by dendritic cells (25), endothelial cells (26), keratinocytes (27) and bronchial epithelial cells (28).

In this study, we examined the expression of CCL17 and CCR4 in diseased periodontal tissues and investigated whether human gingival fibroblasts (HGFs) produce CCL17 when stimulated with proinflammatory cytokines, Th1 cytokines, Th2 cytokines, anti-inflammatory cytokines and toll-like receptor (TLR) ligands in order to elucidate the mechanism of Th2 cell migration into inflamed periodontal tissues.

Material and methods

Gingival tissue biopsy and cell culture

Tissue samples were obtained at surgery from the inflamed gingiva of patients diagnosed with chronic periodontitis, or from the gingiva of clinically healthy subjects. All gingival biopsy sites in the chronic periodontitis group exhibited radiographic evidence of bone destruction, as well as having clinical probing depths greater than 4 mm, with sulcular bleeding on probing; otherwise the patients were systemically healthy. We collected samples after basic periodontal therapy such as scaling. Samples of gingival tissues were obtained from 17 chronic periodontitis patients (4 males and 13 females; average age 61.0 ± 9.8 years, average probing depth 6.33 ± 2.06 mm, average attachment loss: 7.02 ± 2.26 mm) and five healthy control subjects (5 females; average age 31.2 ± 9.8 years, average probing depth 2.4 ± 0.54 mm, average attachment loss: 2.7 ± 0.57 mm). We used two clinically healthy gingival samples and eight chronic periodontitis samples for reverse transcriptase-polymerase chain reaction (RT-PCR), and nine chronic periodontitis samples for immunohistochemical staining. We used HGFs isolated from three clinically healthy gingivae during a routine distal wedge surgical procedure. Gingival specimens were cut into small pieces and transferred to culture dishes. HGFs that grew from the gingivae were cultured on uncoated 100 mm^2 plastic dishes in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (penicillin G, 100 units/mL; streptomycin, 100 mg/mL) at 37°C in humidified air with 5% CO_2. Confluent cells were transferred and cultured for use in the present study. After three to four subcultures by trypsinization, cultures contained homogeneous, slim, spindle-shaped cells growing in characteristic swirls. The cells were used for experiments after five passages.

Informed consent was obtained from all subjects participating in this study. The study was performed with the approval and compliance of the Tokushima University Ethical Committee.

Extraction of RNA and RT-PCR analysis

Total RNA was prepared from biopsied gingival tissue or HGFs using the RNeasy total RNA isolation Kit (Qiagen, Hilden, Germany). Single-stranded cDNA for a PCR template was synthesized from 48 ng of total RNA using a primer, oligo$(dT)_{12–18}$ (Invitrogen, Carlsbad, CA, USA), and SuperScript™ III reverse transcriptase (Invitrogen) in the conditions recommended by the manufacturer. Specific primers were designed from the cDNA sequences for CCL17, CCR4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each cDNA was amplified by PCR using HotStar *Taq* DNA polymerase (Qiagen). The sequences of the primers were as follows: CCL17 forward (5′-ACTGCTCCAGGGATGCCATCGTTTTT-3′), CCL17 reverse (5′-ACAAGGGGATGGGATCTCCCTCACTG-3′), CCR4 forward (5′- AAGAAGAACAAGGCGGTGAAGATG-3′), CCR4

reverse (5′-AGGCCCCTGCAGGTT-TTGAA-3′), GAPDH forward (5′-TG-AAGGTCGGAGTCA ACGGATT-TGGT-3′) and GAPDH reverse (5′-CATGTGGGCCATGAGGTCCAC-CAC-3′). The conditions for PCR were one cycle at 95°C for 15 min, thirty-five cycles at 94°C for 1 min, 59°C for 1 min and 72°C, 1 min, and finally one cycle at 72°C for 10 min. The products were analysed on a 1.5% agarose gel containing ethidium bromide.

Immunohistochemistry

Gingival tissue samples were immediately embedded in the optical cutting temperature (OCT) compound (Miles Laboratories Inc., Elkhart, IN, USA) and quenched and stored in liquid nitrogen. The specimens were cut into 6 μm sections using a cryostat (SFS, Bright Instrument Co. Ltd, Huntingdon, UK) and collected on poly-L-lysine-coated slides. Expression of CCL17 and CCR4 was analysed with specific antibodies: goat anti-human CCL17 antibody (DAKO, Kyoto, Japan; 5 μg/mL) and mouse anti-human CCR4 antibody (R&D Systems, Minneapolis, MN, USA; 10 μg/mL), respectively. An isotype-matched control antibody was used as a negative control. The sections were reacted with specific antibodies overnight at 4°C. After being washed with phosphate-buffered saline (PBS), the sections were incubated with biotinylated anti-mouse and anti-rabbit immunoglobulins (DAKO) or biotinylated anti-goat immunogloblins (DAKO) for 20 min at room temperature and washed with PBS to remove any unreacted antibodies. The sections were then treated with peroxidase-conjugated streptavidin (DAKO) for 10 min, and washed and reacted with 3,3-diamino-benzidine tetrahydrochrolide (DAKO) in the presence of 3% H_2O_2 to develop colour. The sections were counterstained with haematoxylin and mounted with glycerol.

Release of CCL17 by HGFs

HGFs were stimulated with TNF-α (Peprotech, Rocky Hill, NJ, USA), IFN-γ (Peprotech), IL-4 (Peprotech), IL-13 (Peprotech), IL-10 (Peprotech), transforming growth factor (TGF)-β1 (Peprotech), *E. coli* lipopolysaccharide (InvivoGen, San Diago, CA, USA), Pam3CSK4 (TLR2 ligand) (InvivoGen) and CpG DNA (TLR9 ligand) (Hokkaido System Science Co. Ltd, Sapporo, Japan) for 24 h. Supernatants from the cells were collected, and the concentration of CCL17 was measured in triplicate by enzyme-linked immunosorbent assay (ELISA). A CCL17 Duoset ELISA Development system (R&D Systems) was used for the determination. All assays were performed according to the manufacturer's instructions, and cytokine levels were determined using a standard curve prepared for each assay. In selected experiments, HGFs were cultured for 1 h in the presence or absence of SP600125 (0.2–20 μM; Sigma), LY294002 (0.2–20 μM; Calbiochem, La Jolla, CA, USA) and MG-132 (0.2–20 μM; Calbiochem) prior to their incubation with the various stimulants.

Statistical analysis

Statistical significance was analysed with unpaired Student's *t*-test; p values < 0.05 were considered significant. We used STATVIEW software to perform the statistical analysis.

Results

Expression of CCL17 and CCR4 in periodontal tissues

We first examined CCL17 and CCR4 mRNA expression in whole gingival tissues. The CCL17 mRNA was not detected in normal gingival tissues but was detected in all samples of inflamed gingival tissues. The CCR4 mRNA was not detected in normal gingival tissues. We could detect CCR4 mRNA in two of nine samples from patients with periodontal disease (Fig. 1). Next, we carried out immunohistochemical staining to investigate the expression of CCL17 and CCR4 in diseased periodontal tissues (Fig. 2). Epithelial cells in diseased tissues strongly expressed CCL17 (Fig. 2A), and fibroblasts and mononuclear cells also expressed CCL17 (Fig. 2B). We could see CCR4-positive cells in inflamed gingival tissues (Fig. 2C). Expression of CCL17 was detected in the same area that was infiltrated by CCR4-positive cells (Fig. 2D). CD3-positive cells were the mainly infiltrating cell type, and a few CD14-positive cells were present in the areas shown in Fig. 2C and D (data not shown). We could detect CCR4 expression in three of nine samples, and CCL17 expression in four of nine samples. We could not detect CCR4 and CCL17 expression in normal gingival tissues (data not shown).

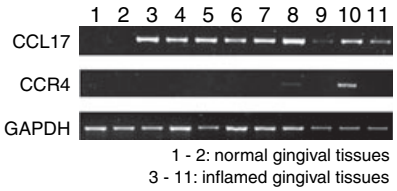

Fig. 1. Reverse transcriptase-polymerase chain reaction analysis of CCL17 and CCR4 mRNA expression in diseased periodontal tissue. Total RNA was prepared from two clinically healthy gingival samples (pocket depth, 2 mm; lanes 1 and 2) and nine diseased gingival samples (pocket depth, 4–10 mm; lanes 3–11). The expression of CCL17, CCR4 and GAPDH mRNA in periodontal tissues was analysed by RT-PCR as described in the Materials and methods.

The combination of TNF-α with Th2 cytokines induced CCL17 production in HGFs

In order to examine the effects of cytokines on the expression of CCL17 by HGFs, we used the RT-PCR and ELISA techniques. We focused on the Th2 cytokines, IL-4 and IL-13, because CCL17 is one of the Th2 chemokines. Single stimuli of TNF-α, IL-4 or IL-13 did not induce CCL17 mRNA expression and release by HGFs. In contrast, the combination of TNF-α with IL-4 or IL-13 induced CCL17 mRNA expression (Fig. 3A) and release by HGFs in an IL-4 or IL-13 dose-dependent fashion (Fig. 3B).

Interferon-γ modulated CCL17 release by TNF-α and Th2 cytokine-stimulated HGFs

Next, we examined the effects of IFN-γ on the release of CCL17 induced by

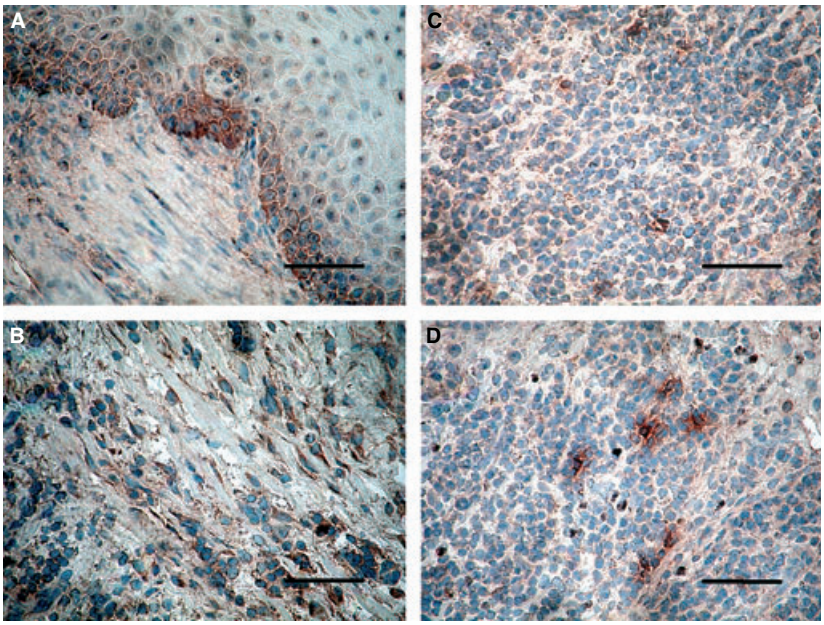

Fig. 2. Immunostaining for CCL17 and CCR4 in diseased periodontal tissues. Immunohistochemical staining of human diseased periodontal tissues with anti-CCL17 antibody (A, B and D) or anti-CCR4 antibody (C). Panels C and D are serial sections. Scale bar represents 50 μM.

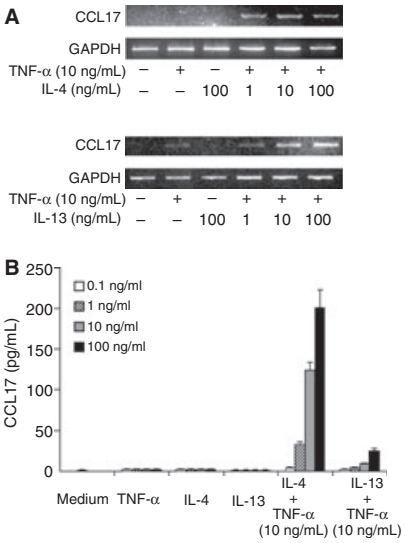

Fig. 3. Expression of CCL17 by HGFs. (A) HGFs were treated with TNF-α (10 ng/mL) with or without IL-4 (1–100 ng/mL) or IL-13 (1–100 ng/mL) for 4 h. Total RNA was isolated, and RT-PCR was carried out for CCL17 and GAPDH. Similar results were obtained in three experiments. (B) Cells were stimulated with IL-4 (0.1–100 ng/mL), TNF-α (0.1–100 ng/mL), IFN-γ (10 ng/mL), IL-4 (0.1–100 ng/mL), IL-13 (0.1–100 ng/mL), and IL-13 (0.1–100 ng/mL) + TNF-α (10 ng/mL) for 24 h. The expression levels of CCL17 in the supernatants were measured with ELISA. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values.

TNF-α and Th2 cytokine-stimulated HGFs, since both Th1 and Th2 cells are found in diseased periodontal tissues. A low concentration of IFN-γ (0.1 ng/mL) strongly enhanced the release of CCL17 by TNF-α with TR2 cytokines stimulated-HGFs (Fig. 4A and B). In contrast, a high dose of IFN-γ inhibited CCL17 release by TNF-α and IL-13-stimulated HGFs (Fig. 4B).

Effects of anti-inflammatory cytokines on CCL17 release by HGFs

Next, we examined the effects of the anti-inflammatory cytokines IL-10 and TGF-β1 on the release of CCL17 induced by TNF-α- plus IL-4-stimulated HGFs. The TGF-β1 inhibited CCL17 release by HGFs (Fig. 5A). Meanwhile, IL-10 did not modulate CCL17 release by TNF-α- plus IL-4-stimulated HGFs (Fig. 5B).

Effects of TLR ligand on CCL17 release by HGFs

Next, we examined the effects of TLR ligands, namely *E. coli* lipopolysaccharide (TLR4 ligand), Pam3CSK4 (TLR2 ligand) and CpG DNA (TLR9 ligand), on CCL17 release by HGFs, because periodontal disease is characterized by chronic inflammation associated with the presence of bacteria in the oral cavity. The *E. coli* lipopolysaccharide and Pam3CSK4 inhibited CCL17 release by HGFs (Fig. 6A and B). In contrast, CpG DNA enhanced CCL17 release by TNF-α- plus IL-4-stimulated HGFs (Fig. 6C).

Involvement of c-Jun NH_2 terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3K) and nuclear factor κB (NF-κB) in the production of CCL17 by HGFs stimulated with TNF-α and IL-4

It has been reported that TNF-α treatment activates JNK, PI3K and NF-κB pathways in fibroblasts (29,30). Therefore, in order to investigate whether the activation of JNK, PI3K and NF-κB is required for the production of CCL17 in response to TNF-α and IL-4, the effects of several inhibitors on the production of CCL17 by HGFs were examined (Fig. 7). At a concentration of 20 μM, MG-132, a cell-permeable peptide–aldehyde protease inhibitor that blocks the activation of NF-κB by acting on the proteasome, completely prevented the production of CCL17 in response to TNF-α and IL-4. SP600125, a selective JNK inhibitor, partly inhibited CCL17 production by HGF stimulated with TNF-α and IL-4. A high concentration of LY294002, a selective PI3K inhibitor, almost completely inhibited CCL17 release by HGFs.

Discussion

Local inflammatory immune reactions to periodontal pathogens seem to be crucial in protecting the host from infections, but may lead to pathological changes in host tissues (3,4). Selective migration of different cell types to gingival tissues is apparently related to

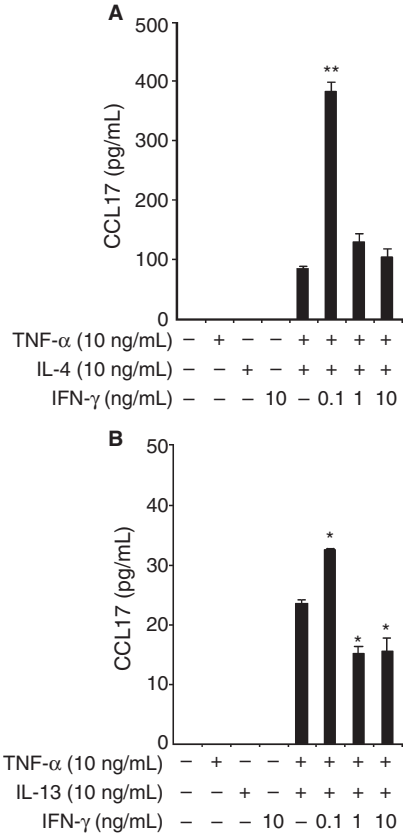

Fig. 4. Interferon-γ modulated the TNF-α- and IL-4 or IL-13-induced release of CCL17 by HGFs. HGFs were treated with TNF-α (10 ng/mL) and IL-4 (10 ng/mL; A) or IL-13 (10 ng/mL; B) with or without IFN-γ (0.1, 1 or 10 ng/mL), and the supernatants were collected after 24 h. The expression levels of CCL17 in the supernatants were measured by ELISA. Data are representative of HGFs from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values. $*p < 0.05$, $**p < 0.01$, significantly different from the TNF-α plus IL-4 or IL-13-stimulated HGFs.

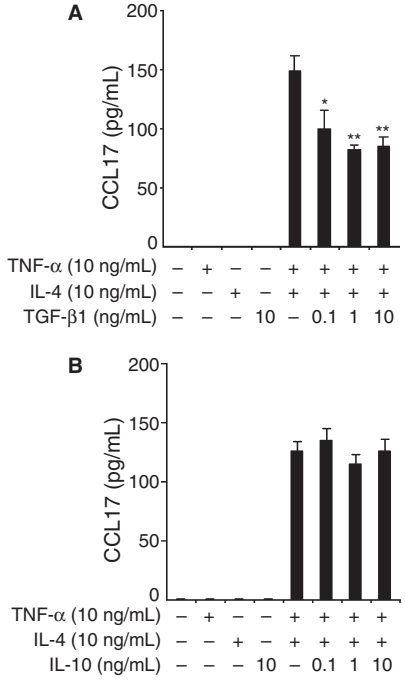

Fig. 5. Anti-inflammatory cytokines modulated the TNF-α plus IL-4-induced release of CCL17 by HGFs. (A) HGFs were treated with TNF-α (10 ng/mL) and IL-4 (10 ng/mL) with or without TGF-β1 (0.1, 1 or 10 ng/mL), and the supernatants were collected after 24 h. The expression levels of CCL17 in the supernatants were measured by ELISA. (B) HGFs were treated with TNF-α (10 ng/mL) and IL-4 (10 ng/mL) with or without IL-10 (0.1, 1 or 10 ng/mL), and the supernatants were collected after 24 h. The expression levels of CCL17 in the supernatants were measured by ELISA. Data are representative of HGFs from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values. $*p < 0.05$, $**p < 0.01$, significantly different from the TNF-α plus IL-4-stimulated HGFs.

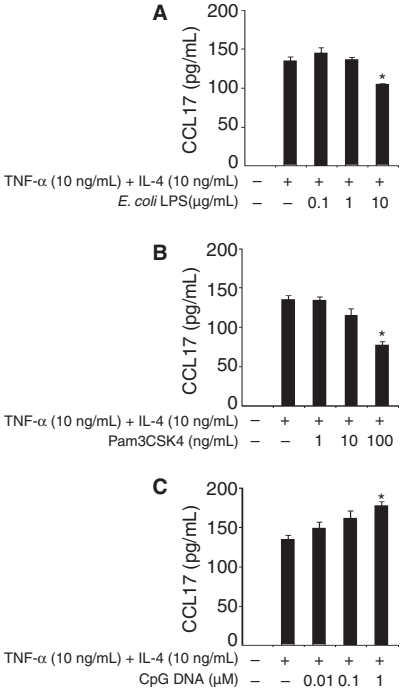

Fig. 6. TLR ligands modulated the TNF-α plus IL-4-induced release of CCL17 by HGFs. The HGFs were treated with TNF-α (10 ng/mL) plus IL-4 (10 ng/mL) with or without *Escherichia coli* lipopolysaccharide (LPS; 0.1, 1 or 10 μg/mL; A), Pam3CSK4 (1, 10 or 100 ng/mL; B) or CpG DNA (0.01, 0.1 or 1 μM; C), and the supernatants were collected after 24 h. The expression levels of CCL17 in the supernatants were measured by ELISA. Data are representative of HGFs from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values. $*p < 0.05$, significantly different from the TNF-α plus IL-4-stimulated HGFs.

the immunopathogenesis of periodontal disease (5). Of the chemokines, we focused on CCL17 because it has been reported to be involved in pathological damage in some diseases (20–24), and it is unknown whether CCL17 is expressed in periodontal tissues or not.

CC Chemokine ligand 17 is involved in the accumulation of Th2 cells (18). It has been reported that Th2 cells are involved in the pathogenesis of periodontal disease (8). Moreover, we found here that TNF-α with IL-4 or IL-13 enhanced the production of CCL17, and a high dose of IFN-γ inhibited CCL17 release by HGFs stimulated with TNF-α and IL-13. These results suggest that CCL17 release by HGFs may control the migration of Th2 cells into diseased periodontal tissues. Furthermore, IL-4 and IL-13, which are produced by Th2 cells, induced the release of CCL17 by HGFs, thereby progressively shifting the Th1–Th2 balance in the Th2 direction. Meanwhile, IFN-γ, which is produced by Th1 cells, inhibited the expression of CCL17 by HGFs in periodontal tissues, which might decrease the migration of Th1 cells. These results could explain why the production of CCL17 is important for control of the Th1–Th2 balance in diseased periodontal tissues.

In this report, we showed that a low dose of IFN-γ enhanced CCL17 production by HGFs. Yu *et al.* (31) reported that IFN-γ (100 ng/mL) enhanced CCL17 production by IL-4- and TNF-α-stimulated NG1RGB cells (a human skin fibroblast cell line), though IFN-γ (100 ng/mL) inhibited TNF-α plus IL-13-induced CCL17 production by HGFs. Fukuda *et al.*

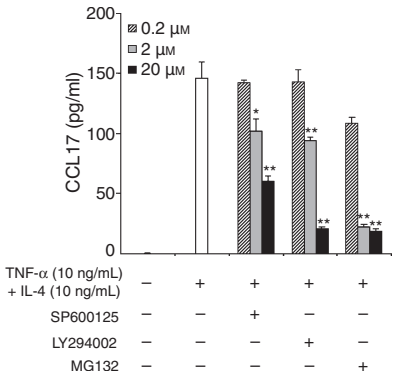

Fig. 7. Effects of a JNK inhibitor, a PI3K inhibitor and a NF κB inhibitor on the TNF-α plus IL-4-stimulated CCL17 release by HGFs. Cells were pre-incubated with SP600125 (0.2, 2 or 20 μM), LY294002 (0.2, 2 or 20 μM) or MG-132 (0.2, 20 or 20 μM) for 1 h and then incubated with TNF-α (10 ng/mL) and IL-4 (10 ng/mL). After 24 h incubation, supernatants were collected and CCL17 expression was measured by ELISA. Data are representative of HGFs from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values. $*p < 0.05$, $**p < 0.01$, significantly different from the TNF-α plus IL-4-stimulated HGFs without inhibitors.

(32) revealed differential expression of CCL17 by cytokine-stimulated human fibroblasts from cornea, skin and lung. These reports and ours indicate that cytokine regulation of CCL17 expression differs among fibroblasts derived from different tissues, such as the cornea, skin, lung and periodontal tissue.

It has been reported that each cell type expresses different chemokine receptors. For example, Th2 cells express CCR4 and CCR8 (33), and natural killer cells express CCR1, CCR4, CCR7, CXCR3, CXCR4, CX3CR1 and CXCR6 (34). Several chemokines are expressed in periodontal diseased tissues, for example, CCL2 (CCR1 ligand), CCL3 (CCR1 and CCR5 ligand), CCL5 (CCR1 and CCR5 ligand), CCL20 (CCR6 ligand), CXCL10 (CXCR3 ligand), CXCL12 (CXCR4 ligand), CXCL16 (CXCR6 ligand) and fractalkine (CX3CR1 ligand; 11–13,35–37). These previous reports and our results suggest that more than one chemokine controls one type of cell, so the chemokine system involved in periodontal disease is complex. Further investigations are necessary to clarify the chemokine system in periodontal tissue.

Our report revealed that *E. coli* lipopolysaccharide and Pam3CSK4 inhibited CCL17 production by HGFs, although CpG DNA enhanced CCL17 production. It has been reported that TLR4 ligand enhanced Th1 chemokine release by fibroblasts (38), and TLR2 ligand directly triggered Th1 effector functions (39). Our findings seem reasonable because TLR2 and TLR4 ligands inhibited Th2 chemokine production by HGFs. Recently, it has been reported that CpG DNA strongly inhibited the effector phase of inflammatory arthritis, which is known to be a Th1-related disease (40). The CpG DNA might inhibit the Th1 response because it induced Th2-type chemokines, such as CCL17. Further investigation is necessary to reveal how TLR ligands control Th1 and Th2 responses.

We demonstrated that a JNK inhibitor, a PI3K inhibitor and a NF-κB inhibitor inhibited CCL17 production. It has been reported that a NF-κB inhibitor reduced CCL17 production by keratinocytes (41). Their report agrees with ours. On the other hand, this is the first report to reveal that a JNK inhibitor and a PI3K inhibitor reduce CCL17 production by HGFs; Heijink et al. reported that a p38 mitogen-activated protein kinase (MAPK) inhibitor and an extracellular regulated kinase (ERK) inhibitor reduced CCL17 production by epithelial cells (42). A p38 MAPK inhibitor and an ERK inhibitor did not show a significant reduction of CCL17 release by HGFs in our experiments (data not shown). These results show that signal transduction pathways related to CCL17 are different in different cell types, and the CCL17 production by HGFs is mediated by JNK, PI3K and NF-κB pathways.

In conclusion, CCL17 is expressed by HGFs in diseased periodontal tissues. The expression is controlled by various cytokines and TLR ligands. The CCL17 produced by HGFs may play a role in the attraction of Th2 cells to diseased tissue and the exacerbation of periodontal disease. However, further study is necessary to clarify the role of CCL17 in the development of periodontal disease.

Acknowledgements

This study was supported by a grant-in-aid from the Ministry of Education, Science, and Culture of Japan.

References

1. Dzink JL, Tanner AC, Haffajee AD, Socransky SS. Gram negative species associated with active destructive periodontal lesions. *J Clin Periodontol* 1985; **12:**648–659.
2. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
3. Kjeldsen M, Holmstrup P, Bendtzen K. Marginal periodontitis and cytokines: a review of the literature. *J Periodontol* 1993;**64:**1013–1022.
4. Wilson M, Reddi K, Henderson B. Cytokine-inducing components of periodontopathogenic bacteria. *J Periodont Res* 1996;**31:**393–407.
5. Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. *J Periodontol* 2005;**76:**2033–2041.
6. Jotwani R, Cutler CW. Multiple dendritic cell (DC) subpopulations in human gingiva and association of mature DCs with CD4+ T-cells *in situ*. *J Dent Res* 2003;**82:**736–741.
7. Taubman MA, Kawai T. Involvement of T-lymphocytes in periodontal disease and in direct and indirect induction of bone resorption. *Crit Rev Oral Biol Med* 2001;**12:**125–135.
8. Seymour GJ, Gemmell E, Reinhardt RA, Eastcott J, Taubman MA. Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *J Periodont Res* 1993; **28:**478–486.
9. Taubman MA, Haffajee AD, Socransky SS, Smith DJ, Ebersole JL. Longitudinal monitoring of humoral antibody in subjects with destructive periodontal diseases. *J Periodont Res* 1992;**27:**511–521.
10. Kawai T, Eisen-Lev R, Seki M, Eastcott JW, Wilson ME, Taubman MA. Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *J Immunol* 2000;**164:**2102–2109.
11. Hosokawa Y, Nakanishi T, Yamaguchi D *et al*. Macrophage inflammatory protein 3α-CC chemokine receptor 6 interactions

play an important role in $CD4^+$ T-cell accumulation in periodontal diseased tissue. *Clin Exp Immunol* 2002;**128:**548–554.

12. Hosokawa Y, Nakanishi T, Yamaguchi D, Nakae H, Matsuo T. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, in periodontal diseased tissue. *Clin Exp Immunol* 2005;**139:**506–512.
13. Hosokawa Y, Hosokawa I, Ozaki K *et al*. CXCL12 and CXCR4 expression by human gingival fibroblasts in periodontal disease. *Clin Exp Immunol* 2005;**141:**467–474.
14. Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. Increase of CCL20 expression by human gingival fibroblasts upon stimulation with cytokines and bacterial endotoxin. *Clin Exp Immunol* 2005;**142:**285–291.
15. Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. CXC chemokine ligand 16 in periodontal diseases: expression in diseased tissues and production by cytokine-stimulated human gingival fibroblasts. *Clin Exp Immunol* 2007; **149:**146–154.
16. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J Biol Chem* 1997;**272:**15036–15042.
17. Bonecchi R, Bianchi G, Bordignon PP *et al*. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998;**187:**129–134.
18. Imai T, Nagira M, Takagi S *et al*. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999; **11:**81–88.
19. Katakura T, Miyazaki M, Kobayashi M, Herndon DN, Suzuki F. CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. *J Immunol* 2004;**172:**1407–1413.
20. Sekiya T, Yamada H, Yamaguchi M *et al*. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy* 2002;**57:**173–177.
21. Jahnz-Rozyk K, Targowski T, Paluchowska E, Owczarek W, Kucharczyk A. Serum thymus and activation-regulated chemokine, macrophage-derived chemokine and eotaxin as markers of severity of atopic dermatitis. *Allergy* 2005;**60:**685–688.
22. Wenzel J, Henze S, Worenkamper E *et al*. Role of the chemokine receptor CCR4 and its ligand thymus- and activation-regulated chemokine/CCL17 for lymphocyte recruitment in cutaneous lupus erythematosus. *J Invest Dermatol* 2005; **124:**1241–1248.
23. Yoneyama H, Harada A, Imai T *et al*. Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. *J Clin Invest* 1998;**102:**1933–1941.
24. Kawasaki S, Takizawa H, Yoneyama H *et al*. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* 2001;**166:**2055–2062.
25. Imai T, Yoshida T, Baba M, Nishimura M, Kakizaki M, Yoshie O. Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein-Barr virus vector. *J Biol Chem* 1996;**271:**21514–21521.
26. Campbell JJ, Haraldsen G, Pan J *et al*. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 1999;**400:**776–780.
27. Vestergaard C, Yoneyama H, Murai M *et al*. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest* 1999;**104:**1097–1105.
28. Sekiya T, Miyamasu M, Imanishi M *et al*. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* 2000;**165:**2205–2213.
29. Reinhard C, Shamoon B, Shyamala V, Williams LT. Tumor necrosis factor α-induced activation of c-jun N-terminal kinase is mediated by TRAF2. *EMBO J* 1997;**16:**1080–1092.
30. Westra J, Brouwer E, Bos R *et al*. Regulation of cytokine-induced HIF-1α expression in rheumatoid synovial fibroblasts. *Ann N Y Acad Sci* 2007;**1108:**340–348.
31. Yu B, Koga T, Urabe K *et al*. Differential regulation of thymus- and activation-regulated chemokine induced by IL-4, IL-13, TNF-α and IFN-γ in human keratinocyte and fibroblast. *J Dermatol Sci* 2002;**30:**29–36.
32. Fukuda K, Fujitsu Y, Seki K, Kumagai N, Nishida T. Differential expression of thymus- and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) by human fibroblasts from cornea, skin, and lung. *J Allergy Clin Immunol* 2003;**111:**520–526.
33. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* 2000; **18:**593–620.
34. Inngjerdingen M, Damaj B, Maghazachi AA. Expression and regulation of chemokine receptors in human natural killer cells. *Blood* 2001;**97:**367–375.
35. Hanazawa S, Kawata Y, Takeshita A *et al*. Expression of monocyte chemoattractant protein 1 (MCP-1) in adult periodontal disease: increased monocyte chemotactic activity in crevicular fluids and induction of MCP-1 expression in gingival tissues. *Infect Immun* 1993;**61:** 5219–5224.
36. Gemmell E, Carter CL, Seymour GJ. Chemokines in human periodontal disease tissues. *Clin Exp Immunol* 2001;**125:**134–141.
37. Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Levels of interleukin-1β, -8, and -10 and RANTES in gingival crevicular fluid and cell populations in adult periodontitis patients and the effect of periodontal treatment. *J Periodontol* 2000;**71:**1535–1545.
38. Proost P, Vynckier AK, Mahieu F *et al*. Microbial Toll-like receptor ligands differentially regulate CXCL10/IP-10 expression in fibroblasts and mononuclear leukocytes in synergy with IFN-γ and provide a mechanism for enhanced synovial chemokine levels in septic arthritis. *Eur J Immunol* 2003;**33:**3146–3153.
39. Imanishi T, Hara H, Suzuki S, Suzuki N, Akira S, Saito T. Cutting edge: TLR2 directly triggers Th1 effector functions. *J Immunol* 2007;**178:**6715–6719.
40. Wu HJ, Sawaya H, Binstadt B *et al*. Inflammatory arthritis can be reined in by CpG-induced DC–NK cell cross talk. *J Exp Med* 2007;**204:**1911–1922.
41. Komine M, Kakinuma T, Kagami S, Hanakawa Y, Hashimoto K, Tamaki K. Mechanism of thymus- and activation-regulated chemokine (TARC)/CCL17 production and its modulation by roxithromycin. *J Invest Dermatol* 2005; **125:**491–498.
42. Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ, Vellenga E. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J Immunol* 2007;**178:**7678–7685.