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Cytokines differentially regulate CXCL10 production by interferon-γ-stimulated or tumor necrosis factor-α-stimulated human gingival fibroblasts

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Background and Objective: CXC chemokine 10 (CXCL10) activates CXC chemokine receptor 3 (CXCR3) and attracts activated T-helper 1 cells. In this study we examined the effects of cytokines on CXCL10 production by human gingival fibroblasts.

Material and Methods: Human gingival fibroblasts were exposed to pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor-α), a T-helper 1 cytokine (interferon-γ), T-helper 2 cytokines (interleukin-4, interleukin-13), T-helper 17 cytokines (interleukin-17A, interleukin-22) and regulatory T-cell cytokines (interleukin-10, transforming growth factor-β1) for 24 h. CXCL10 production by human gingival fibroblasts was examined by enzyme-linked immunosorbent assay.

Results: Human gingival fibroblasts produced CXCL10 protein upon stimulation with interleukin-1β, tumor necrosis factor-α and interferon-γ. Treatment of human gingival fibroblasts with interferon-γ in combination with tumor necrosis factor-α or interleukin-1β resulted in a synergistic production of CXCL10. However, interleukin-4 and interleukin-13 inhibited CXCL10 production by interferon-γ-stimulated or tumor necrosis factor-α-stimulated-human gingival fibroblasts. On the other hand, interleukin-17A and interleukin-22 enhanced CXCL10 production by human gingival fibroblasts treated with interferon-γ and inhibited CXCL10 production by tumor necrosis factor-α-stimulated human gingival fibroblasts. Furthermore, the anti-inflammatory cytokine, interleukin-10, inhibited CXCL10 production by both interferon-γ- and tumor necrosis factor-α-stimulated human gingival fibroblasts, but transforming growth factor-β1 enhanced interferon-γ-mediated CXCL10 production by human gingival fibroblasts.

Conclusion: These results mean that the balance of cytokines in periodontally diseased tissue may be essential for the control of CXCL10 production by human gingival fibroblasts, and the production of CXCL10 might be important for the regulation of T-helper 1 cell infiltration in periodontally diseased tissue.

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Periodontitis is a chronic bacterial infection of tooth-supporting structures. It causes destruction of periodontal connective tissues and bone. Oral plaque bacteria, including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythensis*, are recognized as etiologic agents in periodontitis. The initiation and progression of periodontitis result from the host response to plaque bacteria. Immunohistochemical studies reveal massive infiltration of inflammatory cells, including T and B cells and macrophages, in the region affected by periodontitis. In addition, high levels of cytokines and chemokines, such as interleukin-1β, tumor necrosis factor-α, interferon-γ, interleukin-4, interleukin-17A, CCL5, CCL20 and fractalkine, were detected in inflamed gingival tissues and gingival crevicular fluid (1–6).

Gingival fibroblasts, the major cell type in periodontal connective tissues, provide a tissue framework for tooth anchorage. Until recently, gingival fibroblasts were presumed to be immunologically inert. Now, however, researchers recognize their active role in host defense. Upon stimulation with cytokines, as well as with bacterial pathogens, human gingival fibroblasts secrete various soluble mediators of inflammation, such as interleukin-1β, interleukin-6 and interleukin-8 (7–10), and up-regulate the expression of human leukocyte antigen-DR, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (11). These fibroblast-derived mediators and surface antigens are thought to play an important role in the periodontal inflammatory response.

The CXC chemokine CXCL10 was discovered as an interferon-γ-inducible protein of 10 kDa in monocytic U937 cells (12). CXCL10 attracts activated T-helper 1 cells through interaction with CXC chemokine receptor 3 (CXCR3) (13,14). CXCL10 shares this receptor, and hence biological activity, with two more-recently identified CXC chemokines, CXCL9 and CXCL11 (15–17). *In vivo*, enhanced levels of CXCL10 have been reported in several inflammatory diseases that are predominantly associated with a T-helper 1 phenotype. It is reported that CXCL10 and CXCR3 are present in inflamed gingival tissues (18,19). However, it is unknown whether or not human gingival fibroblasts are related to CXCL10 production in inflamed gingival tissues.

Recently, several studies demonstrated that T-helper 1 cells are involved in bone resorption in the oral cavity. Kawai and colleagues reported that *A. actinomycetemcomitans* 29-kDa outer membrane protein and lipopolysaccharide activation of T-helper 1-type T cells appeared to trigger inflammatory periodontal bone resorption (20). Stashenko and colleagues reported that intrapulpal challenge with viable *P. gingivalis* results in massive periapical bone destruction during a systemic T-helper 1 response (21).

In this study we investigated the effects of various cytokines, including pro-inflammatory cytokines, T-helper 1-type cytokines, T-helper 2-type cytokines, T-helper 17-type cytokines and anti-inflammatory cytokines, on the production of CXCL10 by human gingival fibroblasts.

Material and methods

Cells and culture conditions

Human gingival fibroblasts were prepared from the explants of clinically normal gingiva from different three patients (three women, 26–40 years of age) during a routine distal wedge surgical procedure and after obtaining informed consent. The explants were cut into pieces and cultured in 100-mm-diameter tissue-culture dishes containing Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KA, USA), 50 IU/mL of penicillin and 50 μg/mL of streptomycin, with a medium change every 3 d for 10–15 d until confluent cell monolayers were formed. The cells were detached by incubation in 0.25% trypsin-EDTA, washed with phosphate-buffered saline and subcultured in plastic flasks. After three to four subcultures, homogeneous, slim spindle-shaped cells grown in characteristic swirls were obtained. The cells were used as confluent monolayers at subculture levels five to 15. Human gingival fibroblasts were stimulated with interleukin-1β (Peprotech, Rocky Hill, NJ, USA), tumor necrosis factor-α (Peprotech), interferon-γ (Peprotech), interleukin-4 (Peprotech), interleukin-13 (Peprotech), interleukin-17A (Peprotech), interleukin-22 (Peprotech), interleukin-10 (Peprotech) and transforming growth factor-β1 (Peprotech) for 24 h. At predetermined time-points, cell-free supernatants were harvested and stored at −80°C for cytokine determination.

Cytokine determination

The concentration of CXCL10 in the culture supernatant was measured by enzyme-linked immunosorbent assay. Duoset (R&D Systems, Minneapolis, MI, USA) was used to detect CXCL10. The assay was performed according to the manufacturer's instructions. In brief, 100 μL of standard CXCL10 and sample supernatants were placed, respectively, in the 96-well polystyrene microplate precoated with anti-CXCL10 immunoglobulin. CXCL10 was detected by a horseradish peroxidase-labelled monoclonal antibody to CXCL10. Then 100 μL of anti-CXCL10 conjugates were placed in each well and incubated for 2 h at room temperature. The microplate was washed to remove unbound enzyme-labeled antibodies. We used the TMB peroxidase EIA substrate kit (Bio-Rad Laboratories, Hercules, CA, USA) to determine the amount of horseradish peroxidase bound to each well. The reaction was stopped by the addition of sulfuric acid. The plates were read at 450 nm. The data were determined by using a standard curve prepared for each assay.

Statistical analysis

Statistical significance was analyzed using the Student's *t*-test. A *p*-value of < 0.05 was considered significant.

Results

CXCL10 release by single cytokine-stimulated human gingival fibroblasts

It has been reported that various types of cytokines are produced by cells in inflamed gingival tissues. Therefore, we stimulated human gingival fibroblasts using pro-inflammatory cytokines, a T-helper 1 cytokine, T-helper 2 cytokines and T-helper 17 cytokines. As shown in Fig. 1, interferon-γ was the most efficient inducer of CXCL10 in human gingival fibroblasts in this experiment. Tumor necrosis factor-α moderately, and interleukin-1β slightly, induced CXCL10 release by human gingival fibroblasts in a dose-dependent manner. Interleukin-4, interleukin-13, interleukin-17A and interleukin-22 induced only very low levels of CXCL10 production by human gingival fibroblasts. Reverse transcription–polymerase chain reaction analysis revealed that interleukin-1β, tumor necrosis factor-α and interferon-γ induced CXCL10 mRNA expression in human gingival fibroblasts (data not shown).

Synergistic effects between tumor necrosis factor-α and interferon-γ on the production of CXCL10 by human gingival fibroblasts

In diseased periodontal tissues, various cytokines simultaneously activate cells through interaction with their corresponding cytokine receptors. Therefore, induction of CXCL10 in human gingival fibroblasts was studied after stimulation with different combinations of cytokines. In Fig. 2, we simultaneously stimulated human gingival fibroblasts with tumor necrosis factor-α and interferon-γ. We showed the synergistic induction of CXCL10 by interferon-γ and tumor necrosis factor-α (Fig. 2).

Effects of interleukin-1β on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts

Next, we examined the production of CXCL10 after treatment of human gingival fibroblasts with different combinations of interleukin-1β and tumor necrosis factor-α/interferon-γ. Interleukin-1β combined with interferon-γ provided a synergistic induction of CXCL10 by human gingival fibroblasts. On the other hand, interleukin-1β combined with tumor necrosis factor-α produced an additive effect on CXCL10 induction by human gingival fibroblasts (Fig. 3).

Effects of T-helper 2 cytokines on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts

Single stimuli of interleukin-4 and interleukin-13 did not induce CXCL10 production by human gingival fibroblasts. However, it is uncertain whether interleukin-4 and interleukin-13 modulate cytokine-induced CXCL10. Next, we stimulated human gingival fibroblasts with combinations of T-helper 2 cytokines and interferon-γ or tumor necrosis factor-α (Fig. 4). Both interleukin-4 and interleukin-13 inhibited CXCL10 production induced by interferon-γ or tumor necrosis factor-α in a dose-dependent manner.

Effects of T-helper 17 cytokines on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts

Single stimuli of interleukin-17A and interleukin-22 did not induce CXCL10 release by human gingival fibroblasts, similarly to the situation with T-helper 2 cytokines. We examined the effects of T-helper 17 cytokines on CXCL10 production by cytokine-stimulated human gingival fibroblasts. T-helper 17 cytokines enhanced CXCL10 production by interferon-γ-stimulated human gingival fibroblasts in a dose-dependent manner, unlike T-helper 2 cytokines (Fig. 5A,B). On the other hand, interleukin-17A and interleukin-22 inhibited CXCL10 production by tumor necrosis factor-α-stimulated human gingival fibroblasts, as observed in T-helper 2 cytokines (Fig. 5C,D).

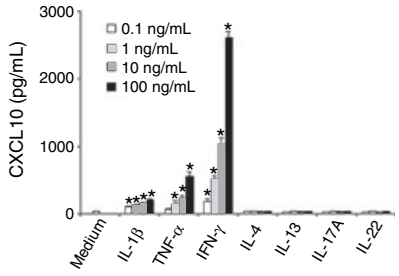

Fig. 1. Release of CXCL10 by human gingival fibroblasts. CXCL10 induction in human gingival fibroblasts by a single stimulus. Human gingival fibroblasts were incubated for 24 h at 37°C with interleukin-1β, tumor necrosis factor-α, interferon-γ, interleukin-4, interleukin-13, interleukin-17A or interleukin-22. The culture medium was removed and assayed for CXCL10 release using enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation ($n = 3$; Student's t-test, $^{*}p < 0.05$ vs. medium). Similar results were obtained in three identical experiments. IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

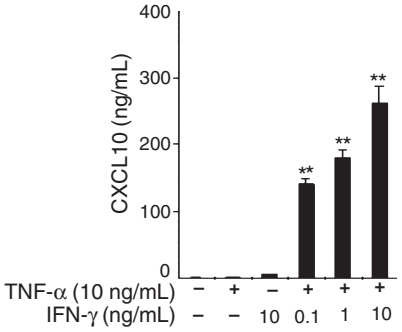

Fig. 2. Synergistic effects of tumor necrosis factor-α and interferon-γ on the production of CXCL10 in human gingival fibroblasts. Human gingival fibroblasts were incubated for 24 h with varying concentrations of interferon-γ in combination with tumor necrosis factor-α (10 ng/mL). The culture medium was removed and assayed for CXCL10 release using enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation ($n = 3$; Student's t-test, $^{**}p < 0.01$ vs. tumor necrosis factor-α single-stimulated). Similar results were obtained in three identical experiments. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

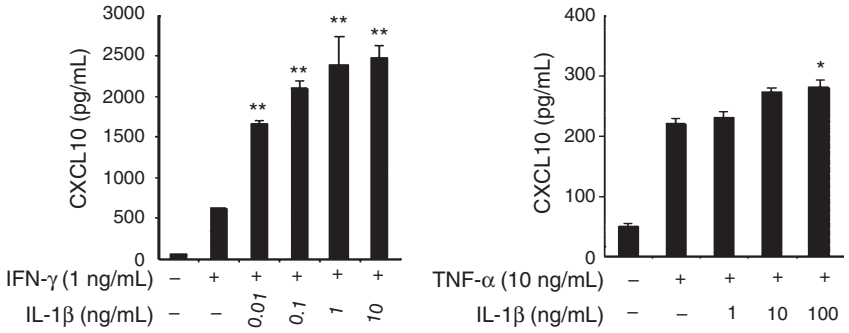

Fig. 3. Effects of interleukin-1β on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts. Human gingival fibroblasts were incubated for 24 h with varying concentrations of interleukin-1β in combination with interferon-γ (1 ng/mL) or tumor necrosis factor-α (10 ng/mL). The culture medium was removed and assayed for CXCL10 release using enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation ($n = 3$; Student's t-test, $*p < 0.05$, $**p < 0.01$ vs. interferon-γ or tumor necrosis factor-α single-stimulated). Similar results were obtained in three identical experiments. IFN-γ, interferon-γ; IL, interleukin.

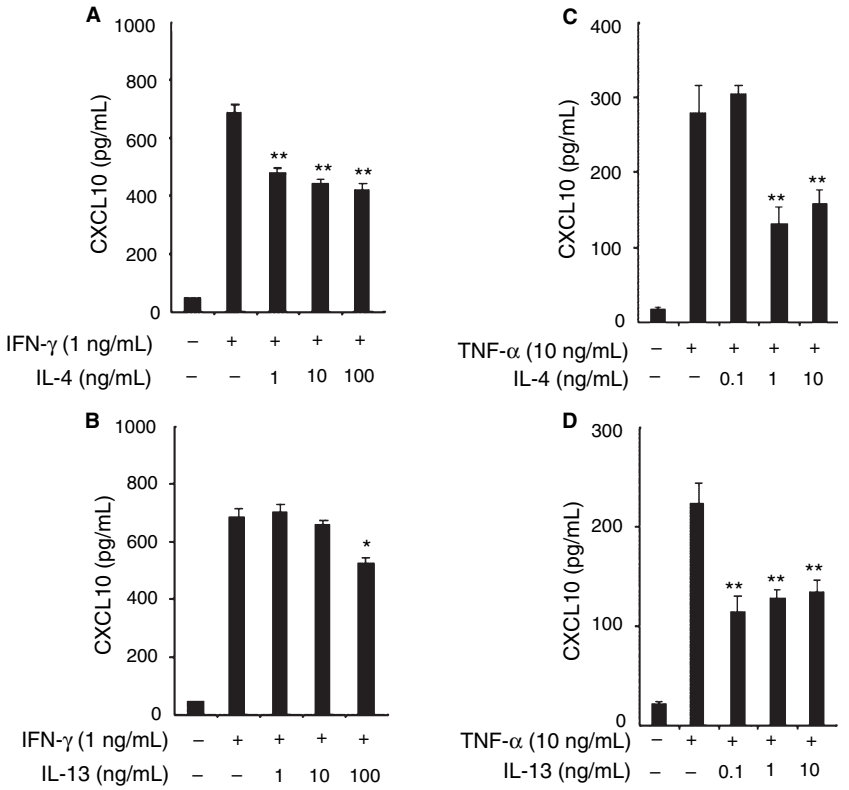

Fig. 4. Effects of T-helper 2 cytokines on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts. Human gingival fibroblasts were incubated for 24 h with varying concentrations of interleukin-4 or interleukin-13 in combination with interferon-γ (1 ng/mL) or tumor necrosis factor-α (10 ng/mL). The medium was removed and assayed for CXCL10 release using enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation ($n = 3$; Student's t-test, $*p < 0.05$, $**p < 0.01$ vs. interferon-γ or tumor necrosis factor-α single-stimulated). Similar results were obtained in three identical experiments. IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

Effects of anti-inflammatory cytokines on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts

Interleukin-10 and transforming growth factor-β1 are known as the anti-inflammatory cytokines. We hypothesized that interleukin-10 and transforming growth factor-β1 would inhibit CXCL10 production by human gingival fibroblasts. As expected, interleukin-10 inhibited CXCL10 production by interferon-γ-stimulated-human gingival fibroblasts (Fig. 6A). However, contrary to expectations, transforming growth factor-β1 enhanced the CXCL10 production by interferon-γ-stimulated human gingival fibroblasts in a dose-dependent manner (Fig. 6B). Interleukin-10 and transforming growth factor-β1 inhibited CXCL10 production by tumor necrosis factor-α-treated human gingival fibroblasts (Fig. 6C,D).

Discussion

The inflamed gingival tissue of periodontal disease is characterized by an infiltration of inflammatory cells, including T cells. It has been reported that T-helper 1 cells are related to bone resorption in diseased periodontal tissues (20,21). The interaction of CXCR3 and their ligands may be critical in perpetuating the local T-helper 1 immune response. In the present study, we demonstrated that human gingival fibroblasts are able to secrete the CXCR3-agonistic chemokine, CXCL10, when stimulated with the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α, or with the T-helper 1 cytokine, interferon-γ. Therefore, human gingival fibroblasts appear to be an important cellular source of this T-helper 1-associated chemokine in diseased periodontal tissues. Moreover, it is known that tumor necrosis factor-α (not interferon-γ) is the main inducer of CXCL10 by skin fibroblasts (22). In the present study, interferon-γ was found to be a more potent inducer of CXCL10 than tumor necrosis factor-α. These differ-

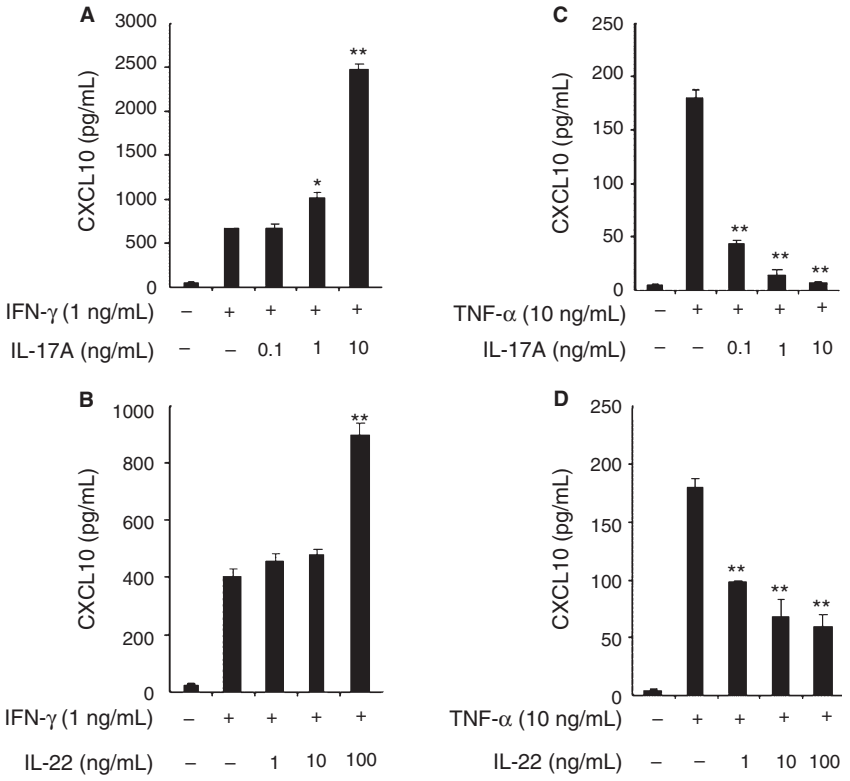

Fig. 5. Effects of T-helper 17 cytokines on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts. Human gingival fibroblasts were incubated for 24 h with varying concentrations of interleukin-17A or interleukin-22 in combination with interferon-γ (1 ng/mL) or tumor necrosis factor-α (10 ng/mL). The culture medium was removed and assayed for CXCL10 release using enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation ($n = 3$; Student's t-test, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. interferon-γ or tumor necrosis factor-α single-stimulated). Similar results were obtained in three identical experiments.

ences might be explained by the different type fibroblasts used.

Ueno and colleagues reported that synovial fibroblast cell lines derived from arthritis patients were able to secrete significant amounts of CXCL10 when stimulated with a combination of interferon-γ and tumor necrosis factor-α/interleukin-1β (23), and their study findings are supported by the results of the present study. This means that the presence of both the T-helper 1 cytokine (interferon-γ) and pro-inflammatory cytokines (interleukin-1β and tumor necrosis factor-α) in inflamed gingival tissues induce the production of large amounts of CXCL10 by human gingival fibroblasts and directs the periodontally diseased tissue towards the T-helper 1 condition.

Interleukin-4 enhanced CXCL10 release by tumor necrosis factor-α or interferon-γ-treated HaCaT cells (24). In the present study, interleukin-4 and interleukin-13 inhibited the production of CXCL10 by interferon-γ-stimulated and tumor necrosis factor-α-stimulated-human gingival fibroblasts. These results are totally different from the results of previous studies using HaCaT cells, and the differences might be a result of the cell type used. The T-helper 2 cytokines (interleukin-4 and interleukin-13) might control the T-helper 1 response by inhibiting CXCL10 production by human gingival fibroblasts in diseased periodontal tissue.

Recently, a new CD4-effector T-cell subset called T-helper 17 has been discovered (25). It has been reported that the T-helper 1 cytokine, interferon-γ, and the T-helper 2 cytokine, interleukin-4, inhibit the function and differentiation of T-helper 17 (26). However, it is still uncertain whether the T-helper 17 cytokines (interleukin-17A and interleukin-22) can modify the T-helper 1/T-helper 2 balance in peripheral tissues. In this report, we found that interleukin-17A and interleukin-22 enhanced the interferon-γ-induced production of CXCL10, but inhibited the tumor necrosis factor-α-induced production of CXCL10 by human gingival fibroblasts. This means that the production of CXCL10 by human gingival fibroblasts will be up-regulated when both interferon-γ and interleukin-17A/interleukin-22 are present in the same diseased periodontal tissues. Therefore, T-helper 17 cells might lean towards the T-helper 1 condition in inflamed gingival tissues when T-helper 1 cells are also present in diseased tissues. On the other hand, T-helper 17 cells might inhibit the migration of T-helper 1 cells without interferon-γ in inflamed gingival tissues. These results reveal that the effects of T-helper 17 cytokines on the T-helper 1/T-helper 2 balance might be dependent on whether or not interferon-γ exists in inflamed gingival tissues.

It is known that interleukin-10 and transforming growth factor-β1 are anti-inflammatory cytokines. These cytokines inhibit the production of pro-inflammatory cytokines by several cell types (27,28). In this report, interleukin-10 inhibited interferon-γ-induced and tumor necrosis factor-α-induced CXCL10 production by human gingival fibroblasts, but transforming growth factor-β1 enhanced interferon-γ-induced CXCL10. Cheeran and colleagues reported that treatment with interleukin-10, but not with transforming growth factor-β1, inhibits cytomegalovirus-induced CXCL10 production by microglial cells (29). The results of their report are similar to the results obtained in the present study. It has been reported that transforming growth factor-β1 is involved in T-helper 17 differentiation (25). The effect of transforming growth factor-β1 is similar to that of interleukin-17A

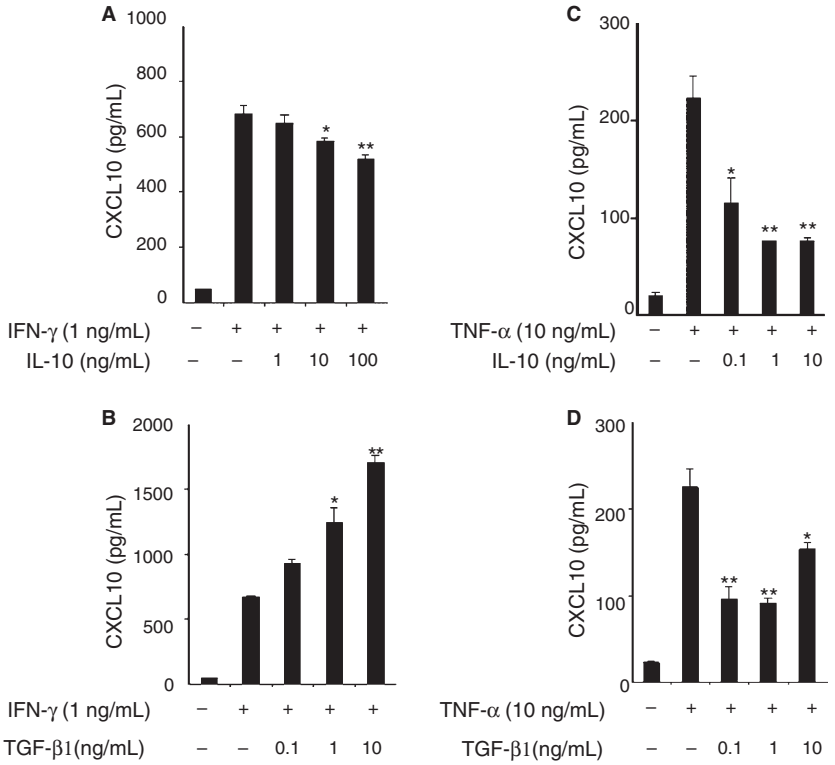

Fig. 6. Effects of anti-inflammatory cytokines on CXCL10 production by tumor necrosis factor-α-stimulated or interferon-γ-stimulated human gingival fibroblasts. Human gingival fibroblasts were incubated for 24 h with varying concentrations of interleukin-10 or transforming growth factor-β1 in combination with interferon-γ (1 ng/mL) or tumor necrosis factor-α (10 ng/mL). The culture medium was removed and assayed for CXCL10 release using enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation ($n = 3$; Student's t-test, $*p < 0.05$, $**p < 0.01$ vs. interferon-γ or tumor necrosis factor-α single-stimulated). Similar results were obtained in three identical experiments.

and interleukin-22. Transforming growth factor-β1 might act similarly to T-helper 17 cytokines in respect to CXCL10 production by human gingival fibroblasts in inflamed gingival tissues.

Finally, we discovered that human gingival fibroblasts are the source of CXCL10, and that the production of CXCL10 is modified by various cytokines in inflamed gingival tissues. In particular, interferon-γ and pro-inflammatory cytokines/T-helper 17 cytokines greatly enhanced the production of CXCL10 by human gingival fibroblasts. Determination of the level of these cytokines in inflamed gingival tissues or crevicular fluid might be useful for assessing the likelihood of progression of periodontal disease because the T-helper 1 condition is related to bone resorption in periodontal tissues.

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