Potential of the *Tannerella forsythia* S-layer to Delay the Immune Response

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Abstract

The periodontal pathogen *Tannerella forsythia* possesses a glycosylated S-layer as an outermost cell decoration. While the S-layer provides a selection advantage to the bacterium in the natural habitat, its virulence potential remains to be investigated. In the present study, the immune responses of human macrophages and gingival fibroblasts upon stimulation with wild-type *T. forsythia* and an S-layer-deficient mutant were investigated. The mRNA expression levels of the pro-inflammatory mediators IL-1β, TNF-α, and IL-8 were analyzed by qPCR, and the production of the corresponding cytokines was investigated by ELISA. The S-layer-deficient *T. forsythia* mutant induced significantly higher levels of pro-inflammatory mediators compared with wild-type *T. forsythia*, especially at the early phase of response. Analysis of these data suggests that the S-layer of *T. forsythia* is an important virulence factor that attenuates the host immune response to this pathogen by evading the bacterium's recognition by the innate immune system.

Keywords

Tannerella forsythia; S-layer; immunology; cytokine production; macrophages

INTRODUCTION

The "red complex bacteria", including *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*, are strongly associated with clinical measurements of periodontitis (Socransky *et al*., 1998; Kinane, 2001; Holt and Ebersole, 2005) and, additionally, pose a potential risk for the development of systemic disorders (Beck *et al*., 2000).

Among the identified virulence factors of *T. forsythia* are cell-surface proteolytic enzymes (Moncla *et al*., 1990; Saito *et al*., 1997), glycosidases (Hughes *et al*., 2003), envelope lipoproteins (Hasebe *et al*., 2004), lipopolysaccharides (Bodet *et al*., 2006a; Bodet and Grenier, 2010), surface antigens (Sharma *et al*., 1998), and the S(surface)-layer (Kerosuo,

1988; Lee *et al*., 2006). While S-layers, as outermost bacterial cell-surface layers (Sleytr *et al*., 2010), generally provide a selection advantage to the bacteria, the S-layer of *T. forsythia* has been shown to mediate adhesion/invasion to human gingival epithelial cells and to epidermal carcinoma cells of the mouth (Sabet *et al*., 2003; Sakakibara *et al*., 2007); its involvement in hemagglutination is reported to be contradictory (Sakakibara *et al*., 2007). In addition, a significantly elevated serum IgG level to the S-layer was found in early-onset periodontitis patients, indicating an increased interaction between the host adaptive immune mechanisms and *T. forsythia* during disease progression (Yoneda *et al*., 2003). Despite these findings, the role of the S-layer in the host response to *T. forsythia* remains largely unknown.

The aim of the present study was to investigate how deletion of the S-layer from *T. forsythia* influences the cellular immune response. The experimental set-up included *T. forsythia* wild-type cells and an S-layer-deficient mutant acting as stimuli for macrophages as integral parts of the innate immune system, and for human gingival fibroblasts (HGFs), as the most abundant cells in the periodontium, playing a crucial role in the host response (Ara *et al*., 2009). To characterize the elicited immune response, we determined the release of the pro-inflammatory mediators IL-1β, TNF-α, and IL-8 by ELISA and analyzed the expression of the corresponding genes by qPCR. A possible scenario of how the *T. forsythia* S-layer may contribute to evading the host immune defense is discussed.

MATERIALS & METHODS

Bacterial Strain and Growth Conditions

T. forsythia ATCC 43037 (*Tf*wt) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). An S-layer-deficient *T. forsythia* mutant (*Tf tfsAB*) was obtained upon insertional inactivation of the S-layer genes (Sakakibara *et al*., 2007). *T. forsythia* cells were grown anaerobically at 37°C for 4 days in tryptic soy broth (30 g/L; Gerbu, Gaiberg, Germany), supplemented with yeast extract (5 g/L; Becton Dickinson, Heidelberg, Germany), phytone peptone (5 g/L; Becton Dickinson), cysteine (0.2 g/L; Sigma, Vienna, Austria), horse serum (20 mL/L; PAA, Linz, Austria), hemin (2.5 μg/mL, Sigma), menadione (2 μg/mL, Sigma), and N-acetylmuramic acid (10 μg/mL, Sigma). Cells were harvested by centrifugation (15 min, $4500 \times g$), and the OD_{600} was set to 1.0 with MEM medium containing 5% FCS (PAA) before stimulation, corresponding to ~10^9 cells/mL of MEM medium.

Ultrathin-sectioning of *T. forsythia* Cells

Ultrathin-sectioning of bacterial cells and electron microscopy were performed as described previously (Messner *et al*., 1986).

Cell Lines

The U937 monocytic cell line was purchased from ATCC. HGFs were isolated from the gingival tissue of periodontally healthy patients undergoing routine third molar tooth extraction (Qu *et al*., 2010). Patients were informed before the surgical procedures and gave written agreement. The study protocol was approved by the Ethics Committee of the

Medical University of Vienna. U937 cells and HGFs were cultured in RPMI1640 medium (Invitrogen, Vienna, Austria) and Dulbecco's modified Eagle's medium (DMEM; Invitrogen), respectively, supplemented with 10% of FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For differentiation into adherent macrophages, U937 cells were seeded (10^6 cells/mL) and treated with phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO, USA) at a concentration of 0.2 μg/mL for 72 hrs.

Stimulation of Macrophages and Human Gingival Fibroblasts

Adherent U937 macrophages and HGFs were seeded in a 24-well plate at a density of 10^5 cells *per* well containing 0.5 mL of RPMI1640 and DMEM medium without FBS, respectively. Subsequently, either viable *Tf*wt (10^7 cells/mL) or viable *Tf ∆tfsAB* cells (10^7 cells/mL) were added to the medium to mimic the native scenario of infection. This corresponds to a multiplicity of infection of 50 bacteria *per* mammalian cell, which is recommended for minimizing proteolytic degradation of cytokines (Bodet *et al.*, 2005, 2006b). Cells stimulated with 1 μg/mL of *P. gingivalis* LPS (Invitrogen) as well as non-stimulated cells were taken as controls. Each experimental group included 4 wells. After stimulation for either 3 or 24 hrs, the cellular mRNA expression levels of IL-1β, TNF-α, and IL-8 in macrophages and GFBs as well as the content of corresponding proteins in the conditioned medium were determined. Experiments were repeated at least 3 times.

Viability Test of Mammalian Cells

Cells were seeded at a density of 10^4 cells *per* well containing 0.1 mL of corresponding medium without FBS and stimulated with *Tf* wt and *Tf ∆tfsAB* cells for 24 hrs. Each experimental group included 8 wells. After stimulation, a 10-μL quantity of MTT dye (5 mg/mL in PBS) was added, and culture plates were incubated at 37°C for 4 hrs. Subsequently, the medium was discarded, a 100-μL quantity of DMSO was added, and the OD_{550} was measured on a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Quantitative PCR

The mRNA expression levels of IL-1β, TNF-α, and IL-8 were determined by qPCR (Bertl *et al.*, 2009), with the GAPDH encoding gene as internal reference. Isolation of mRNA from 10^5 cells, each, and transcription into cDNA was performed with the TaqMan® Gene Expression Cells-to-CT™ kit (Ambion/Applied Biosystems, Foster City, CA, USA). qPCR was performed on an ABI Prism SDS 7000 device (Applied Biosystems) in paired reactions with Taqman® gene expression assays with the following ID numbers (all from Applied Biosystems): IL-1β, Hs01555413_m1; TNF-α, Hs99999043_m1; IL-8, Hs00174103_m1; and GAPDH, Hs99999905_m1. qPCR reactions were performed in triplicate with the following thermocycling conditions: 95°C for 10 min, 40 cycles, each for 15 sec at 95°C and at 60°C for 2 min. The point at which the PCR product was first detected above a fixed threshold (cycle threshold, C_t) was determined for each sample. Changes in the expression of target genes were calculated with the $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t = (C_t^{target} - C_t^{GAPDH})_{sample} - (C_t^{target} - C_t^{GAPDH})_{control}$, with an untreated sample as a control.

Determination of Cytokines

The levels of IL-1β, TNF-α, and IL-8 in the conditioned medium were determined by ELISA with Ready-SET-Go kits (Ebioscience, San Diego, CA, USA). For measurement, IL-1β and TNF-α after three-hour stimulation were undiluted, IL-1β and TNF-α after 24-hour stimulation and IL-8 after three-hour stimulation were diluted 1:5, and IL-8 after 24-hour stimulation was diluted 1:20. The detection limit for all cytokines was 2 pg/mL.

Statistical Analysis

Statistical differences were analyzed by ANOVA's statistic, and paired comparisons were performed by Tukey's *post hoc* test. Statistical analyses were performed with the SPSS 17.0 program. Data are expressed as mean ± SD. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Electron Microscopy of *T. forsythia* Wild-type and S-layer-deficient Cells

Transmission electron microscopy of ultrathin-sectioned *Tf* wt cells indicated the presence of a ~10-nm-thick S-layer completely surrounding the cells, while the S-layer was completely missing on *Tf* *tfsAB* cells (Figs. 1A, 1B).

Viability of Macrophages and Human Gingival Fibroblasts

The viability of U937 macrophages and HGFs was significantly increased upon stimulation with both *Tf* wt and *Tf* *tfsAB* mutant cells (Fig. 2), while *P. gingivalis* LPS did not increase cell viability. At the same time, no significant difference in the effects of the 2 types of *T. forsythia* on viability of macrophages and HGFs was observed.

Cytokine Expression in U937 Macrophages

Changes in the levels of IL-1β, TNF-α, and IL-8 in U937 macrophages upon stimulation with *Tf* wt, *Tf* *tfsAB*, and *P. gingivalis* LPS were determined by qPCR and ELISA (Fig. 3). After both three- and 24-hour stimulation, all stimuli induced significantly higher expression levels of pro-inflammatory mediators in U937 macrophages compared with non-stimulated cells. After 3 hrs of stimulation, macrophages treated with *Tf* *tfsAB* exhibited significantly higher mRNA expression levels of all 3 cytokines in comparison with macrophages stimulated with *Tf*wt (Fig. 3A). This observation was supported by the measurements of cytokine levels in conditioned medium, showing significantly increased IL-1β and TNF-α production by U937 macrophages stimulated with *Tf* *tfsAB* compared with macrophages stimulated with *Tf*wt (Fig. 3B). A similar tendency was observed also for IL-8, although the difference was not statistically significant. Both types of *T. forsythia* cells induced significantly lower cytokine expression levels in comparison with *P. gingivalis* LPS after 3 hrs of stimulation. After 24 hrs of stimulation, however, the difference in cytokine expression between differently stimulated cells was no longer observed (Figs. 3A, 3C).

IL-8 Expression in Human Gingival Fibroblasts

The IL-8 expression level in HGFs was significantly increased upon stimulation with *Tf*wt, *Tf tfsAB*, and *P. gingivalis* LPS in comparison with non-stimulated cells after both 3 and 24 hrs of stimulation (Fig. 4). HGFs stimulated with *Tf tfsAB* exhibited a significantly higher IL-8 mRNA expression level than those stimulated with the wild-type species (Figs. 4A, 4B). However, no significant difference in IL-8 production between HGFs stimulated with *tfsAB* and *Tf* wt was observed by cytokine measurement (Figs. 4C, 4D). The mRNA levels of IL-1β and TNF-α as well as the levels of the corresponding cytokines in conditioned medium were below the methodological detection limit (data not shown).

DISCUSSION

We investigated the role of the S-layer of the periodontal pathogen *T. forsythia* in the host immune responses of macrophages and HGFs by determining the release of pro-inflammatory mediators. The study focused on the determination of changes in the expression of IL-1β, TNF-α, and IL-8, which are released by macrophages during the early phase of host cell stimulation and are associated with the acute phase of host response (Le and Vilcek, 1987). Both IL-1β and TNF-α may directly stimulate bone resorption *in vitro* and *in vivo* (Mundy, 1993) or stimulate production of prostaglandin E$_2$ (Nakao *et al*., 2002; Rausch-Fan *et al*., 2005), which, in turn, is a potent stimulator of bone resorption (Offenbacher *et al*., 1993). IL-8 attracts neutrophils into the inflamed tissue, promoting the development of acute inflammation (Baggiolini *et al*., 1994).

T. forsythia at the concentration used in the present study had no cytotoxic effect on either macrophages or HGFs. An MTT assay (Mosmann, 1983) indicated that viability of both mammalian cell types was significantly increased by *T. forsythia*. In contrast, *P. gingivalis* LPS, which was used as a control, did not have such an effect. Thus, it can be speculated that while LPS activates Toll-like receptors, *T. forsythia* could be internalized by macrophages and stimulate intracellular receptors. Although the exact mechanism underlying increased viability is currently unclear, it might be important for providing long-lasting viability of host cells, which is critical for an effective immune response and appropriate bacterial clearance (Marriott *et al*., 2005).

The main observation of this study is that after 3 hrs of stimulation, macrophages treated with S-layer-deficient *T. forsythia* produced significantly higher levels of pro-inflammatory mediators than macrophages treated with wild-type *T. forsythia*, whereas after 24 hrs of stimulation, these differences were no longer observed. Thus, it is conceivable that the S-layer of *T. forsythia* delays the host immune response to this pathogen. The responses of macrophages upon stimulation with *T. forsythia* are complex, also involving autocrine responses to produced cytokines. In parallel, degradation of cytokines takes place. Obviously, the contributions of these processes are substantially higher after 24 hrs of stimulation than after 3 hrs of stimulation, which may be manifested in time-dependent differences of results.

The increased virulence activity of S-layer-deficient *T. forsythia* compared with that of the wild-type species was also confirmed in experiments with HGFs. Particularly, a *Tf tfsAB*

mutant induced a significantly higher mRNA expression level of IL-8 compared with *Tf*wt. A similar trend was observed by the measurement of the IL-8 level in HGFs' conditioned medium, although in this case, the difference was not statistically significant. The differences observed for HGFs upon stimulation with different kinds of *T. forsythia* are especially important, because these cells are primary cells and, thus, partially mimic the situation in periodontal pockets. Expression of IL-1β and TNF-α in HGFs could not be detected, which is in agreement with a previous study with HGFs (Kent *et al.*, 1996).

The functional importance of the S-layer for the modulation of the host immune response to *T. forsythia* is currently unclear. Since the primary aim of a host immune response is the removal of invading pathogens, one can assume that the S-layer of *T. forsythia* is a strategy to evade recognition by the innate immune system. Indeed, the S-layer of *T. forsythia* attenuates the host response at the initial phase and, thus, delays the clearance of this pathogen by the immune system. Several components of *T. forsythia* could be involved in the immune response to this pathogen. Particularly, LPS and bacterial DNA of *T. forsythia* were shown to induce production of pro-inflammatory cytokines by human macrophages, with the IL-8 secretion level of LPS from *T. forsythia* being about 1.5 times the effect of LPS from *P. gingivalis* (Bodet *et al.*, 2006a; Sahingur *et al.*, 2010). Considering the cell envelope architecture of *T. forsythia*, in which a rough LPS (G. Posch, O. Holst, C. Schäffer, manuscript in preparation) is proposed to serve as anchor for the S-layer to the outer membrane (Noonan and Trust, 1997), it is conceivable that the S-layer shields the LPS from recognition by the immune system, at least at the early stage of infection. The later response could be induced by some intracellular structures, for instance, bacterial DNA, and could be similar for both *T. forsythia* species. The exact mechanisms responsible for the differences in virulence activity of S-layer-deficient and wild-type *T. forsythia* remain to be further investigated.

ACKNOWLEDGMENTS

Abbreviations

DMSO	dimethylsulfoxide
FBS	fetal bovine serum
GAPDH	glycerinaldehyde-3-phosphate-dehydrogenase
HGFs	human gingival fibroblasts
LPS	lipopolysaccharide
MEM	minimal essential medium
MTT	3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
OD	optical density

| **PBS** | phosphate-buffered saline |
qPCR	quantitative polymerase chain-reaction
SD	standard deviation
***Tf* wt**	*Tannerella forsythia* ATCC 43037
Tf tfsAB	*Tannerella forsythia* ATCC 43037 S-layer mutant

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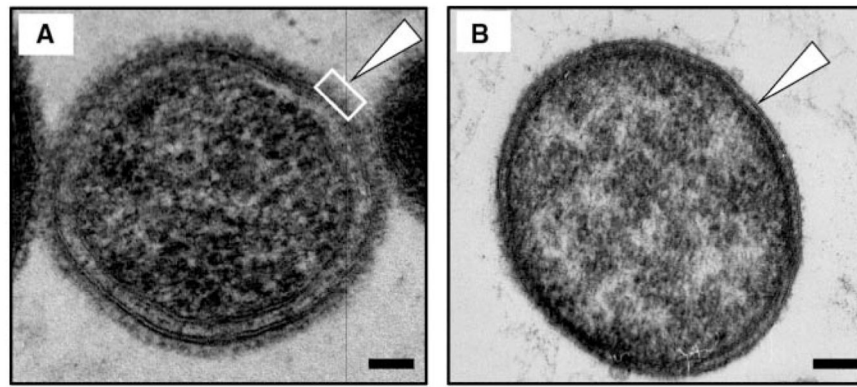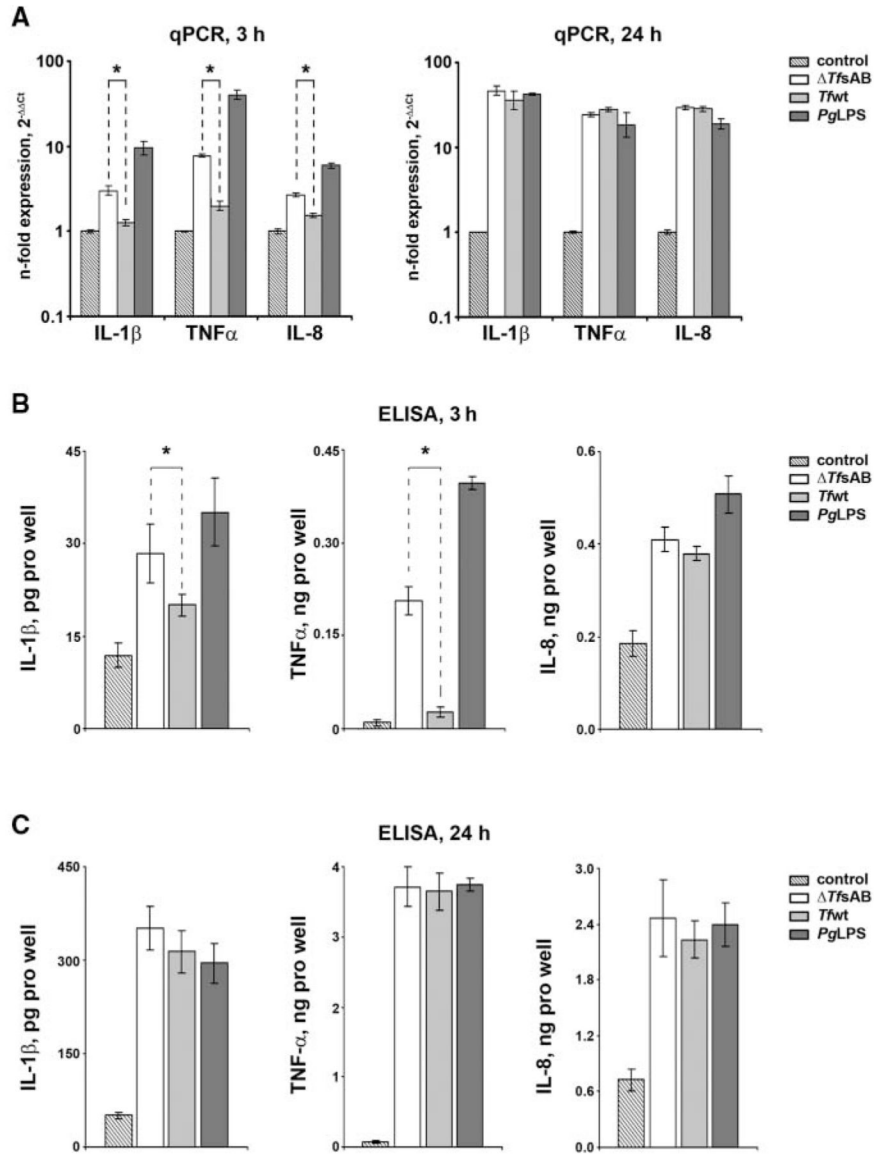
Figure 1.
TEM micrographs of an ultrathin-sectioned (**A**) *T. forsythia* ATCC 43037 wild-type cell and
(**B**) an S-layer-deficient *T. forsythia* Δ*tfsAB* mutant cell. Open triangles point to the
outermost surface of either cell. The S-layer-covered cell surface is indicated by an open
square. Bars, 100 nm.
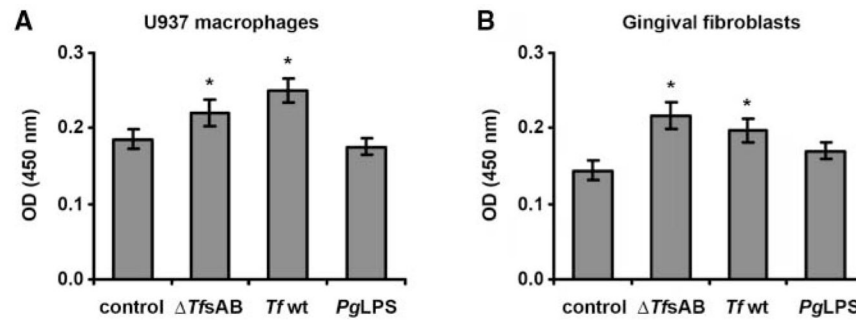
Figure 2.
Viability of U937 macrophages (**A**) and human gingival fibroblasts (**B**) upon stimulation with *T. forsythia* ATCC 43037. Cells were stimulated with wild-type *T. forsythia* (10^7 cells/mL), *T. forsythia tfsAB* mutant (10^7 cells/mL), or *P. gingivalis* LPS (1 µg/mL) for 24 hrs, and the cell viability was measured in an MTT assay. Each value represents the mean value ± SD of 8 values measured in one representative assay. A similar tendency was also observed in the other viability experiments. *Significantly different from control, with *P* < 0.01.

Figure 3.
Cytokine expression in U937 macrophages upon stimulation with *T. forsythia* ATCC 43037. U937 macrophages were stimulated with wild-type *T. forsythia* (10^7 cells/mL), *T. forsythia tfsAB* mutant cells (10^7 cells/mL), and *P. gingivalis* LPS (1 μg/mL) for 3 and 24 hrs, and the mRNA expression levels of IL-1β, TNF-α, and IL-8 (**A**), as well as the content of corresponding proteins in conditioned medium (**B,C**), were measured by qPCR and ELISA, respectively. Changes in the gene expression are presented by the relative amount of mRNA with the formula $2^{(-\Delta\Delta Ct)}$, with non-stimulated macrophages as a control. Cytokine levels measured in the conditioned medium of non-stimulated macrophages were subtracted from those measured in conditioned medium of stimulated cells. The data are given as mean value ± SD of 4 different wells originating from one representative experiment. A similar tendency was also observed in the other qPCR experiments. *Significantly different, with *P* < 0.01.
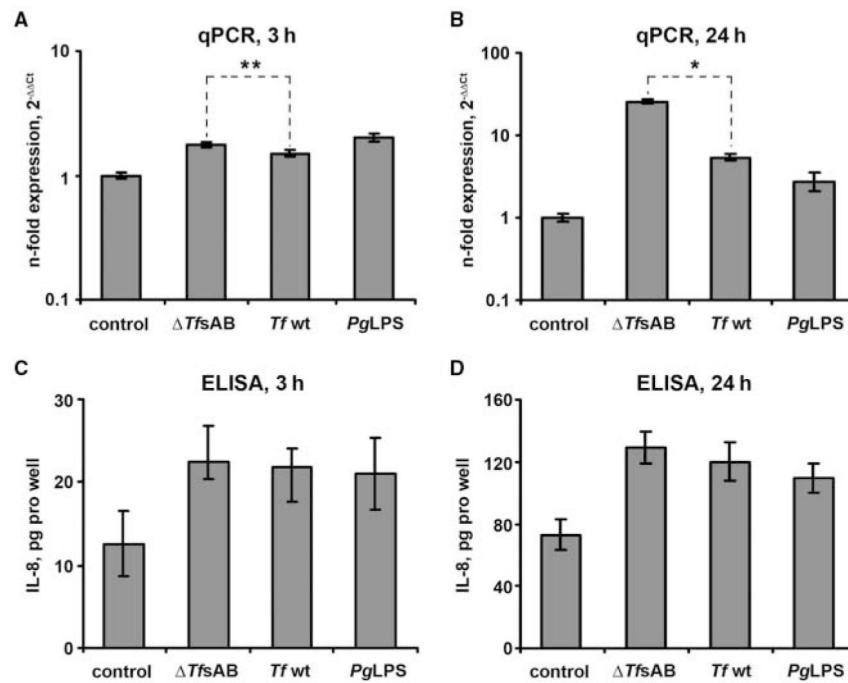
Figure 4.
IL-8 expression in human gingival fibroblasts upon stimulation with *T. forsythia* ATCC 43037. Gingival fibroblasts were stimulated with wild-type *T. forsythia* (10^7 cells/mL), tfsAB *T. forsythia* mutant cells (10^7 cells/mL), or *P. gingivalis* LPS (1 μg/mL) for either 3 or 24 hrs, and the change in the IL-8 expression level was measured by qPCR (**A,B**) and ELISA (**C,D**). Data are presented as in Fig. 3. *,** Significantly different, with $P < 0.01$ and $P < 0.05$, respectively.