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Regulation of IL-24 by *Tannerella forsythia* in oral keratinocytes

구강 각화 세포에서의 *Tannerella forsythia*에 의한 IL-24의 조절

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고연경

Abstract

Regulation of IL-24 by *Tannerella forsythia* in oral keratinocytes

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Objectives

Interleukin-24 (IL-24) is a member of the interleukin-10 family and binds to the IL-24 receptors (IL-20R2/IL-20R1 and IL-20R2/IL-22R1). Unlike IL-19 and IL-20, which are cytokines that share the same receptors, IL-24 can be glycosylated to be biologically active. IL-24 was highly expressed in inflammatory diseases such as inflammatory

bowel disease (IBD), psoriasis, rheumatoid arthritis (RA) and keratitis.

Tannerella forsythia is a gram-negative bacteria and is known to be a causative agent of periodontal disease. Our preliminary proteomic study showed that *T. forsythia* significantly increased IL-24 in human oral keratinocytes (HOK-16B cells) after 48 h incubation.

Several virulence factors and their pathogenic mechanisms of *T. forsythia* have been studied, but no studies have been conducted on IL-24.

The objective of this study was to examine the factors involved in the induction of IL-24 in HOK-16B cells by *T. forsythia* and the effect of IL-24 on the expression of pro-inflammatory factors.

Methods

HOK-16B cells were treated with *T. forsythia* and *Streptococcus oralis* (*S. oralis*) at various MOIs (0, 100, 500) for 12 to 48 hr. The mRNA level of IL-24 was measured by real-time RT-PCR and the protein level was measured by immunoblotting. To determine whether

reactive oxygen species (ROS) was associated with IL-24 production, ROS generation by *T. forsythia* was measured using 2',7'-dichlorofluorescin diacetate (DCF-DA) and the regulation of IL-24 expression and glycosylation by ROS were evaluated by using a ROS inhibitor N-acetylcysteine (NAC). To analyze the regulation of IL-24 by cytokines and MAPKs signaling pathway, IL-6 and TNF-α, which are inflammatory cytokines, and MAPKs inhibitors were added into HOK-16B cells and the expression of IL-24 was analyzed by real-time RT-PCR and immunoblotting. To assess whether IL-24 can induce pro-inflammatory factors, HOK-16B cells were treated with recombinant IL-24 and the expression of IL-1α, IL-8, CXCL10, and MCP-1 was analyzed by real-time RT-PCR.

Results

T. forsythia induced the expression of IL-24 in HOK-16B cells and secretion of glycosylated IL-24 into the culture supernatants, but *S.*

oralis did not. *T. forsythia* activated ERK and p38 in HOK-16B cells and inhibition of ERK and p38 activation resulted in reduced expression of IL-24 and secretion of glycosylated IL-24. *T. forsythia* increased the level of ROS and IL-6 expression in HOK-16B cells. IL-6 was a strong inducer of IL-24 expression and glycosylation. IL-24 expression and glycosylation were decreased when treated with NAC, an ROS inhibitor, and inhibitors of p38 and ERK. Recombinant IL-24 induced IL-1α, IL-8, CXCL10, and MCP-1 in HOK-16B cells, but not IL-6.

Conclusion

In this study, glycosylated IL-24 was induced by *T. forsythia* in HOK-16B cells and it was regulated by IL-6 via ROS and MAPK activation. IL-24 was able to increase pro-inflammatory factors. These results indicate that *T. forsythia*-induced IL-24 may be involved in periodontal pathogenesis.

Keywords : Periodontitis, *Tannerella forsythia*, IL-24, ROS, MAPKs

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Contents

Ⅰ. Introduction

Interleukin-24 (IL-24) is a member of the interleukin-10 family (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29) [1]. IL-10 family cytokines can promote innate immune responses upon viral and bacteria infection [21]. IL-24 binds to two different heterodimeric IL-20 receptor complexes (IL-20R2/IL-20R1 and IL-20R2/IL-22R1) [9] and these receptors shared with IL-19 and IL-20. Binding to the receptors, it leads to the activation of STAT3 [4].

IL-19 and IL-20 are expressed monocytes and non-immune tissue cells under inflammatory conditions and involved in a pathogenesis of a few inflammatory diseases [11]. These cytokines, due to the shared same receptors, are quite overlapping in function [12].

IL-24 has been detected in severe inflammation regions of inflammatory diseases, such as inflammatory bowel disease (IBD), psoriasis, rheumatoid artritis (RA) and keratitis. IL-24 stimulates mucin (MUC) gene expression via JAK1/STAT3 activation,

contributing to a protective role in the mucosa from IBD patients [4]. In psoriasis, TNF-induced IL-24 expression depends on ROS and MEK-ERK signaling and mediates psoriasis relevant cytokines expression [6]. IL-24 is also found in higher amounts in synovial fluid samples from patients with RA and spondyloarthropathy (SpA) [7]. In keratitis, *Pseudomonas aeruginosa* has been shown to induce the early expression of IL-24, which is associated with suppression of early protective mucosa immune response [5].

Signal 1 receptor (Sig1R) is expressed in cancer cells, but not in normal cells. IL-24 binds to Sig1R and induces tumor suppressor activities by inhibition of angiogenesis, sensitization to chemotherapy, and induction of cancer-specific apoptosis [2, 3].

IL-24 has distinctive structural characteristics. IL-24 is glycosylated in cells and glycosylated IL-24 can maintain solubility and bioactivity. IL-24 possesses three consensus N-linked glycosylation sites site Ⅰ (Asn-85), site Ⅱ (Asn-99), and site Ⅲ (Asn-126). Unlike IL-24, IL-20 has no post-translational N-linked glycosylation signals and

IL-19 possesses two consensus sites, but only one is utilized [17]. When the glycosylation sites of IL-24 is mutated, it loses the ability of secretion from cells and binding to receptors of cancer cells and non-cancer cells [17].

Periodontitis is a set of inflammation of periodontal tissues surrounding the teeth. The symptoms are swollen, redness and bleeding of the gums, causing tooth loss in severe cases [23]. Periodontitis is initiated by bacteria in subgingival crevice.

Tannerella forsythia is a gram-negative bacteria and belongs to red complex bacteria together with *Porphyromonas gingivalis* and *Treponema denticola*. These bacteria are known to be the major causative agents of periodontal disease [23]. *Tannerella*, especially *T. forsythia* was abundantly observed in severe periodontitis patients with bleeding on probing at sampled sites and it was highly relevant with periodontitis status [24]. However, studies on the precise role of *T. forsythia* in periodontal pathogenesis have yet to be conducted. There are several virulence factors of *T. forsythia* identified so far:

Trpsin-like and PrtH protease, sialidases SiaH and NanH, a leucin-rich repeat cell-surface-associated and secreted protein BspA, an apoptosis-inducing activity, alpha-D-glucosidase and N-acetyl-beta-glucosaminidase, a hemagglutinin, components of the bacterial S-layer, and methylglyoxal production [22]. So it is important to figure out the potential role of *T. forsythia* in periodontitis.

On preliminary proteomic analysis of HOK-16B cells stimulated with *T. forsythia* revealed that IL-24 expression was approximately 680,000 times greater than that of the control group without bacterial treatment (Fig 1). The role of IL-24 in periodontitis has not been elucidated.

The aim of this study was to investigate IL-24 expression and the regulation of IL-24 by *T. forsythia* in HOK-16B cells.

Compound	p (Corr)	p	Regulation	FC (abs)	FC
Interleukin-24 OS=Homo	0.002871	2.60E-05	up	688902.4	688902.4
U6 snRNA-associated Sm	1.01E-05	8.75E-09	up	264912.4	264912.4
Fanconi anemia group G	0.009453	1.99E-04	up	20210.42	20210.42
HCG201155, isoform CRA	3.17E-04	1.16E-06	up	12521.27	12521.27
Trafficking protein particl	0.006734	1.01E-04	up	10426.98	10426.98
U3 small nucleolar ribonu	0.034824	0.001405069	up	8954.836	8954.836
COX assembly mitochonc	0.008001	1.43E-04	up	7847.7	7847.7

Figure 1. Proteomic analysis of HOK-16B cells treated with *T. forsythia*

Ⅱ. Materials and Methods

2.1. Bacterial culture and growth condition

T. forsythia (ATCC 43037) was grown in New Oral Spirochete (NOS) broth supplemented with N-acetylmuramic acid (0.01 μg/ml) and vitamin K (0.02 μg/ml) under the anaerobic condition (10% CO_2 , 10% H_2 , and 80% N_2) at 37℃ for 2 days.

S. oralis (ATCC 30537) was grown in Tryptone Soya Broth under the aerobic condition at 37℃ for 1 day.

For following experiments, overnight cultured bacteria were washed with DPBS and used for infection.

2.2. Cells culture

HOK-16B cells were cultured in KGM medium (PromoCell GmbH, Heidelberg, Germany) supplemented with 0.15 mM $CaCl_2$, 30 μg/ml Gentamicin (Gibco, Paisley, UK), and 15 ng/ml mycoplasma removal agent (MRA, MP Biomedicals, New Zealand).

HGFs were cultured in DMEM medium (WELGENE Cat. LM 001-05, Korea) supplemented with 10% Fetal bovine serum (FBS, Gibco BRL, Paisley, UK) and 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) in a humidified 5% CO2 atmosphere at 37℃.

For the following experiments, cells were detached using trypsin/EDTA (Gibco BRL) and seeded into experimental culture plates and cultured overnight.

HOK-16B cells (5×10^5 cells/well) and HGFs (2×10^5 cells/well) were seeded into 6-well plates and treated with *T. forsythia* (MOI 100 and 500) for various time points. The cells and culture supernatants were used for RT-PCR, real time RT-PCR and immunoblotting.

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

Total RNA from the cells were extracted with an easy-BLUE™ total extraction kit (iNtRON Biotechnology, Seongnam, Korea). cDNA were synthesized using reverse transcription kit (M-MLV Reverse Transcriptase kit, Promega, Madison, WI, USA) and oligo dT primer (Cosmo GENETECH, Seoul, Korea). For real-time RT-PCR, cDNA was mixed with primer pairs (200 nM each) and 10 μl of Power SYBR® Green Master mix (Appplied Biosystems, MA, USA in a 20 μl reaction volume. Following initial denaturation at 95 ℃ for 5 minutes, cDNA was amplified for 40 cycles of denaturation (95 ℃, 15 sec), annealing (60℃, 15 sec), and extension (72℃, 33 sec) in 7500 Real-Time PCR system (Applied Biosystems).

Glyceraldehydes-3-phosphate dehyrogenase (GAPDH), a housekeeping gene, was used as a control. The gene expression level was normalized against GAPDH and calculated by $2^{-\triangle\triangle ct}$.

The primer sequences used in this study are shown in Table Ⅰ.

Gene Name	Primer sequences	
GAPDH	Forward	5′ - GTC GCC AGC CGA GCC – 3′
	Reverse	5’ - TGA AGG GGT GAT TGA TGG CA – 3′
IL-24	Forward	5′ - TGG ACT TTA GCC AGA CCC TTC T - 3′
	Reverse	5′ - GGG CCC AAA GTG GAA TTC TTG – 3′
IL-20	Forward	5′ - TTG CAA GAC ACA AAG CCT GC - 3′
	Reverse	5′ - TGG TCA GGG GTC TGG TAG TT - 3′
IL-19	Forward	5′ - TGC GTG TTC CTT ACC ACT CAC - 3′
	Reverse	5′ - AAC TTC ATG CCC CGT GGA AC - 3′
SOCS3	Forward	5′ - GCT CCA AGA GCG AGT ACC AG – 3′
	Reverse	5′ - CTG TCG CGG ATC AGA AAG GT – 3′
IL-20R1	Forward	5′ - AAC AGA ACG TGG TCC CAG TG – 3′
	Reverse	5′ - TTC TCA GAA GGC TGA GCA CG – 3′
IL-20R2	Forward	5′ - ATG CAG ACT TTC ACA ATG GTT CTA – 3′
	Reverse	5′ - ATG GCC ACT TCA TCT GTG AGC – 3′
IL-22R1	Forward	5′ - CTC TGC AGC ACA CTA CCC TC – 3′
	Reverse	5′ - ATG TCT TCC AGG GTT AGC CG – 3′
IL-6	Forward	5′ - GAT TCA ATG AGG AGA CTT GCC TGG – 3′
	Reverse	5′ - GCA GGA ACT GGA TCA GGA CTT T – 3′
TNF-α	Forward	5′ - CCT GCT GCA CTT TGG AGT GA – 3′
	Reverse	5′ - CTC AGC TTG AGG GTT TGC TAC A – 3′
IL-1α	Forward	5′ - GTT TAA GCC AAT CCA TCA CTG ATG – 3′
	Reverse	5′ - GAC CTA GGC TTG ATG ATT TCT TCC T – 3′
IL-8	Forward	5′ - CTG TGT GAA GGT GCA GTT TTG C – 3′
	Reverse	5′ - AAC TTC TCC ACA ACC CTC TGC – 3′
CXCL10	Forward	5′ - TTC TTA GTG GAT GTT CTG ACC – 3′
	Reverse	5′ - GTG TTT GGA ATT GTA TGT AGG T – 3′
MCP1	Forward	5′ - CAG CCA GAT GCA ATC AAT GC – 3′
	Reverse	5′ - GTG GTC CAT GGA ATC CTG AA – 3′

Table Ⅰ. Primer sequences used to determine gene expression.

2.4. Agarose gel electrophoresis of PCR products

cDNA was synthesized from RNA template with M-MLV reverse transcriptase kit (Promega, Madison, WI, USA) and it is amplified with target primers and EmeraldAmp® PCR Master Mix (TaKaRa, Shiga, Japan). The amplified PCR products were loaded on 1% agarose gel.

2.5. Immunoblotting

The stimulated cells were washed with ice-cold DPBS and centrifuged at 16,000 g for 5 minutes at 4 ℃ and the pellets were lysed with RIPA buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 50 mM NaF, 1 mM EDTA, 5 μM Na_3 VO_4 , 1 mM PMSF, 1 × complete protease inhibitor cocktail (Roche, Manheim, Germany)]. The lysates were clarified by centrifugation at 16,000 g for 45 minutes at 4℃. The culture supernatants of the cells were collected, and the protein were precipitated with 10% trichloroacetic

acid (TCA, Sigma-Aldrich, MO, USA). After centrifugation at 16,000 g for 10 minutes at 4℃, the pellets were neutralized using 0.1 N NaOH. The cell lysates and TCA-precipitated supernatants were mixed with 5 × sample buffer [1 M Tris HCL (pH 6.8), 50% glycerol, 10% SDS, 5% 2-mercaptoethanol, 0.1% bromphenol blue] and heated at 95℃ for 5 minutes for protein denaturation. The proteins were separated by SDS-PAGE (12% gel) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk for 1 hr at 37℃ and subsequently incubated with following primary antibodies for overnight at 4℃ after washing with 1 x PBST 3 times for 5 minutes. Antibodies used were anti-IL-24 (Abcam, ab182567, Cambridge, UK), anti-actin Ab-5 612656 (BD Biosciences, NJ, USA), anti-phospho-p38 (Thr180/Tyr182) (Cell signaling, No. 9211S, MA, USA), anti-p38 (Thr180/Tyr182) (Cell signaling No. 9212), anti-phospho-p44/42 (Thr202/Tyr204) (Cell signaling No. 9101S), ERK 1 Antibody (K-23) (Santa cruz, No. SC-94, TX, USA),

anti-phospho-SAPK/JNK (Thr183/Tyr185) (Cell signaling No. 9251S), anti-SAPK/JNK (Thr183/Tyr185) (Cell signaling No. 9252S), anti-phospho-NF-κB p65 (Ser536) (93H1) (Cell signaling No. 3033), anti-NF-κB p65 (D14E12) XP® (Cell signaling No. 8242). Membranes were washed with 1 x PBST 3 times for 5 minutes. The membranes were reacted with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG secondary antibodies (R&D systems, Minneapolis, MN, USA). After washing with 1 x PBST, the membranes were detacted using ECL Western blotting substrate (SUPEX, Dyne-Bio, Sungnam, Korea) and MicroChemi (Bio-Imaging Systems).

2.6. Enzyme-linked immuonsorbent assay (ELISA)

The concentration of IL-6 in the culture supernantants was determined using sandwich ELISA Kit (Human IL-6 ELISA MAXTM Standard Set, BioLegned, CA, USA) following the manufacturer's instructions.

2.7. PNGase F treatment

TCA-precipitated supernatants were neutralized with 15 μl 0.1 N NaOH. Nine microliter of neutralized TCA-precipitated proteins were mixed with 1 μl 10 X Glycoprotein Denaturing Buffer (NEW ENGLAND BioLabs, Ipswich, Southeastern England) and heated at 100℃ for 2 minutes for denaturation. After cooling it on ice, the denatured proteins were mixed with 2 μl 10 x Glycobuffer 2 (NEW ENGLAND BioLabs), 2 μl 10% NP-40 (NEW ENGLAND BioLabs), 1 unit of PNGase F (Sigma-Aldrich, Cat. P7367) and 5 μl H_2O to make a total reaction volume of 20 μl reaction volume. Denatured proteins with PNGase F were incubated for 4 hr at 37℃ and was analyzed by Immunoblotting.

2.8. Pro-inflammatory cytokine treatment

HOK-16B cells (5×10^5 cells/well) and HGFs (2×10^5 cells/well) were seed into 6 well-plates and cultured overnight. Cytokines

(Recombinant IL-24, IL-6, TNF-α, IL-17A, IL-1α, IL-1β, IL-4 was purchased from R&D systems) were diluted in serum-free media and treated to cells with the concentration of 10 ng/ml.

2.9. ROS fluorescence measurement

HOK-16B cells (2×10^5 cells/well) were seeded into 96-well plates and cultured overnight. Cells were stained with 10 μM 2',7'-Dichlorofluorescin diacetate (DCF-DA, Sigma-Aldrich) for 30 minutes and washed with serum-free media, then infected with *T. forsythia*. (MOI 100 and 500) 0 to 24 h. The fluorescence was measured using excitation at 485 nm and emission at 520 nm by FLUOStar OPTIMA (BMG Labtechnologies, Inc., Ortenberg, Germany).

2.10. Inhibitor treatment

2.10.1 NAC treatment

10 mM N-Acetyl-L-cysteine (NAC, Sigma-Aldrich) was diluted in

serum-free media containing sodium bicarbonate (Sigma-Aldrich) for neutralizing acidification. HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with 5 mM NAC for 30 minutes before *T. forsythia* infection.

2.10.2 MAPK inhibitor treatment

SB 203580, PD 98059 and SP 600125 (Sigma-Aldrich) were diluted with serum-free media. HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with MAPK inhibitors (10, 30, and 50 μM) for 30 minutes before *T. forsythia* infection and treated to cells 30 minutes before infection.

2.11. Statistical analysis

Statistically significant differences between samples were analyzed with an unpaired, one-tailed Student's t test. The data are shown as the mean ± SD. A p value of < 0.05 was considered statistically significant.

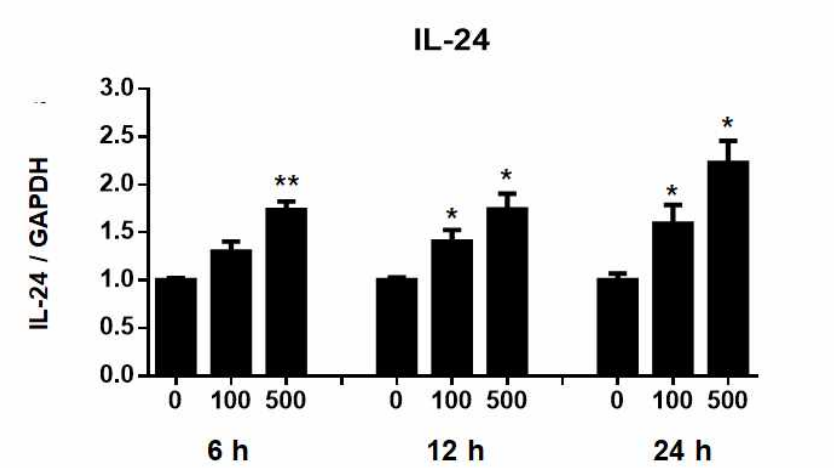
Ⅲ. Results

3.1. Expression of IL-24 and IL-24 receptors in HOK-16B cells and HGFs by *T. forsythia*

The preliminary proteomic analysis showed that IL-24 was the most upregulated protein in lysates of HOK-16B cells treated with *T. forsythia* for 48 hr. To confirm the expression of IL-24 by *T. forsythia* in HOK-16B cells, the mRNA level of IL-24 was analyzed by real-time RT-PCR. *T. forsythia* increased IL-24 mRNA expression in a MOI-dependent manner (Fig 1A). The expression of IL-24 was also analyzed by immunoblotting. The anti-IL-24 antibody recognized protein at 24 kDa in the cell lysates and at 45 kDa in the culture supernatants, suggesting that the secretion form is glycosylated IL-24. (Fig 1B). Increased expression and secretion of IL-24 also were observed in HGFs treated with *T. forsythia*. *S. oralis*, an oral commensal, did not induce the secretion of glycosylated IL-24. (Fig 2).

IL-24 can bind to IL-20 receptors (IL-20R2/IL-20R1 and IL-20R2/IL-22R1) to activate downstream signaling. The expression of the subunits of IL-20 receptors in HOK-16B cells and HGFs was analyzed by real-time RT-PCR. All three subunits, IL20R1, IL-20R2, and IL-22R2, were expressed in both cell types and their expression was not changed in HOK-16B cells by *T. forsythia*. However, the expression of the receptors in HGFs was slightly decreased by *T. forsythia*. (Fig 3).

(A)


IL-24
IL-24 / GAPDH
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
*
*
0 100 500
0 100 500
0 100 500
6 h
12 h
24 h

(B)

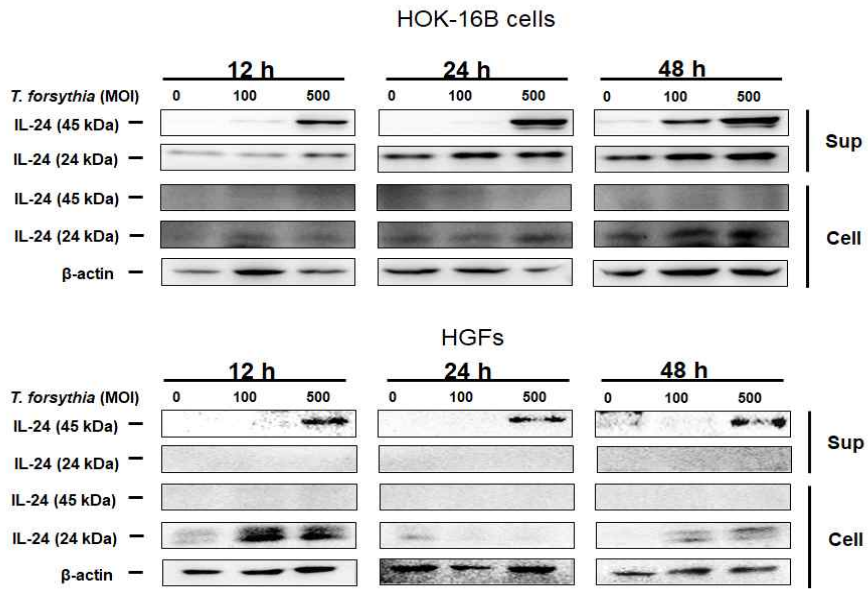

HOK-16B cells
12 h
24 h
48 h
T. forsythia (MOI)
0 100 500
IL-24 (45 kDa)
IL-24 (24 kDa)
IL-24 (45 kDa)
IL-24 (24 kDa)
β-actin
Sup
Cell
HGFs
12 h
24 h
48 h
T. forsythia (MOI)
0 100 500
IL-24 (45 kDa)
IL-24 (24 kDa)
IL-24 (45 kDa)
IL-24 (24 kDa)
β-actin
Sup
Cell

Figure 1. IL-24 expression in HOK-16B cells and HGFs treated with *T. forsythia* (A) HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with *T. forsythia* at various MOIs for 6 h, 12 h, and 24 h. The mRNA level of IL-24 was analyzed by real-time RT-PCR. The data represents the mean ± SD. ($*$: $p < 0.05$, $**$: $p < 0.01$ compared to non-treated control groups) (B) HOK-16B cells (5×10^5 cells/well) and HGFs (2×10^5 cells/well) were seeded into 6-well plates and treated with *T. forsythia* at various MOIs for 12 h, 24 h, and 48 h. The protein level of IL-24 in supernatants and lysates was determined by immunoblotting.

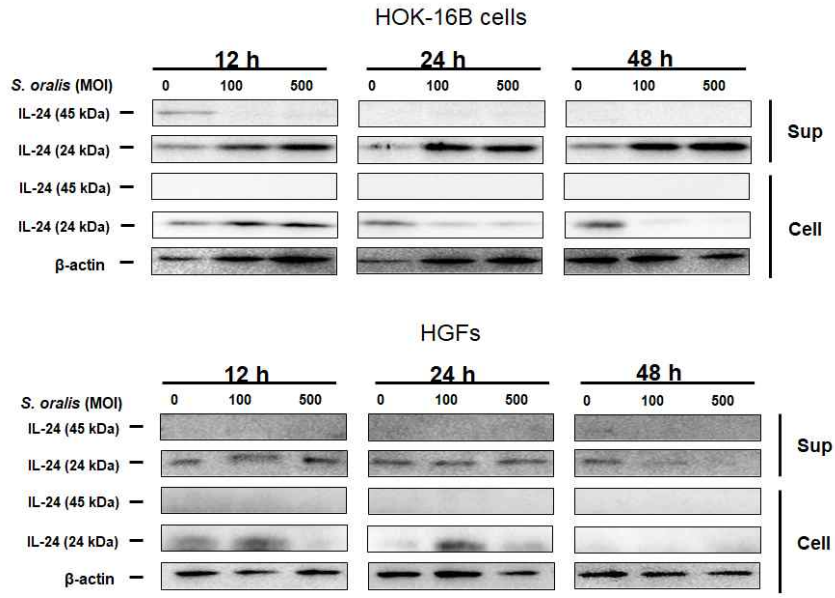


Figure 2. IL-24 expression in HOK-16B cells and HGFs treated with *S. oralis* HOK-16B cells (5×10^5 cells/well) and HGFs (2×10^5 cells/well) were seeded into 6-well plates and treated with *S. oralis* at various MOIs for 12 h, 24 h, and 48 h. The protein level of IL-24 in supernatants and lysates was determined by immunoblotting.

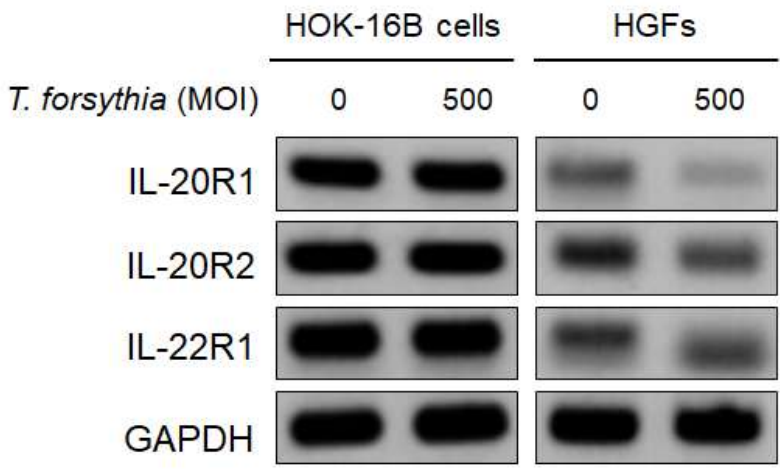


Figure 3. Expression of IL-24 receptors in HOK-16B cells and HGFs. HOK-16B cells (5×10^5 cells/well) and HGFs (2×10^5 cells/well) were seeded into 6-well plates and treated with *T. forsythia* (MOI 500) for 24 h and the expression was analyzed by RT-PCR.

3.2. De-glycosylation of secreted IL-24 by N-glycosidase f

IL-24 can be secreted from cells as a glycosylated form which has a bioavailability and can bind to IL-20 receptors. To confirm glycosylation of secreted IL-24, N-glycosidase f was added to the supernatant of HOK-16B cells, treated with *T. forsythia*. N-glycosidase f is an excellent enzyme for N-linked deglycosylation of glycoproteins [25]. Upon treatment with N-glycosidase f, secreted IL-24 was detected at the position of 24 kDa, but not at 45 kDa by immunoblotting (Fig 4). These results indicate that *T. forsythia* induces secretion of glycosylated IL-24.

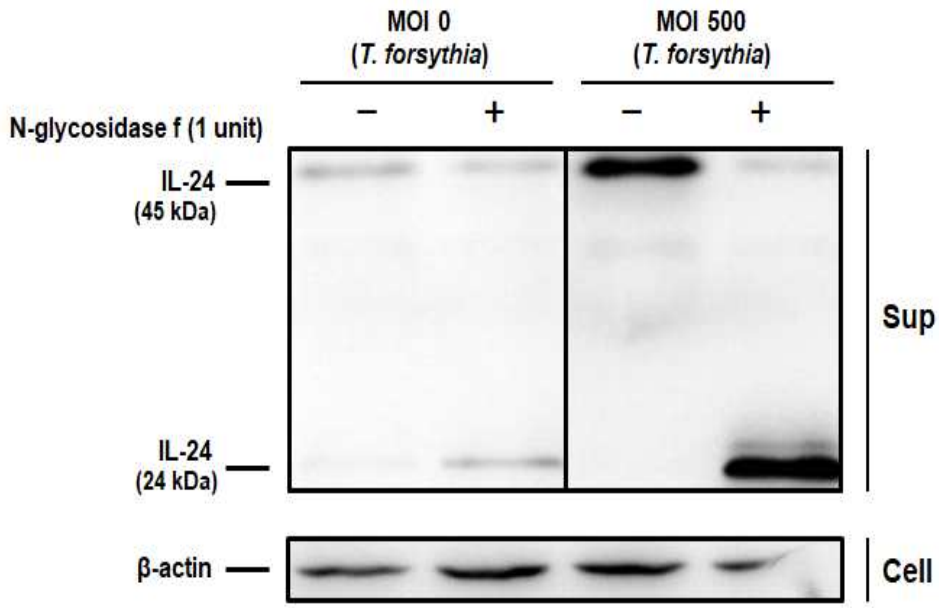


Figure 4. De-glycosylation of secreted IL-24 by N-glycosidase f. HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with *T. forsythia* for 24 h. Culture supernatants were precipitated with TCA and TCA-precipitated supernatants were neutralized with 0.1 N NaOH. Proteins were denaturated in Glycoprotein Denaturing Buffer at 100℃ for 2 minutes and incubated with N-glycosidase f enzyme for 4 h at 37℃. The de-glycosylation of secreted IL-24 was analyzed by immunoblotting.

3.3. Expression of IL-20 cytokines and SOCS3

IL-19 and IL-20 belong to IL-20 cytokines together with IL-24. SOCS3 is involved in a negative regulation of cytokines that signal through the JAK/STAT pathway. The expression level of IL-20 cytokines and SOCS3 was analyzed by RT-PCR. SOCS3, IL-24 and IL-20 were expressed in HOK-16B cells and HGFs, but IL-19 was expressed only in HGFs. SOCS3 and IL-24 expression showed similar bimodal regulation pattern so that their expression was upregulated at 3 h and 24 h, but downregulated at 12 h (Fig 5).

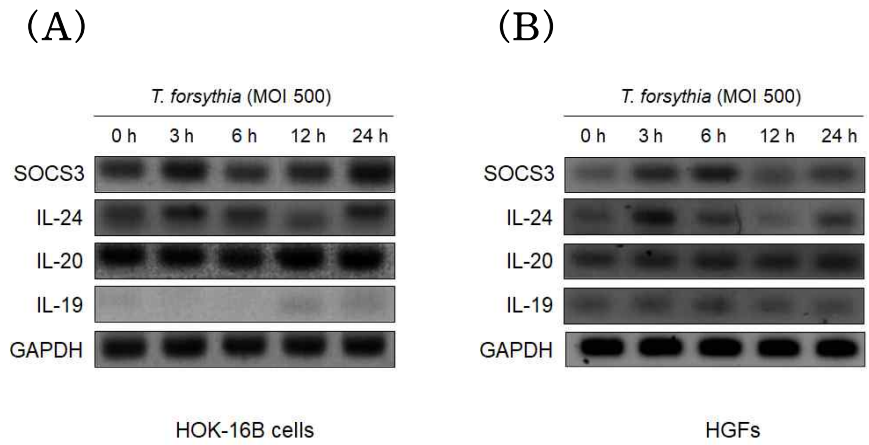


Figure 5. Expression of IL-20 cytokines and SOCS3 mRNA expression by *T. forsythia*. HOK-16B cells (5×10^5 cells/well) and HGFs (2×10^5 cells/well) were seeded into 6-well plates and treated with *T. forsythia* (MOI 500) for 3 h, 6 h, 12 h, and 24 h. The expression level was analyzed by RT-PCR.

3.4. IL-24 regulation by IL-6

To determine which cytokines can induce IL-24, HOK-16B cells were stimulated with various pro-inflammatory cytokines and the mRNA level of IL-24 was analyzed by RT-PCR. IL-6 of tested cytokines induced IL-24 expression as *T. forsythia* did (Fig 6A). To determine the effect of IL-6 and TNF-α on IL-24 expression pattern, HOK-16B cells were stimulated with IL-6 and TNF-α. TNF-α is known as an inducer of IL-24 expression in psoriasis [6]. The mRNA level of IL-24 was analyzed by RT-PCR. IL-6 and TNF-α induced IL-24 at early times and late times (Fig 6B). Secretion of glycosylated IL-24 by IL-6 was confirmed by immunoblotting. Glycosylated IL-24 was increased in a dose-dependent manner by IL-6 (Fig 6C). To examine IL-6 and TNF-α expression by *T. forsythia* in the cells, HOK-16B cells were treated with *T. forsythia*. The mRNA level of IL-6 and TNF-α was analyzed by real-time RT-PCR and the protein level of IL-6 was analyzed by ELISA. IL-6 was increased by *T. forsythia* at the gene and protein level, but

TNF-α was not increased by *T. forsythia* (Fig 7).

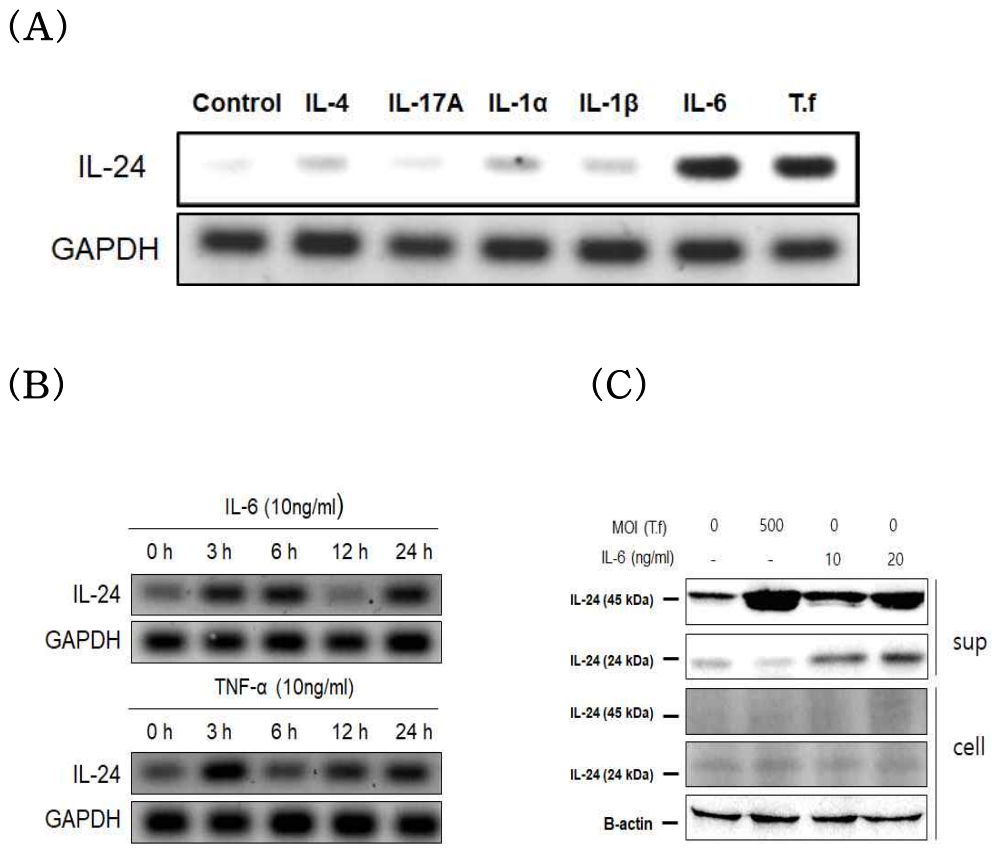
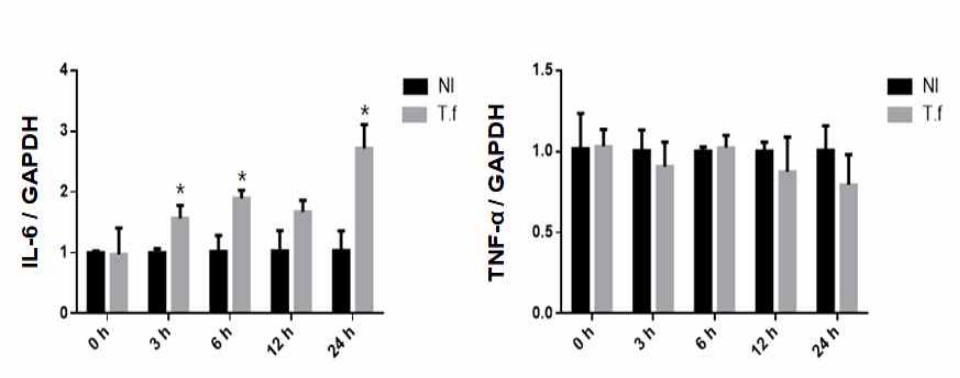


Figure 6. IL-24 expression by pro-inflammatory cytokines in HOK-16B cells (A) HOK-16B cells (5 × 10^5 cells/well) were treated with pro-inflammatory cytokines at a concentration of 10 ng/ml for 24 hr. The cells treated with *T. forsythia* at MOI 500 were used as a positive control. The mRNA level of IL-24 was analyzed by RT-PCR. (B) HOK-16B cells (5 × 10^5 cells/well) were treated with

10 ng/ml of IL-6 and TNF-α for 3 h to 24 h. The mRNA level of IL-24 was analyzed by RT-PCR. (C) HOK-16B cells (5×10^5 cells/well) were treated with 10 and 20 ng/ml of IL-6 for 24 hr. The protein level of IL-24 was analyzed by immunoblotting.

(A)



(B)

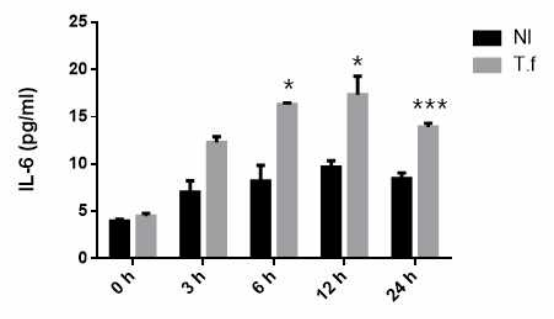


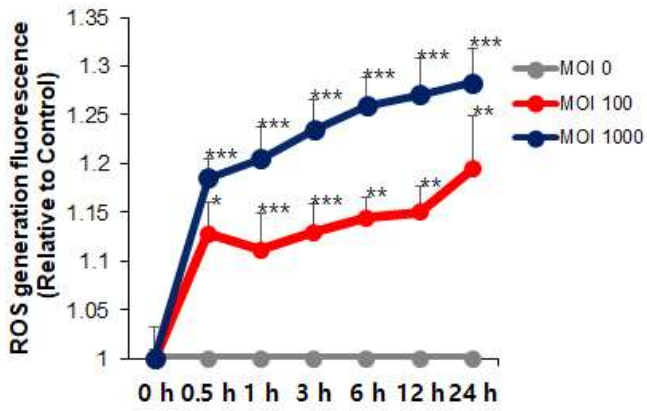
Figure 7. Expression of IL-6 and TNF-α in HOK-16B cells treated with *T. forsythia.* HOK-16B cells (5×10^5 cells/well) were treated with *T. forsythia* for 3 h to 24 h. (A) The mRNA level was analyzed by real-time RT-PCR and (B) the protein level was analyzed by ELISA. The data represents the mean ± SD. (∗ : $p <$

0.05, *** : $p < 0.001$ compared to non-treated control)

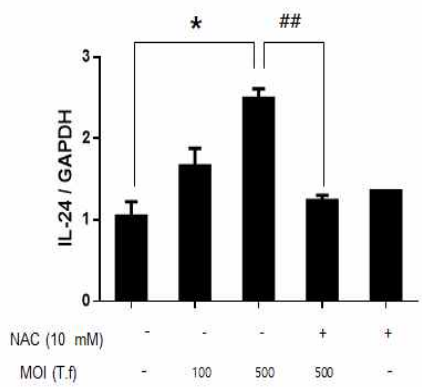
3.5. IL-24 regulation by ROS

ROS is known as an upstream signal of IL-24 induction in psoriasis and can be induced by bacteria. To determine ROS generation by *T. forsythia* in HOK-16B cells, cells were stained with DCF-DA for 30 min before infection. The fluorescence was measured with excitation at 485 nm and emission at 520 nm by FLUOStar OPTIMA. The fluorescence was increased by *T. forsythia* in a MOI and time-dependent manner (Fig 8A). To determine the effect of NAC, a ROS scavenger, on *T. forsythia*-induced IL-24 expression, HOK-16B cells were treated with 10 mM NAC for 30 min before infection. The mRNA and protein level were analyzed by real-time RT-PCR and immunoblotting, respectively. *T. forsythia*-induced IL-24 mRNA and glycosylated IL-24 protein were decreased by NAC (Fig 8B and C).

(A)



(B)



(C)

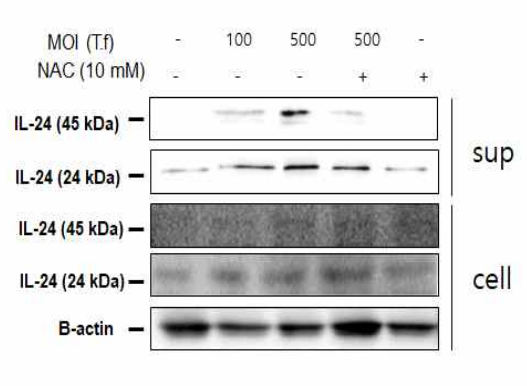
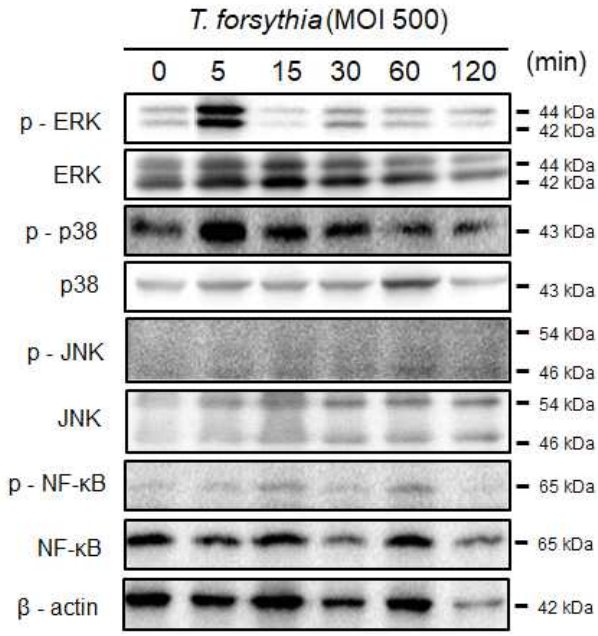


Figure 8. *T. forsythia*-induced IL-24 reduction by a ROS scavenger in HOK-16B cells. (A) HOK-16B cells (2×10^5

cells/well) were seeded into 96-well plates and cultured overnight. Cells were stained with 10 μM DCF-DA for 30 min, washed with serum-free media, and then infected with *T. forsythia for* 24 h. (MOI 100 and 500). The fluorescence was measured with excitation at 485 nm and emission at 520 nm by FLUOStar OPTIMA. The data represents the mean ± SD. (* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$ compared to non-treated control group) (B), (C) HOK-16B cells (5×10^5 cells/well) were treated with 10 mM NAC for 30 minutes before infection. The mRNA level (B) and protein level (C) were analyzed by real-time RT-PCR and immunoblotting, respectively. (* : $p < 0.05$ compared to non-treated control, ## : $p < 0.01$ compared to *T. forsythia*-treated group)

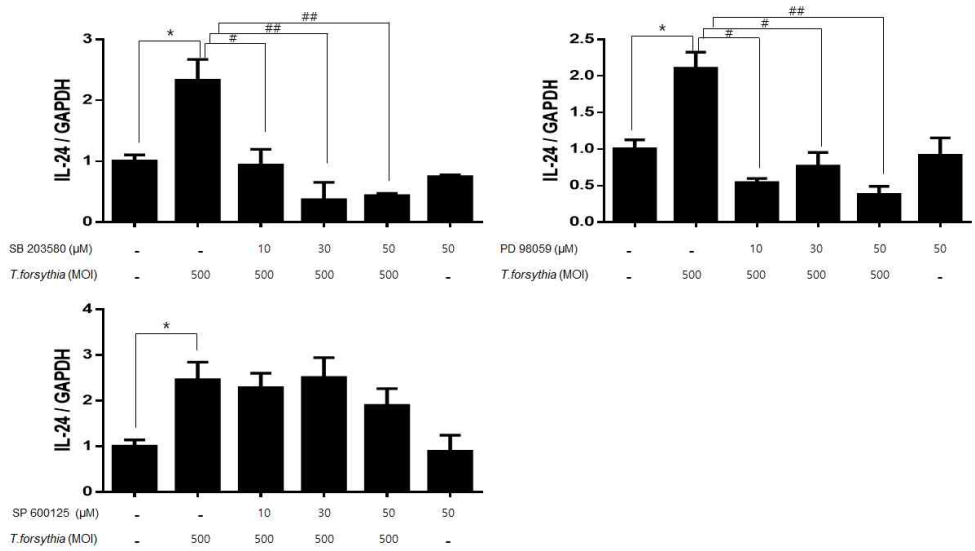
3.6. IL-24 regulation by MAPKs

To determine the signaling pathway for IL-24 expression, HOK-16B cells were treated with *T. forsythia* and analyzed by immunoblotting using antibodies against NF-κB and MAPK. *T. forsythia* activated p38 and ERK, but minimally activated JNK (Fig 9). The bacterium did not activate NF-κB signaling. To examine whether IL-24 expression was regulated by MAPK signaling, MAPK inhibitors were treated for 30 min before infection with *T.forsythia*. The mRNA and protein level were analyzed by real-time RT-PCR and immunoblotting, respectively. *T. forsythia*-induced IL-24 was decreased by SB 203580 (a p38 inhibitor) and PD 98059 (a ERK inhibitor), but was not affected by SP 600125 (a JNK inhibitor) (Fig 10).

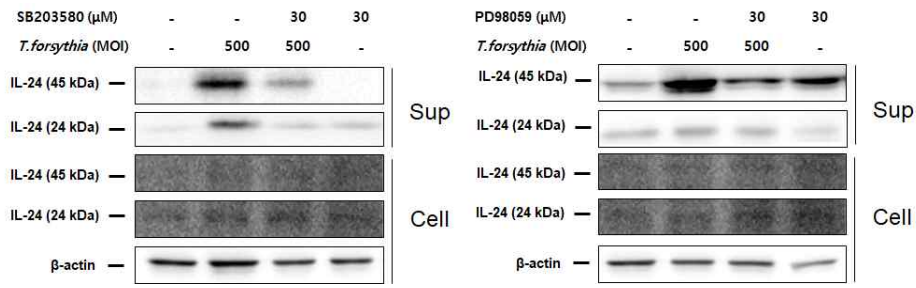


Figrue 9. MAPK activation by *T. forsythia* in HOK-16B cells. HOK-16B cells (5×10^5 cells/well) were seeded into 30 mm plates and treated with *T. forsythia* for 5 min to 120 min. The MAPKs and NF-κB signaling was determined by immunoblotting.

(A)



(B)



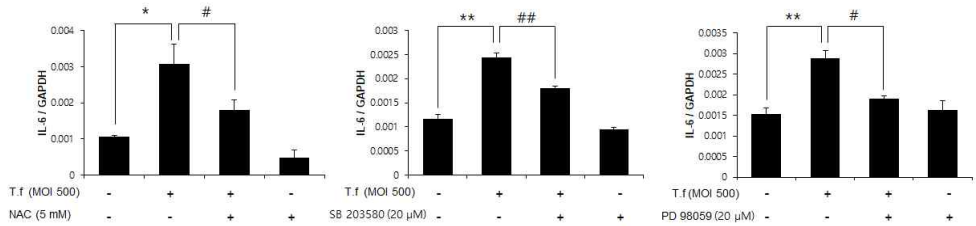
Figrue 10. IL-24 regulation by MAPK signaling (A) HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with various concentrations of MAPK inhibitors for 30 min before *T.*

forsythia infection for 24 h. The mRNA level of IL-24 was analyzed by real-time RT-PCR. The data represents the mean ± SD. (* : $p < 0.05$ compared to non-treated control group, # : $p < 0.05$ and ## : $p < 0.01$ compared to *T. forsythia*-treated group.) (B) HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with 30 μM of MAPK inhibitors for 30 minutes before *T. forsythia* infection for 24 h. The protein level of IL-24 was analyzed by immunoblotting.

3.7. IL-6 regulation by ROS and MAPKs

As described earlier, it was shown that IL-6, ROS and MAPKs were involved in regulation of IL-24. To investigate how these signals can affect each other in cells, the IL-6 regulation by ROS and MAPKs was analyzed. HOK-16B cells were treated with inhibitors of ROS and MAPKs for 30 min and infected with *T. forsythia* for 6 h. The mRNA level and protein level of IL-6 were analyzed by real-time RT-PCR and ELISA, respectively. *T. forsythia* significantly increased IL-6 expression, which was significantly reduced by NAC, SB 203580 and PD 98059 (Fig 11A and B). Although IL-6 was able to induce IL-24 expression (Fig. 6), recombinant IL-24 did not induce IL-6 expression (Fig 12). As well, NAC inhibited activation of p38 and ERK MAPK by *T. forsythia* (Fig 13). Taken together, these results indicate that *T. forsythia*-stimulated ROS induced MAPK activation, leading to IL-6 expression followed by IL-24 expression.

(A)



(B)

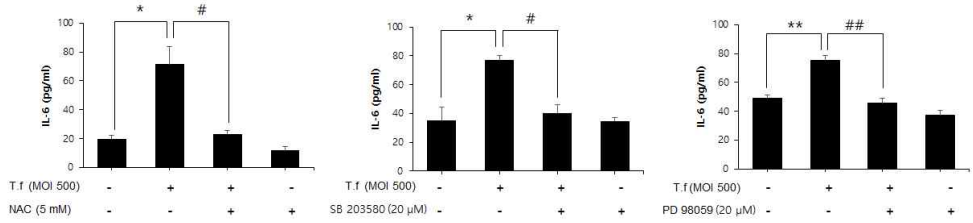


Figure 11. Effect of ROS and MAPKs on *T. forsythia*-induced IL-6 expression. HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with 5 mM NAC or 20 μM of MAPK inhibitors for 30 minutes before *T. forsythia* infection for 12 h. (A) The mRNA level of IL-6 was analyzed by real-time RT-PCR and (B) the protein level of IL-6 was analyzed by ELISA. The data represents the mean ± SD. (* : $p < 0.05$, ** : $p < 0.01$ compared to non-treated control groups, # : $p < 0.05$, ## : $p < 0.01$ compared to

T. forsythia-treated groups.)

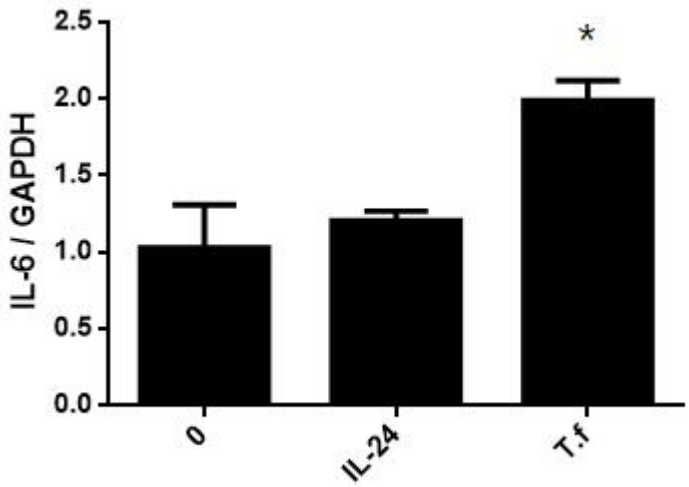


Figure 12. IL-6 expression by recombinant IL-24. HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with 100 ng/ml recombinant IL-24 and *T. forsythia* (MOI 500) for 12 h. The mRNA level of cytokine was analyzed by real-time RT-PCR. The data represents the mean ± SD. (* : $p < 0.05$ compared to non-treated control groups)

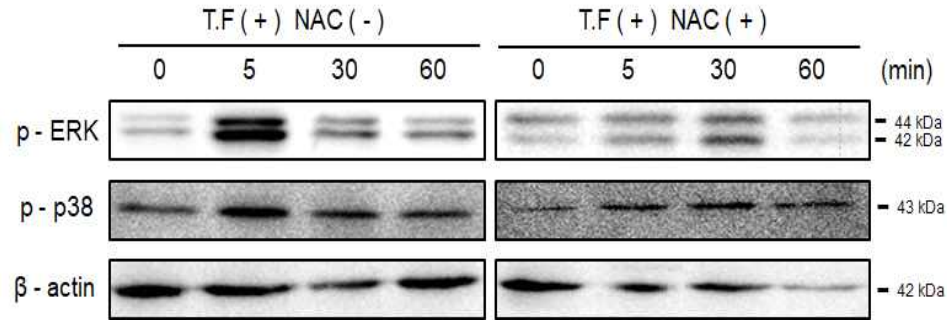


Figure 13. Inhibition of MAPK activation by NAC. HOK-16B cells (5×10^5 cells/well) were seeded into 30 mm plates and treated with10 mM NAC for 30 minutes before infection with *T. forsythia* for 5 min to 60 min. The p-ERK and p-p38 activation was determined by immunoblotting.

3.8. Effect of recombinant IL-24 on pro-inflammatory factors

To investigate the effect of IL-24 on the expression of IL-1α, IL-8, CXCL10 and MCP1, which are expressed in inflamed gingival tissues and relevant to periodontitis, cells were treated with recombinant IL-24 for various times. The mRNA expression of the factors was analyzed by real-time RT-PCR. IL-1α, IL-8, CXCL10 and MCP1 were significantly increased in cells stimulated with recombinant IL-24 (Fig 14).

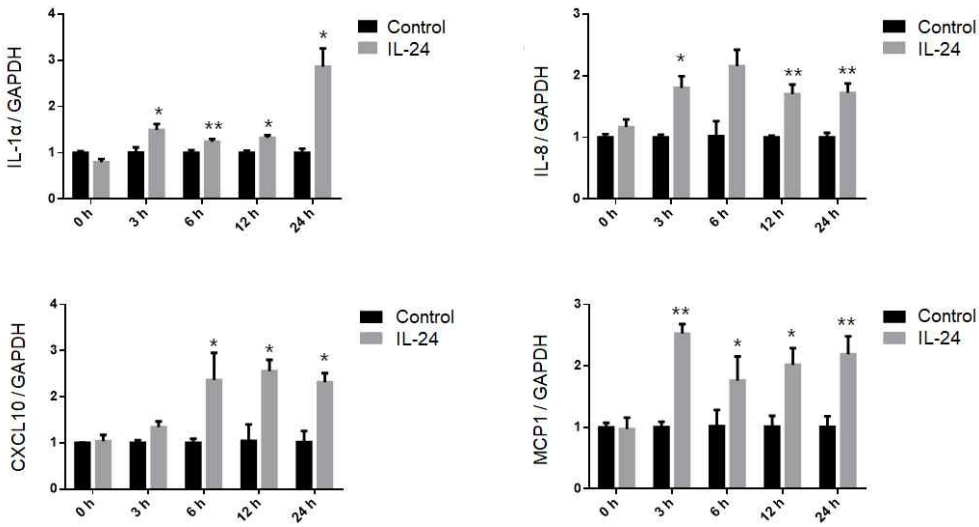


Figure 14. Expression of proinflammatory factors by recombinant IL-24. HOK-16B cells (5×10^5 cells/well) were seeded into 6-well plates and treated with 100 ng/ml recombinant IL-24 for various times. The mRNA level of the factors was analyzed by real-time RT-PCR. The data represents the mean ± SD. (* : $p < 0.05$, ** : $p < 0.01$ compared to non-treated control groups)

Ⅳ. Discussion

In this study, we investigated IL-24 expression and IL-24 induction pathway by *T. forsythia* in HOK-16B cells. IL-24 was increased by *T. forsythia* , a pathogen of periodontitis, but not by *S. oralis*, a oral commensal bacteria. IL-24 was secreted from cells as a glycosylated form and this glycosylated IL-24 has bioactivity that affects cells via binding to IL-20 receptors. Increased ROS and activated MAPKs in response to *T. forsythia* infection increased IL-24 expression through IL-6.

It was reported that several pro-inflammatory cytokines induced IL-24. IL-24 expression was observed in human colonic subepithelial myofibroblasts (SEMFs) and IL-1β significantly enhanced IL-24 mRNA and protein expression, but not IL-17A, TNF-α and IFN-ɣ [4]. IL-1β also induced the expression of SOCS3, IL-24, IL-1β, and IL-6 in primary cultured human corneal epithelial cells [5]. TNFR signaling in NF-κB-deficient keratinocytes induces psoriasis-like skin

disease [6]. However, when we tested the effect of several pro-inflammatory cytokines on IL-24 expression, IL-6 was found to be the major inducer of IL-24 expression in HOK-16B cells.

p38 MAPK regulates IL-24 expression by interfering with destabilization of the 3′UTR of IL-24 mRNA [16]. Inhibition of p38 MAPK in normal human epidermal keratinocytes (NHEK) resulted in downregulation of IL-1β-induced IL-24 [16]. MAPK signaling was activated by *T. forsythia* in HOK-16B cells ~~and~~ while p38 and ERK regulated IL-24 expression but not JNK. We demonstrated that ROS generated by *T. forsythia* in HOK-16B cells activated MAPK signaling and it enhanced IL-24 expression through elevated IL-6 expression. IL-6 was not induced by recombinant IL-24, thus these two cytokines do not affect each other.—Yet, IL-6 was the only inducer of IL-24.

In conclusion, our data demonstrates that IL-24 expression is enhanced after *T. forsythia* infection. IL-24 induced inflammatory cytokines and chemokines which are highly observed in periodontal

inflammation. This is the first step of research process to further study IL-24 in periodontitis and it will become the basis for studying not only IL-24 but also IL-19 and IL-20, which share the same receptor and often are observed together in inflamed region.

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국문요약

구강 각화 세포에서의 *Tannerella forsythia*에 의한 IL-24의 조절

1. 목 적

인터루킨 -24 (IL-24)는 인터루킨 -10 계열의 구성원으로 IL-24 수용체 (IL-20R2 / IL-20R1 및 IL-20R2 / IL-22R1)와 결합한다. 동일한 수용체를 공유하는 사이토카인인 IL-19 및 IL-20과 달리, IL-24는 당화되어서 생물학적 활성을 가질 수 있다. IL-24는 염증성 장 질환 (IBD), 건선, 류마티스 관절염 (RA) 및 각막염과 같은 염증성 질환에서 고도로 발현되었다.

*Tannerella forsythia*는 그람 음성균이며 치주 질환의 원인균으로 알려져 있다. 이전의 프로테옴 연구에서 *T. forsythia*가 48 시간 감염 후 사람 구강 각화 세포 (HOK-16B 세포)에서 IL-24를 유의하게 증가시키는

것으로 나타났다.

*T. forsythia*의 몇 가지 독성 인자와 그 경로 기전이 연구되었지만 IL-24에 대한 연구는 아직 이루어지지 않았다.

본 연구의 목적은 *T. forsythia*에 의한 HOK-16B 세포의 IL-24 유도와 관련된 인자들과 염증성 인자의 발현에 미치는 IL-24의 영향을 조사하는 것이다

2. 방 법

HOK-16B 세포를 *T. forsythia* 및 *Streptococcus oralis* (*S. oralis*)로 12 내지 48 시간 동안 다양한 MOI (0, 100, 500)에서 처리 하였다. IL-24의 mRNA 수준은 실시간 중합효소연쇄반응으로, 단백질 수준은 면역블로팅으로 분석하였다. IL-24 생산에 활성산소종 (reactive oxygen species, ROS)이 관련되는지 알아보기 위해 *T. forsythia*의 ROS 생성을 2′,7′- 디클로로플루오레신 디아세테이트 (DCF-DA)을 사용하여 측정하였고 ROS에 의한 IL-24 발현 및 당화 조절은 ROS 억제제인 N-아세틸 시스테인 (NAC)을 사용하여 평가하였다.

사이토카인 및 MAPK 신호 전달 경로에 의한 IL-24의 조절을 분석하기 위해 염증성 사이토 카인인 IL-6 및 TNF-α와 MAPK 억제제를 HOK-16B 세포에 처리한 후 IL-24의 발현을 실시간 중합효소연쇄반응 및 면역 블로팅으로 분석하였다. IL-24가 염증 유발 인자를 유도 할 수 있는지 알아보기 위해 HOK-16B 세포를 재조합 IL-24로 처리하여 IL-1α, IL-8, CXCL10 및 MCP-1의 발현을 실시간 중합효소연쇄반응으로 분석하였다.

3. 연구결과

*T. forsythia*는 HOK-16B 세포에서 IL-24 발현을 유도하고 배양 상등액으로 당화된 IL-24를 분비하였으나 *S. oralis*에 의해서는 이러한 현상이 일어나지 않았다. *T. forsythia*는 HOK-16B 세포에서 ERK 및 p38을 활성화 시켰으며, 이들의 활성화를 억제했을 때 IL-24의 발현 감소 및 당화된 IL-24의 분비가 감소했다. *T. forsythia*는 HOK-16B 세포에서 ROS 생성과 IL-6 발현을 증가시켰다. IL-6는 IL-24 발현 및 당화의 강력한 유도제였다. IL-24의 발현과 당화는 ROS 억제제인 NAC와, ERK와 p38의 억제제를 처리했을 때 감소하였다. 재조합 단백질 IL-24를 HOK-16B

세포에 처리했을 때 IL-1α, IL-8, CXCL10 및 MCP-1의 발현이 유의하게 증가한 반면 IL-6 발현에는 영향을 주지 않았다.

4. 결 론

당화된 IL-24는 HOK-16B 세포에서 *T. forsythia*에 의해 유도되었으며 ROS와 MAPK 활성화를 통해 유도된 IL-6에 의해 조절되었다. IL-24는 전염증성 인자를 증가시켰으며 이 결과는 *T. forsythia*에 의해 유도 된 IL-24가 치주 병인과 관련이 있음을 시사한다.

주요어 : 치주염, *Tannerella forsythia*, IL-24, ROS, MAPKs

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