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Molecules and Cells

Treponema denticola Suppresses Expression of Human β-Defensin-2 in Gingival Epithelial Cells through Inhibition of TNFα Production and TLR2 Activation

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We previously reported that *Treponema denticola*, a periodontal pathogen, suppressed the expression of human β-defensins (HBDs) and IL-8 in human gingival epithelial cells. To clarify the receptor(s) involved in the suppression of HBD-2, immortalized gingival epithelial (HOK-16B) cells were infected with live or heat-killed *T. denticola* for 24 h, and the expression of HBD-2 was examined by real-time RT-PCR. Live *T. denticola*, but not heat-killed bacteria, suppressed the expression of HBD-2 about 40%. Time courses of suppression revealed that *T. denticola* suppressed HBD-2 expression only at late time points, which was accompanied with the suppression of TNFα production. Neutralization of TNFα with an antibody abrogated the suppressive effect of *T. denticola* on HBD-2. Accordingly, heat-killed *T. denticola* did not suppress TNFα production. Knock-down of toll-like receptor (TLR) 2 *via* RNA interference reversed the suppressive effect of *T. denticola* on the expression of HBD-3, but not on the production of TNFα. Collectively, *T. denticola* suppresses the expression of HBD-2 in gingival epithelial cells by inhibiting the TLR2 axis and TNFα production, which may contribute to the pathogenesis of periodontitis by *T. denticola*.

INTRODUCTION

Treponema denticola, a Gram-negative anaerobic oral spirochete, is one of the well-defined periodontal pathogens. The frequency and amount of *T. denticola* in dental plaque have shown consistent association with the severity of periodontal disease (Socransky et al., 1998; Yoshida et al., 2004). The pathogenic characteristics of *T. denticola* include tissue invasiveness, a highly proteolytic property, and surface proteins that induce cytoskeletal reorganization, membrane blebbing, and the shrinkage of host cells (Ellen and Galimanas, 2005). Although *T. denticola* can induce various innate and adaptive immune responses, it presents immune evasive characteristics at the same time. *T. denticola* is resistant to antimicrobial human β-defensins (HBDs) and phagocytosis by neutrophils, two major defense mechanisms in the gingival sulcus (Brissette and Lukehart, 2002; Ji et al., 2007a). Furthermore, *T. denticola* suppresses antimicrobial oxidative reactions of neutrophils (Sela et al., 1997) and the innate immune mediators of gingival epithelial cells, including IL-8, a neutrophil chemotactant factor, and HBDs (Brissette et al., 2008; Deng et al., 2001; Ji et al., 2007b).

The HBDs are antimicrobial peptides produced by epithelial cells and are involved in the resistance of the epithelial surface to microbial colonization. Whereas HBD-1 is expressed constitutively, the expression of HBD-2 and -3 is upregulated under conditions of infection or inflammation. Microbes recognized through various pattern recognition receptors (PRRs) and proinflammatory cytokines, such as TNFα, IL-1, and IL-17, upregulate the levels of HBD-2 and -3 in epithelial cells (Froy, 2005). Since *T. denticola* is found at the interface of the subgingival plaque and the gingiva (Kigure et al., 1995; Noiri et al., 2001), it is expected to be in direct contact with the gingival epithelium. We have recently shown that *T. denticola* suppresses the expression of HBD-3 by inhibiting the toll-like receptor (TLR) 2 axis in gingival epithelial cells (Shin et al., 2010). The purpose of the present study is to clarify the receptor(s) involved in the suppression of HBD-2 by *T. denticola* in gingival epithelial cells.

MATERIALS AND METHODS

Bacterial culture

The *T. denticola* ATCC 33521 strain obtained from ATCC (USA) was cultured in OMIZ-Pat medium at 37°C under an anaerobic atmosphere (5% H_2, 10% CO_2 and 85% N_2). Heat-killed bacteria were prepared by heating the bacteria at 95°C for 1 h.

Cell culture

The immortalized human gingival keratinocyte HOK-16B cell

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line, a kind gift from Dr. N-H. Park (University of California Los Angeles, USA), was maintained in keratinocyte growth medium containing 0.15 mM calcium and a supplementary growth factor bullet kit (KGM; Clonetics, USA).

Infection of epithelial cells with bacteria

HOK-16B cells were plated at 6×10^4 cells/500 μl/well into 24-well plates 1 day before infection. At 80% confluence, cells were infected with live or heat-killed bacteria at a multiplicity of infection (MOI) of 1000 in KGM containing 2% heat-inactivated human sera (Sigma, USA) and cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2 for 24 h or the indicated time. To study the role of TNFα in HBD expression, HOK-16B cells were pretreated with 1 μg/ml of either neutralizing anti-TNFα or isotype control antibody (R&D systems, USA) for 1 h and infected with live *T. denticola* for 24 h. Recombinant human TNFα at 2 ng/ml (R&D systems) was used as a control to confirm the effect of neutralization. The antibiotics in KGM were removed to prolong the survival of bacteria. Under this culture condition, *T. denticola*, an absolute anaerobe, did not grow, but survived long enough to invade cells. In addition, the viability of HOK-16B cells was not affected. The culture supernatant collected from each well was saved at -80°C for ELISA, and total RNA was extracted using TRIzol (Invitrogen). Experiments were repeated three times.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (2 μg) from HOK-16B cells was subjected to reverse transcription with $(dT)_{18}$ and Superscript II enzyme (Invitrogen) in a 25 μl reaction mix at 42°C for 1 h. Real-time PCR was performed in a 20 μl reaction mix containing 1 μl template cDNA, SYBR Premix Ex *Taq*, ROX Reference Dye (Takara Bio, Japan), and each primer (0.2 μM). Primer sequences are listed in Table 1. All primers were designed to amplify at least two exons in order to prevent the amplification of contaminating gDNA. Amplification was performed in a fluorescence thermocycler (Applied Biosystems 7500 Real-time PCR, USA) under the following conditions: an initial denaturation at 94°C for 1 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and elongation at 72°C for 33 s. The specificity of the PCR product was verified by melting curve analysis and examination on a 3% agarose gel. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified in parallel with the gene of interest. Relative copy numbers compared to GAPDH were calculated using $2^{-\Delta Ct}$. Real-time PCR was performed in triplicate for each RNA sample.

ELISA

The amounts of IL-1α and TNFα secreted into the medium during co-culture with bacteria were measured using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. Fresh KGM was used as a negative control and recombinant IL-1α and TNFα provided with the kit was used as a positive control.

RNA interference (RNAi) in HOK-16B cells and bacterial infection

Stealth™ small interfering (si) RNA duplex oligoribonucleotides against *Homo sapiens* TLR2 (GenBank no. NM_003264) were designed using the BLOCK-iT™ RNAi Designer program (https://rnaidesigner.invitrogen.com/rnaiexpress/) and synthesized by Invitrogen. The oligonucleotide sequences used are as follow: sense 5′-UGAAGCAUCAAUCUCAAGUUCCUCA-3′ and antisense 5′-UGAGGAACUUGAGAUUGAUGCUUCA-3′ for TLR2.

Table 1. Primer sequences used

Primer	Orientation	Sequence (5′- 3′)
GAPDH	Forward	CAGCCTCAAGATCATCAGCA
	Reverse	CCATCCACAGTCTTCTGGGT
HBD-2	Forward	ATCAGCCATGAGGGTCTTGT
	Reverse	GGATCGCCTATACCACCAAA
IL-1α	Forward	GTTTAAGCCAATCCATCACTGATG
	Reverse	GACCTAGGCTTGATGATTTCTTCCT
TNFα	Forward	CAGGGACCTCTCTCTAATCA
	Reverse	AGCTGGTTATCTCTCAGCTC

The BLOIT-iT™ (Invitrogen) fluorescent oligonucleotide, which is not homologous to any known genes, was used as a transfection efficiency detector and a negative control. HOK-16B cells were plated at 4×10^4 cells/well into a 24-well plate. After overnight incubation, cells were transfected with siRNAs using a BLOIT-iT transfection kit (Invitrogen). After culture for 24 h, the cells were re-transfected with siRNA together with live *T. denticola* at an MOI of 1000 for 24 h. Experiments were repeated three times. The *T. denticola*-induced down regulation of HBDs and TNFα was normalized to the expression levels in uninfected cells.

Statistics

The differences between the two groups were analyzed with the two-tailed non-paired Student's *t*-test. Data were considered statistically significant at a *p*-value of < 0.05.

RESULTS

Suppression of HBD-2 expression by live *T. denticola* in gingival epithelial cells occurred at late time points

In our previous report, the suppression of HBD-2 expression by *T. denticola* in HOK-16B cells did not reach statistical significance, although substantial suppression was observed in normal gingival epithelial cells (Shin et al., 2010). Integration of the results of three repeated experiments revealed significant suppression of HBD-2 expression by live *T. denticola*, but not by heat-killed bacteria (Fig. 1A). To understand the mechanism(s) involved in the suppression of HBD-2 expression, time courses of suppression were first examined. The expression of HBD-2 was suppressed only at late time points, 12 and 24 h, after infection (Fig. 1B). In early time points, transient up-regulation of HBD-2 by *T. denticola* was examined.

Neutralization of TNFα reverses the suppression of *T. denticola* on HBD-2 expression

The fact that HBD-2 expression was suppressed only in the late time points suggested potential regulation through other proteins, such as cytokines. Since gingival epithelial cells express substantial amounts of TNFα and IL-1α, the effect of *T. denticola* infection on the production of TNFα and IL-1α was examined. *T. denticola* substantially suppressed TNFα production from 3 h of infection throughout the culture, but not IL-1α (Fig. 2A). Suppression of TNFα mRNA expression preceded that of protein (Fig. 2B), suggesting that the reduced TNFα protein was not the result of degradation by bacterial proteases. Interestingly, the level of IL-1α transcripts was also reduced at 3, 6, and 24 h of *T. denticola* infection, although a slight increase in the IL-1α protein was found. Cycloheximide, an inhibitor of protein biosynthesis, is often used to prove the involvement of

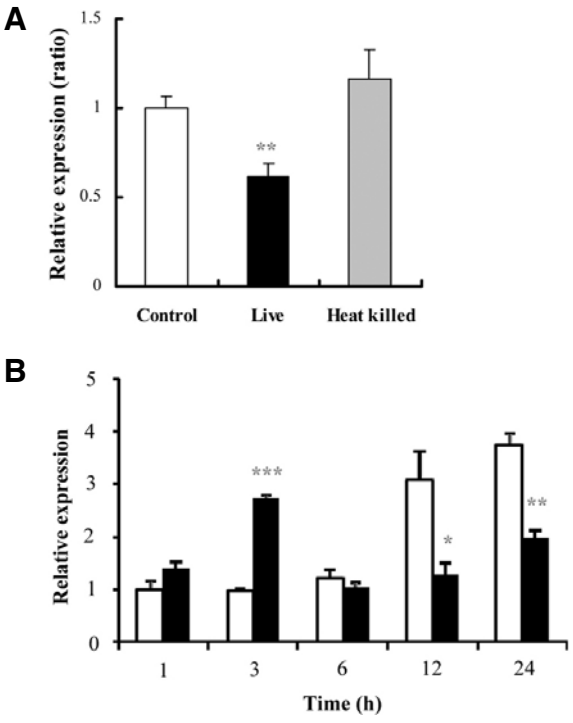

Fig. 1. Live *T. denticola* suppressed the expression of HBD-2 at late time points. (A) HOK-16B cells were infected with live or heat-killed *T. denticola* at an MOI of 1000 for 24 h, and the expression of HBD-2 was examined by real-time RT-PCR. The means ± the standard errors of the mean (SEM) of nine real-time RT-PCR reactions are expressed as the fold induction compared to those of the control culture without bacteria. (B) HOK-16B cells were infected with live *T. denticola* at an MOI of 1000 for 1, 3, 6, 12, and 24 h. The levels of HBD-2 transcripts were evaluated by real-time RT-PCR. The means ± the SEM of the relative copy numbers are expressed as the fold induction compared to those of the control culture without bacteria at 1 h. Representative of two independent experiments with similar results is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus no infection.

newly produced protein. However, it was too toxic to HOK-16B cells (data not shown), even at 0.1 μg/ml, one tenth the suggested concentration (Chun et al., 2001). Instead, the effect of *T. denticola* on HBD-2 expression was examined in the presence of a neutralizing antibody to TNFα. Compared with the isotype control antibody, the neutralizing antibody to TNFα reversed the suppressive effect of *T. denticola* as well as the inductive effect of recombinant human TNFα on HBD-2 expression (Fig. 3).

Heat-killed *T. denticola* does not suppress TNFα production

Because reduced TNFα production was involved in the suppression of HBD-2, the ability of heat-killed *T. denticola* to suppress TNFα was evaluated. In contrast to live *T. denticola*, which reduced the level of TNFα secretion more than 50%, heat-killed bacteria did not suppress TNFα production (Fig. 4).

Knock-down of TLR2 reverses the suppressive effect of *T. denticola* on HBD-2 expression

We recently demonstrated that the suppression of HBD-3 by *T. denticola* in gingival epithelial cells is mediated by the inhibition of TLR2 activation (Shin et al., 2010). Thus, whether or not TLR2 is also involved in the suppression of HBD-2 was examined. Transfection with TLR2-specific siRNA decreased the level of TLR2 transcripts and proteins by 75% and 30%, respectively, but had no effect on TLR4 expression (Figs. 5A and 5B). Knockdown of TLR2 RNA reversed the suppressive effect of *T. denticola* on HBD-2 expression (Fig. 5C). Interestingly, the reduced production of TNFα by *T. denticola* was not restored by TLR2 RNAi (Fig. 5D).

DISCUSSION

We recently reported that *T. denticola* suppresses the expression of HBD-3 by inhibition of the TLR2 axis (Shin et al., 2010). We now present that not only the TLR2 axis, but also the TNFα pathway is involved in the suppression of HBD-2 expression by *T. denticola*.

TLR2 has a role in the upregulation of HBD-2 and -3 in oral epithelial cells (Ji et al., 2009). Because live *T. denticola* can

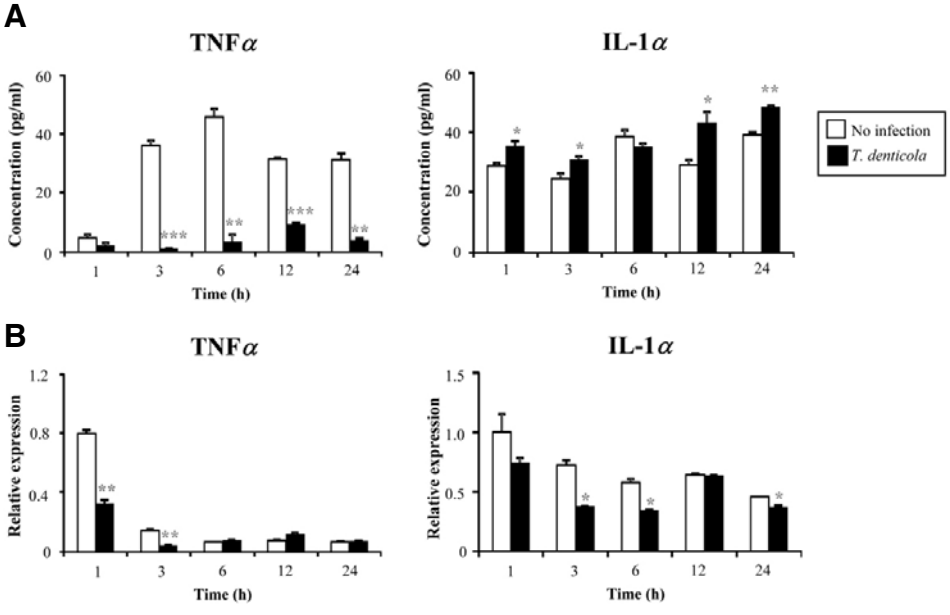

Fig. 2. *T. denticola* infection suppressed the accumulation of TNFα but not that of IL-1α. Culture supernatants and total RNA samples obtained from Fig. 1B were subjected to ELISA analysis of TNFα and IL-1α proteins (A) and real-time RT-PCR to examine their transcripts (B), respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus no infection.

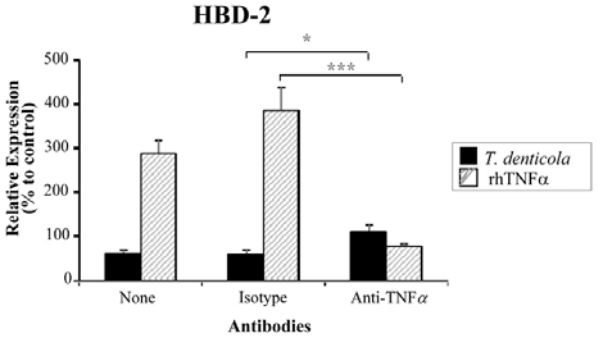

Fig. 3. Neutralization of TNFα reversed the suppressive effect of *T. denticola* on HBD-2 expression. HOK-16B cells were infected with live *T. denticola* (MOI 1000) or treated with human recombinant TNFα (2 ng/ml) for 24 h in the absence or presence of either isotype or anti-TNFα antibodies (1 μg/ml). The levels of HBD-2 transcripts were evaluated by real-time RT-PCR. The means ± the SEM of nine real-time RT-PCR assays are expressed as the relative expression percentage in *T. denticola*-infected cells compared to the control culture without bacteria. *, $P < 0.05$; ***, $P < 0.001$ versus isotype antibody.

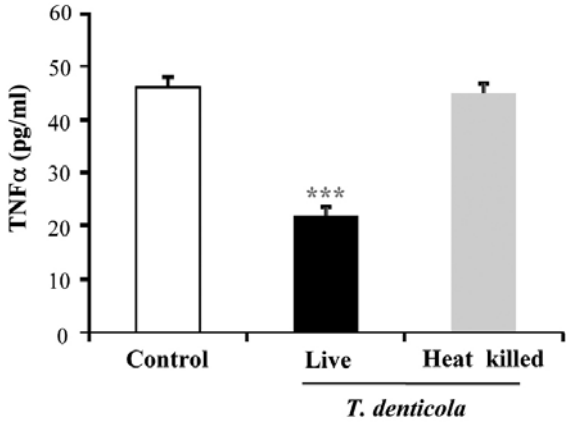

Fig. 4. Heat-killed *T. denticola* did not suppress TNFα production. Culture supernatant collected in Fig. 1A was subjected to ELISA analysis of TNFα. ***, $P < 0.001$ versus no infection.

inhibit Pam3CSK-mediated activation of TLR2 in CHO/CD14/TLR2 reporter cells (Shin et al., 2010), the reversal of the suppressive effects of *T. denticola* on HBD-2 expression in the present study and on HBD-3 expression in the previous study (Shin et al., 2010) by knock-down of TLR2 suggests the involvement of the TLR2 axis in HBD-2 and -3 suppression. Although inhibition of the TLR2 axis was associated with the suppression of both HBD-2 and -3, time responses for the regulation of the two genes were different: whereas the suppression of HBD-3 was observed from a very early time point, i.e., 1 h of infection with *T. denticola* (Shin et al., 2010), the suppression of HBD-2 was found only at late time points (12 h and 24 h). The suppressive effect of *T. denticola* on the levels of HBDs seems to appear when the basal levels of HBDs increase. We previously suggested that the interaction of HBD-3 and TLR2 in gingival epithelial cells may form a positive feedback loop (Shin et al., 2010), based on the known function of HBD-3 as a ligand for TLR1/2 (Funderburg et al., 2007). Indeed, the level of HBD-3 transcripts rapidly increased with time in the control cells (Shin et al., 2010). In contrast, the basal level of HBD-2 expression increased from 12 h, allowing lag time that is required for the production of HBD-3, an endogenous TLR2 ligand. This differential regulation of HBD-2 and -3 in gingival epithelial cells also coincides with our previous observation that TLR2 mediates the induction of HBD-2 and -3 by *Fusobacterium nucleatum*, but thresholds for the two HBD genes are different (Ji et al., 2009). The differential regulation of HBD-2 and -3 may contribute to the differential localization of them in oral epithelia reported by others: whereas HBD-2 is highly expressed in the differentiated granular layers, HBD-3 is expressed in basal and spinous layers (Lu et al., 2005). In our system using HOK-16B cells and normal gingival epithelial cells of equivalent differentiation status, the basal level of HBD-3 is higher than HBD-2 and HBD-3 is more sensitive to TLR2 stimulation than HBD-2 (Ji et al., 2007; 2009). The differentiation status of HOK-16B is close to basal layer cells and epithelial cells in differentiated states may regulate HBD-2 and -3 in different manner.

Control cells without bacterial infection accumulated TNFα in their culture medium during early time points. A slight decrease in the level of TNFα at 12 h coincided with the time when the level of HBD-2 transcript increased. Thus, the consumption of TNFα by cells could have contributed to the increased HBD-2 expression. However, cells infected with *T. denticola* hardly accumulated TNFα in the medium, explaining only the negligible increase in HBD-2 expression. Neutralization of TNFα with an antibody reversed the suppressive effect of *T. denticola* on HBD-2 expression, confirming the involvement of the TNFα pathway. The fact that heat-killed *T. denticola* suppresses neither TNFα production nor HBD-2 expression, also supports the involvement of TNFα in HBD-2 suppression by *T. denticola*.

The reduced level of TNFα is not likely due to the abundant proteases in *T. denticola* because the level of TNFα transcripts also decreased compared with that in control cells, and the level of IL-1α was not reduced by *T. denticola* infection. The reason why the level of IL-1α was rather slightly increased despite a decrease in its transcripts by *T. denticola* infection is not clear at this point. Interestingly, the suppressive effect of *T. denticola* on TNFα expression was not reversed by knock-down of TLR2. Thus, TNFα expression seems to be independent of TLR2 signaling axis and how *T. denticola* suppresses TNFα expression warrants further investigation.

The most intriguing question is which molecule(s) in *T. denticola* are responsible for the inhibition of the TLR2 axis and TNFα expression. We have previously shown that the suppressive effects of *T. denticola* on HBD expression, TLR2 activation, and inflammatory signaling pathways require live bacteria: neither heat-killed bacteria nor bacterial lysates were effective (Ji et al., 2007b; Shin et al., 2010). In the present study, heat-killed bacteria did not suppress TNFα production either. Collectively, heat-labile inhibitor(s) targeting intracellular events are most likely to be involved. Some pathogenic bacteria, such as *Brucella melitensis* and uropathogenic *Escherichia coli*, contain TIR-containing proteins that impede signaling through multiple TLRs by binding to the MyD88 adaptor protein (Cirl et al., 2008). *T. denticola* may similarly hijack the MyD88 adaptor protein. Although the suppressive effect of *T. denticola* on TNFα production was not dependant on the TLR2 pathway, other TLRs may be involved. Alternatively, the heat-labile inhibitor(s) of *T. denticola* may target further downstream of the TLR2 pathway such as how the type III secretion system effectors OspF and OspG of *Salmonella flexneri* modify the mitogen-activated protein kinase and NF-κB pathways to reduce the host inflammatory response (Arbibe et al., 2007; Kim et al., 2005).

In conclusion, *T. denticola* suppresses the expression of

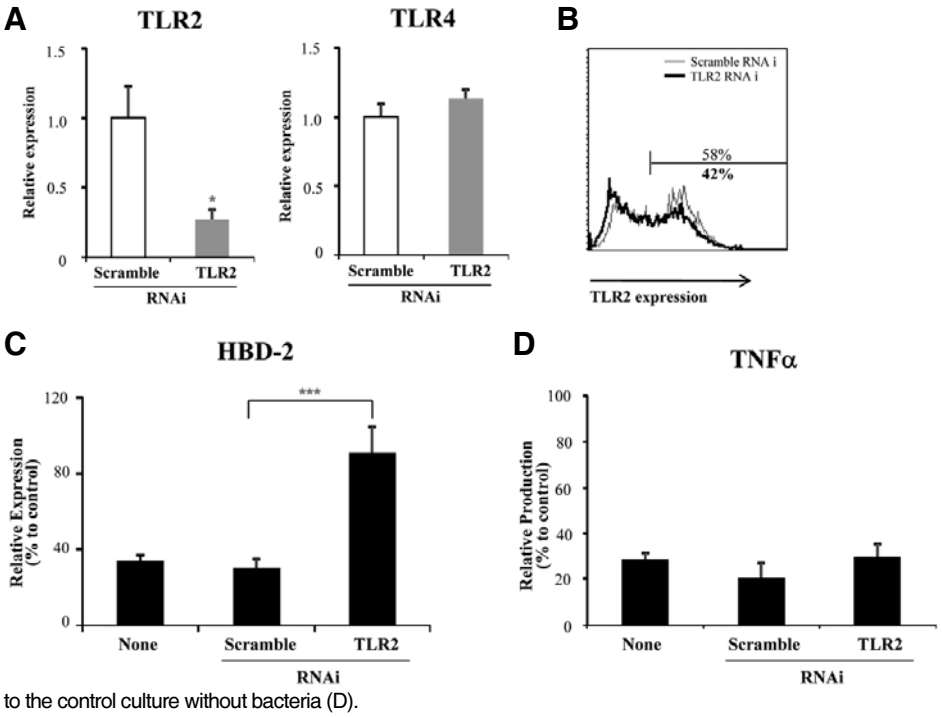

Fig. 5. Knock-down of TLR2 reversed the suppressive effect of *T. denticola* on HBD-2 expression, but not on TNFα production. HOK-16B cells were transfected with control or TLR2-specific siRNA. After a 24-h incubation, the cells were infected with live *T. denticola* at an MOI of 1,000 for 24 h. Gene silencing was assessed by real-time RT-PCR (A) and flow cytometry (B) The 24 h after transfection. The levels of HBD-2 transcripts were evaluated by real-time RT-PCR. The means ± the SEM of nine real-time RT-PCR assays are expressed as the relative expression percentage in *T. denticola*-infected cells compared to the control culture without bacteria (C). ***, $P < 0.001$ versus scramble RNAi. The level of TNFα protein in culture supernatant was examined by ELISA. The means ± the SEM of nine wells are expressed as the percentage in *T. denticola*-infected cells compared to the control culture without bacteria (D).

HBD-2 in gingival epithelial cells by inhibiting the TLR2 axis and TNFα production. The suppression of TNFα production, together with the suppression of IL-8 production, is expected to result in the reduced recruitment of neutrophils. Antimicrobial peptides and neutrophils are two major defense mechanisms in the gingival sulcus. Paralysis of these host responses by *T. denticola* at the surface of gingival epithelia would contribute to the pathogenesis of periodontitis by securing its niche in the gingival sulcus.

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