



# Distal NF- $\kappa$ B binding motif functions as an enhancer for nontypeable *H. influenzae*-induced DEFB4 regulation in epithelial cells



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## ABSTRACT

Among the antimicrobial molecules produced by epithelial cells, DEFB4 is inducible in response to pro-inflammatory signals such as cytokines and bacterial molecules. Nontypeable *Haemophilus influenzae* (NTHi) is an important human pathogen that exacerbates chronic obstructive pulmonary disease in adult and causes otitis media and sinusitis in children. Previously, we have demonstrated that DEFB4 effectively kills NTHi and is induced by NTHi via TLR2 signaling. The 5'-flanking region of DEFB4 contains several NF- $\kappa$ B binding motifs, but their NTHi-specific activity remains unclear. In this study, we aimed to elucidate molecular mechanism involved in DEFB4 regulation, focusing on the role of the distal NF- $\kappa$ B binding motif of DEFB4 responding to NTHi. Here, we show that the human middle ear epithelial cells up-regulate DEFB4 expression in response to NTHi via NF- $\kappa$ B activation mediated by I $\kappa$ K $\alpha$ / $\beta$ -I $\kappa$ B $\alpha$  signaling. Deletion of the distal NF- $\kappa$ B binding motif led to a significant reduction in NTHi-induced DEFB4 up-regulation. A heterologous construct containing the distal NF- $\kappa$ B binding motif was found to increase the promoter activity in response to NTHi, indicating a NTHi-responding enhancer activity of the distal NF- $\kappa$ B binding motif. Furthermore, electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that the p65 domain of NF- $\kappa$ B binds to the distal NF- $\kappa$ B binding motif in response to NTHi. Taken together, our results suggest that NTHi-induced binding of p65 NF- $\kappa$ B to the distal NF- $\kappa$ B binding motif of DEFB4 enhances NTHi-induced DEFB4 regulation in epithelial cells.

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## 1. Introduction

DEFB4, previously known as human  $\beta$ -defensin 2, is a small cationic antimicrobial peptide produced by a variety of epithelial cells [1–3]. DEFB4 predominantly kills Gram-negative bacteria by increasing permeability of the bacterial membrane through pore formation [4]. Unlike DEFB1, DEFB4 is inducible in response to inflammatory stimuli such as pro-inflammatory cytokines [3,5,6] and bacterial molecules [7–9]. A variety of signaling pathways is known to form a network for transcriptional regulation of DEFB4, including SRC-dependent ERK signaling [3], IL-17-dependent JAK signaling [6], and NF- $\kappa$ B signaling mediated by toll/IL-1 receptor [10] and protease-activated receptor 2 [11].

The gene regulatory region of the DEFB4 locus within 3000 base pairs upstream from the exon 1 contains several NF- $\kappa$ B binding motifs involved in DEFB4 regulation, including one distal and two proximal motifs [5,8,12]. Interestingly, a specific NF- $\kappa$ B binding motif contributing to transcriptional regulation of DEFB4 varies

with the type of inflammatory stimuli. For example, *Fusobacterium nucleatum*-induced DEFB4 regulation is independent from NF- $\kappa$ B signaling [13] while *Helicobacter pylori* depends on NF- $\kappa$ B for DEFB4 regulation [14]. Furthermore, there exists controversy regarding the enhancer activity of the distal NF- $\kappa$ B binding motif of DEFB4. The distal NF- $\kappa$ B binding motif (–2193/–2184) of DEFB4 is known to significantly contribute to *Pseudomonas aeruginosa*-induced DEFB4 regulation, but not to IL-1 $\beta$ - or LPS-induced DEFB4 regulation [15,16], which led us to focus on the enhancer of DEFB4 responding to NTHi.

NTHi is a small Gram-negative bacterium and an opportunistic pathogen existing as a commensal organism in the human nasopharynx [17]. Unlike *Haemophilus influenzae* type b, NTHi is nontypeable since it lacks a polysaccharide capsule used for typing and rarely causes life-threatening infections [18]. Nevertheless, NTHi is a clinically important pathogen that exacerbates chronic obstructive pulmonary disease in adults, and causes otitis media and sinusitis in children [19,20]. In contrast to lipopolysaccharide (LPS) utilized in other studies [5,8,16], NTHi contains an unique atypical endotoxin, lipooligosaccharide (LOS), structurally different from LPS of the common Gram-negative bacteria. NTHi is predominantly recognized by TLR2 signaling [21] while LPS triggers TLR4 signaling [22]. In our prior study, we demonstrated that DEFB4

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has a potent antimicrobial effect on NTHi [23,24]. We also showed that the middle ear epithelial cells up-regulate DEFB4 in response to NTHi via TLR2/MyD88 signaling and p38 MAPK signaling [7,24]. NF- $\kappa$ B is known to be involved in transcriptional regulation of DEFB4 [5,8,15,16]; however, it remains unclear if the distal NF- $\kappa$ B binding motif of DEFB4 functions as an enhancer for NTHi-induced DEFB4 regulation.

Here, we present that NF- $\kappa$ B activation mediates NTHi-induced DEFB4 regulation through I $\kappa$ K $\alpha$ / $\beta$ -I $\kappa$ B $\alpha$  signaling. Furthermore, we demonstrate that the NTHi-activated nuclear components interact with the distal NF- $\kappa$ B binding motif to augment DEFB4 up-regulation in response to NTHi. This study will enable us to better understand molecular mechanism involved in transcriptional regulation of DEFB4 in epithelial cells.

## 2. Experimental procedures

### 2.1. Plasmid construction, transfection and luciferase assay

The 5'-flanking region (−2625 to +1) of the DEFB4 gene was subcloned to the pGL3-basic (Promega, Madison, WI) as previously described [3], which was named as pDEFB4/−2625/luc. Nested deletions of pDEFB4/−2625/luc (1.7, 1.1 and 0.7 kb) were obtained using the Erase-a-Base<sup>®</sup> System (Promega) in which exonuclease III was used to specifically digest DNA from a blunt restriction site of BstZ171 (New England Biolabs, Ipswich, MA) and the opposite end was protected from digestion by a 4-base 3' overhang restriction site of KpnI (New England Biolabs). 200 bp-sized segments of the DEFB4 5'-flanking region (−2503 to −2301, −2324 to −2121, −2131 to −1919, −1938 to −1732 and −1751 to −1642) were subcloned upstream of pTAL-luc, a vector containing the luciferase gene with a TATA-like promoter region from the Herpes simplex virus thymidine kinase (TK) promoter (Clontech, Mountain View, CA). pNF $\kappa$ B-luc was purchased from Clontech, containing multiple copies of the NF- $\kappa$ B consensus sequence fused to pTAL-luc. A transdominant mutant I $\kappa$ K $\alpha$ / $\beta$ -I $\kappa$ B $\alpha$  [(S32/36A)] was previously described [21,25], and pcDNA 3.1(+) was purchased from Invitrogen. For luciferase assay, cells were seeded into six-well plates at a density of  $1.5 \times 10^5$  cell/well and transfected at ~60% confluence. pRL-TK vector (Promega) was co-transfected to normalize for transfection efficiency. Luciferase activity was measured using a luminometer (Pharmingen, La Jolla, CA) after adding harvested cell lysate to the luciferase substrate (Promega). Results were expressed as fold-induction of luciferase activity, taking the value of the non-treated group as 1.

### 2.2. Electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays

Cells were treated with NTHi lysate for 1.5 and 3 h after overnight starvation. Nuclear protein was extracted using the NE-PER<sup>®</sup> Nuclear extraction reagent (Pierce Biotechnologies) and the protein concentration was determined using a BCA<sup>™</sup> protein assay kit (Pierce Biotechnologies) [25]. 5'-Biotin labeled double-stranded oligonucleotide probes were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) as follows: wild type NF- $\kappa$ B 1 (5'-gttactttgggacttccccagctatg-3') and mutated NF- $\kappa$ B 1 (5'-gttactttctagcttccccagctatg-3'). In vitro binding of NF- $\kappa$ B to the DEFB4 promoter was determined using the LightShift<sup>®</sup> chemiluminescent EMSA kit (Pierce Biotechnologies) according to the manufacturer's instruction. Briefly, nuclear proteins (4  $\mu$ g) were incubated with a biotin-labeled target DNA probe (20 fmol) in 20  $\mu$ l of binding buffer for 20 min at RT. Samples were applied to 6% polyacrylamide gels under native conditions in high ionic strength buffer and electrophoresis was performed. As a positive and a negative

control, a biotinylated Epstein–Barr nuclear antigen (EBNA) control DNA (5'-TAGGCATATGCTA-3') was applied with or without the EBNA extract. To detect biotin-labeled DNA, a 1:300 dilution of Streptavidin-HRP conjugate was applied to the membranes for 15 min. After washing, the chemiluminescent substrate was added and the signal was detected with exposure to X-ray films. To determine if NTHi induces NF- $\kappa$ B p65 to bind with an enhancer in vivo, ChIP assays were performed using Chip-IT<sup>™</sup> Express (Active Motif, Carlsbad, CA). Briefly, cells were exposed to NTHi lysate for 2 h and fixed with 1% formaldehyde for 10 min. After lysing cells, we collected the nuclei pellet and chromatin was sheared with the provided enzyme cocktail. 10  $\mu$ l of the sheared DNA samples were named as "Input DNA" for use as controls in PCR analysis. 1  $\mu$ g of anti-NF- $\kappa$ B p65 antibody and 25  $\mu$ l of protein G magnetic beads were added to 10  $\mu$ g of sheared DNA samples and incubated for 4 h at 4 °C. The samples were placed on the magnetic stand to pellet beads and the supernatant was discarded carefully. After washing, the pelleted beads were resuspended and named as "Chip DNA." After purifying Chip DNA and Input DNA, PCR analysis was performed with the specific primer pairs as follows: NF- $\kappa$ B1 (−2369/−2108: 5'-agtacagcagcagtgatagtgcca-3' and 5'-ttggtgctgatgtctgagcctt-3'), the positive control (NF- $\kappa$ B2, −687/−423: 5'-ttctcagaggaaggaagtgggcat-3' and 5'-acagtctcaggccaattgagagc-3') and the negative control (−1775/−1400: 5'-tcagcacacaaggaaacaaagccc-3' and 5'-agcatgggtggcttacactgtcat-3').

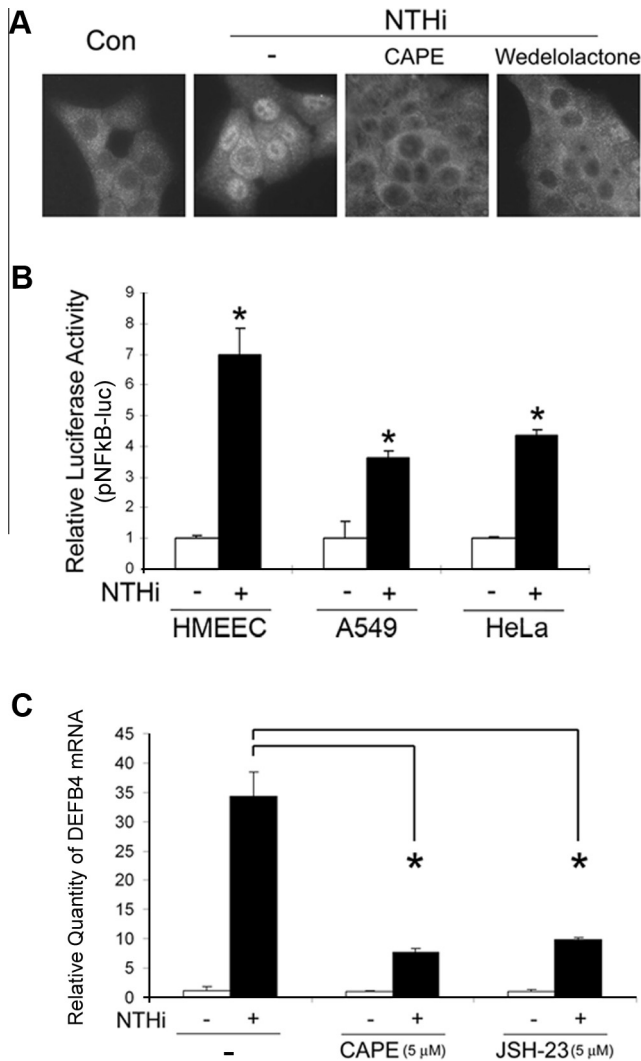
## 3. Results

### 3.1. NF- $\kappa$ B activation is required for NTHi-induced DEFB4 regulation in human epithelial cells

NF- $\kappa$ B is critically involved in transcriptional regulation of DEFB4 induced by proinflammatory cytokines and LPS [5,8,16]. NTHi is known to up-regulate DEFB4 expression via TLR2/MyD88 signaling [23] and activate NF- $\kappa$ B via TLR2/TAK1 signaling [21]. Hence, we sought to determine if NF- $\kappa$ B activation is required for NTHi-induced DEFB4 up-regulation in human epithelial cells. The HMEEC cells were exposed to the NTHi lysate, and immunolabeling was performed using an anti-p65 NF- $\kappa$ B antibody. p65 NF- $\kappa$ B was found to translocate into the nucleus upon exposure to the NTHi lysate (Fig. 1A). Moreover, NTHi-induced NF- $\kappa$ B translocation was blocked by CAPE (an inhibitor of NF- $\kappa$ B activation) and Wedelolactone (a selective and irreversible inhibitor of I $\kappa$ K $\alpha$  and  $\beta$  kinase activity). To investigate NTHi-induced NF- $\kappa$ B activation, luciferase assays were performed using a pNF $\kappa$ B-luc construct containing multiple copies of the NF- $\kappa$ B consensus sequence fused to pTAL-luc. As shown in Fig. 1B, NTHi increases NF- $\kappa$ B activity in human epithelial cell lines such as HMEEC (a human middle ear cell line), A549 (a human lung adenocarcinoma epithelial cell line) and HeLa (a human cervical carcinoma cell line). Next, we sought to determine if NF- $\kappa$ B activation is required for NTHi-induced DEFB4 up-regulation. Cells were pre-treated with chemical inhibitors of NF- $\kappa$ B activation, CAPE and JSH-23, before exposure to the NTHi lysate. Real-time quantitative PCR analysis showed that NTHi-induced DEFB4 up-regulation is inhibited more than 70% by either CAPE or JSH-23 (Fig. 1C).

### 3.2. I $\kappa$ K $\alpha$ / $\beta$ -mediated I $\kappa$ B $\alpha$ phosphorylation is involved in NTHi-induced DEFB4 regulation

Since I $\kappa$ K $\alpha$ / $\beta$ -I $\kappa$ B $\alpha$  signaling has been shown to mediate NTHi-induced NF- $\kappa$ B activation [21] we sought to determine if epithelial cells suppress NTHi-induced DEFB4 up-regulation upon exposure to chemical inhibitors of I $\kappa$ K $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$ . Real-time quantitative PCR analysis showed that NTHi-induced DEFB4



**Fig. 1.** NF- $\kappa$ B activation is required for NTHi-induced DEFB4 up-regulation. (A) Immunolabeling shows that p65 NF- $\kappa$ B is translocated into the nucleus of the HMEEC cells in response to the NTHi lysate. NTHi-induced NF- $\kappa$ B translocation is blocked by CAPE and Wedelolactone. (B) Luciferase assays show that NF- $\kappa$ B activity is up-regulated upon exposure to the NTHi lysate in the A549, HeLa and HMEEC cells. (C) Real-time quantitative PCR analysis shows that NTHi-induced DEFB4 up-regulation is inhibited by CAPE and JSH-23. \* $p < 0.05$ . Results were expressed as fold-induction, taking the value of the non-treated group as 1. The experiments were performed in triplicate and repeated twice. Values are given as the mean  $\pm$  standard deviation ( $n = 3$ ).

up-regulation is inhibited by Wedelolactone and MG132 (a reversible proteasome inhibitor) in a dose-dependent manner (Fig. 2A and B). Furthermore, transfection of the dominant negative construct of I $\kappa$ B $\alpha$  resulted in inhibition of NTHi-induced DEFB4 up-regulation more than 35% (Fig. 2C). Next, we performed immunoblotting to determine NTHi-induced phosphorylation of I $\kappa$ K $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$ . As shown in Fig. 2D and E, I $\kappa$ K $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$  were found to be phosphorylated upon exposure to the NTHi lysate. There was a significant increase in I $\kappa$ B $\alpha$  phosphorylation within 5 min of NTHi exposure (Fig. 2E). Taken together, it is suggested that I $\kappa$ K $\alpha$ / $\beta$ –I $\kappa$ B $\alpha$  signaling contributes to NTHi-induced DEFB4 up-regulation.

### 3.3. The distal NF- $\kappa$ B binding site responds to NTHi resulting in DEFB4 regulation

NF- $\kappa$ B binding motifs are differentially activated according to the type of cells and stimulants. The distal NF- $\kappa$ B binding site of

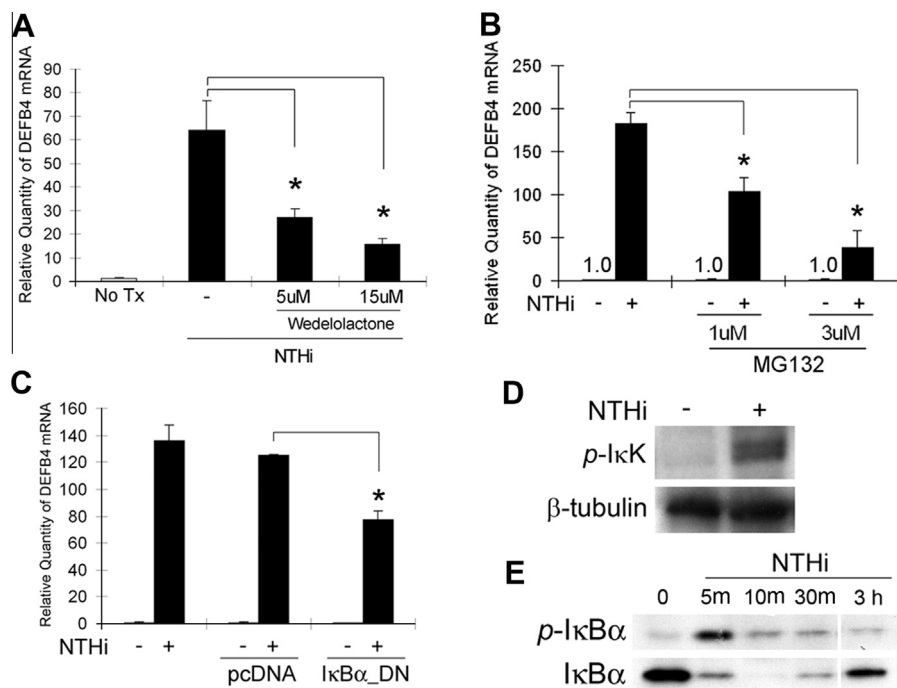
DEFB4 rarely responds to LPS in mononuclear phagocytes [16] but significantly contributes to DEFB4 regulation induced by *P. aeruginosa* in keratinocytes [15]. However, it is poorly understood if the distal NF- $\kappa$ B binding site of DEFB4 responds to NTHi that expresses atypical LPS, lipooligosaccharide [26]. To determine whether a distal NF- $\kappa$ B binding site serves as an enhancer for NTHi-induced DEFB4 regulation, a series of 5' deletion constructs from the 5'-flanking region of DEFB4 were generated by nested deletion using exonuclease III. After transfection with each of the 5' deletion constructs, the HMEEC cells were exposed to the NTHi lysate. Luciferase assays showed that deletion of the distal NF- $\kappa$ B binding site (NF- $\kappa$ B1) surprisingly leads to a reduction more than 60% in NTHi-induced DEFB4 up-regulation, indicating an enhancer activity of NF- $\kappa$ B1 (Fig. 3A). Since this result appeared to disagree with the previous reports [5,16], we prepared heterologous constructs containing segments of the DEFB4 promoter region in order to define the NTHi-responding enhancer precisely. Five of 0.2 kb-sized segments derived from the DEFB4 5'-flanking region (from –1642 to –2503) were subcloned to the upstream of the pTAL-luc vector, a vector containing the firefly luciferase gene with a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. As shown in Fig. 3B, we found that a –2324/–2121 segment containing the NF- $\kappa$ B1 site increases a promoter activity in response to NTHi. Taken together, these results suggest that the distal NF- $\kappa$ B binding site responds to NTHi, resulting in enhancement of NTHi-induced DEFB4 induction.

### 3.4. The p65 NF- $\kappa$ B binds to the distal NF- $\kappa$ B motif of DEFB4 in response to NTHi

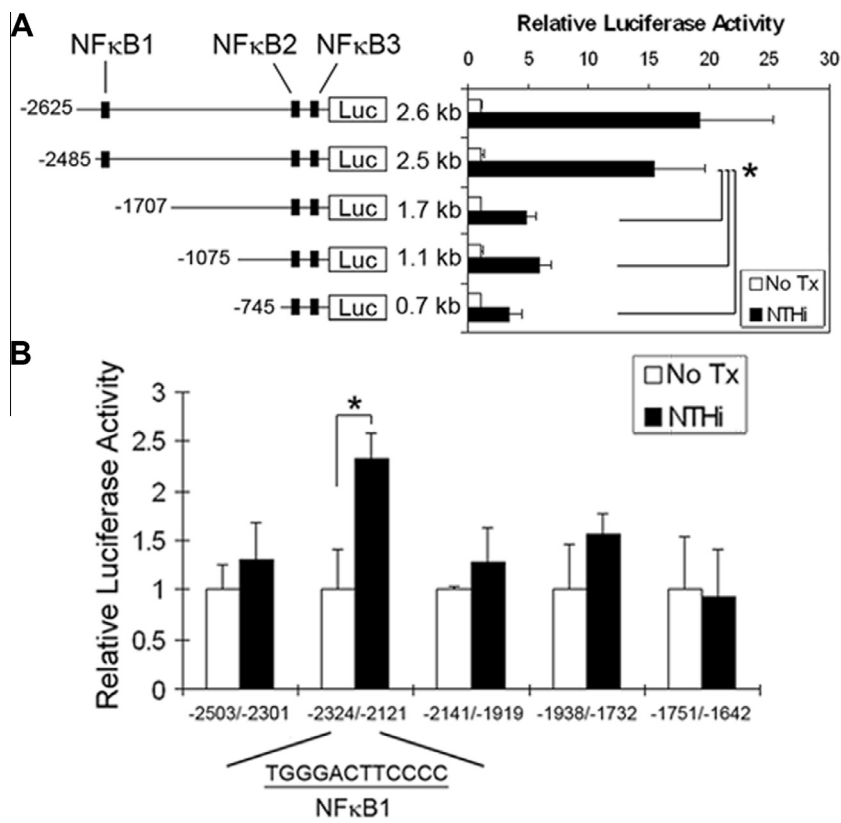
Since we have previously demonstrated that the distal NF- $\kappa$ B binding site of MCP-1/CCL2 responds to NTHi in the inner ear fibrocytes [25], we sought to determine if the NF- $\kappa$ B1 site of DEFB4 interacts with the NTHi-treated nuclear protein in vitro. We performed electrophoretic mobility shift assays using biotinylated oligonucleotides (26 bp) that contain either the wild type or the mutated NF- $\kappa$ B1 DNA sequences. As shown in Fig. 4A, the nuclear extract of NTHi-exposed cells appeared to interact with the wild type NF- $\kappa$ B1, but not with the mutated NF- $\kappa$ B1. To investigate in vivo binding capacity of NF- $\kappa$ B1 motif to p65 NF- $\kappa$ B of NTHi-treated cells, we next performed chromatin immunoprecipitation assays using an anti-p65 NF- $\kappa$ B antibody and the primers spanning NF- $\kappa$ B1. As shown in Fig. 4B, NTHi was found to induce protein/DNA binding in the DNA segment spanning NF- $\kappa$ B1 or NF- $\kappa$ B2 (a positive control), but not in the negative control segment lacking the NF- $\kappa$ B consensus sequences. Altogether, these results indicate that the distal NF- $\kappa$ B motif interacts with p65 NF- $\kappa$ B in response to NTHi and actively serves as an enhancer for NTHi-induced DEFB4 regulation in epithelial cells.

## 4. Discussion

In this study, we investigated the mechanism involved in DEFB4 regulation induced by NTHi in human epithelial cells. We found that the p65 domain of NF- $\kappa$ B translocates into the nucleus in response to NTHi, and this nuclear translocation was blocked by the treatment with NF- $\kappa$ B inhibitors. We also demonstrated that the HMEEC cells activate I $\kappa$ K $\alpha$ / $\beta$ –I $\kappa$ B $\alpha$  signaling in response to NTHi, and I $\kappa$ K $\alpha$ / $\beta$ –I $\kappa$ B $\alpha$  signaling is essential for up-regulation of DEFB4. In addition, we found that deletion of the distal NF- $\kappa$ B binding motif of DEFB4 significantly reduces NTHi-induced DEFB4 up-regulation, indicating its enhancer activity for DEFB4 transcription. These findings support that the distal NF- $\kappa$ B binding motif of DEFB4 functions as a NTHi-responding enhancer for transcriptional regulation of DEFB4.

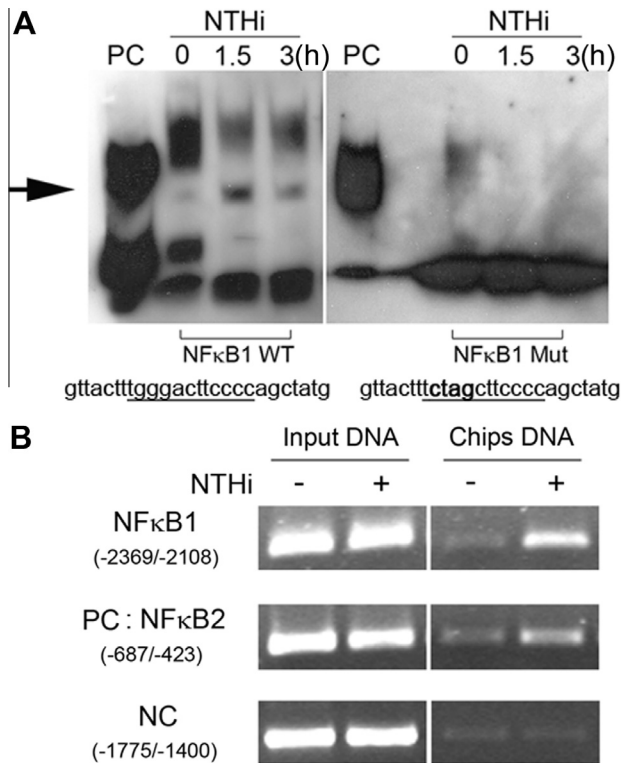


**Fig. 2.** IκK-dependent IκBα phosphorylation is involved in NTHi-induced DEFB4 up-regulation. Real-time quantitative PCR analysis shows that NTHi-induced DEFB4 up-regulation is inhibited by Wedelolactone (A) and MG132 (B) in a dose-dependent manner. (C) It is noted that transfection of the dominant negative construct of IκBα inhibits NTHi-induced DEFB4 up-regulation. \**p* < 0.05. Results were expressed as fold-induction, taking the value of the non-treated group as 1. Values are given as the mean ± standard deviation (*n* = 3). Immunoblotting shows that both IκKα/β (D) and IκBα (E) are phosphorylated upon exposure to the NTHi lysate.



**Fig. 3.** Distal NF-κB binding site functions as an enhancer. (A) Luciferase assays show that deletion of the distal NF-κB binding site (NF-κB1) suppresses NTHi-induced DEFB4 up-regulation. NF-κB2 and NF-κB3: proximal NF-κB binding sites. Luc: luciferase-coding sequences. (B) 0.2 kb-sized segments of DEFB4 5' flanking region (–2503/–2301, –2324/–2121, –2141/–1919, –1938/–1732 and –1751/–1642) were subcloned upstream of the pTAL-luc vector. It is noted that NTHi up-regulates the promoter activity of the –2324/–2121 segment containing NF-κB1. \**p* < 0.05. Results were expressed as fold-induction, taking the value of the non-treated group as 1. Values are given as the mean ± standard deviation (*n* = 3).





**Fig. 4.** Binding of p65 NF-κB to the enhancer region of DEFB4 is required for in NTHi-induced DEFB4 up-regulation. (A) Electrophoretic mobility shift assays show that the NTHi-treated nuclear extract has an affinity for binding to the wild type NF-κB1 (WT), not to the mutated NF-κB1 (Mut). PC: a positive control. (B) Chromatin immunoprecipitation assays show that NTHi induces binding of p65 NF-κB to the DNA segments spanning NF-κB1, not to the negative control segment without NF-κB binding sites (NC). The NF-κB2-spanning segment was used as a positive control. Input DNA: a DNA sample before immunoprecipitation. Chips DNA: DNA sample after immunoprecipitation with an anti-p65 NF-κB antibody.

Within 3000 base pairs upstream of exon 1 in the gene regulatory region of DEFB4, there are specific binding motifs for a variety of transcription factors such as AP-1, STAT, NF-IL6 and NF-κB [16,27]. Particularly, two proximal NF-κB binding motifs (–186/–205 and –572/–596) have been shown to be essentially contribute to transcriptional regulation of DEFB4; however, they are activated differentially according to the specific type of cells and stimulants. For instance, both proximal NF-κB binding motifs are involved in LPS-induced DEFB regulation in mononuclear phagocytes [16] whereas IL-1β and *Escherichia coli* Nissle 1917 require only one proximal NF-κB binding motif (–186/–205) for DEFB4 regulation in pulmonary and intestinal epithelial cells [8].

Previously, it has been reported that the distal NF-κB binding motif (–2193/–2184) of DEFB4 insignificantly functions as an enhancer for LPS-induced DEFB4 up-regulation in mononuclear phagocytes [16] and pulmonary epithelial cells co-cultured with mononuclear phagocytes [5]. On the contrary, our result showed that the distal NF-κB binding motif of DEFB4 responds to NTHi, resulting in enhancement of NTHi-induced DEFB4 up-regulation. In agreement with our findings, the distal NF-κB binding motif was also found to be involved in *P. aeruginosa*-induced DEFB4 regulation in keratinocytes [15], suggesting that activities of NF-κB binding motifs vary with the types of cells and inflammatory stimulants. Further studies are needed to reveal how each stimulant differentially activates transcriptional factors and enhancers to regulate DEFB4 transcription.

Consistent with the study of LPS-induced DEFB4 regulation [5], our data showed the contribution of IκKα/β–IκBα signaling to NTHi-induced DEFB4 regulation. However, inhibition of IκKα/β

and IκBα did not completely block NTHi-induced DEFB4 up-regulation, suggesting either the involvement of IκKα/β-independent activation of NF-κB and/or NF-κB-independent regulation of DEFB4. In addition to NF-κB, DEFB4 regulation requires a diversity of transcriptional factors such as AP-1 [8], NF-IL-6 [27] and myeloid Elf-1-like factor [28]. The AP-1 binding motif (TRE, –121/127) of DEFB4 plays an important role in DEFB4 regulation induced by *E. coli* Nissle 1917 in intestinal epithelial cells [8] and *P. aeruginosa* in keratinocytes [15]. In this study, we did not investigate the role of AP-1, but it is expected that AP-1 significantly contributes to DEFB4 regulation in response to NTHi.

Unlike a single component of bacteria, LPS, the NTHi lysate contains a variety of bacterial products including HMW proteins [29], p6 protein [30] and lipooligosaccharide [18]. LPS is recognized by TLR4 signaling [22], but NTHi involves multiple receptor signaling pathways such as TLR2 signaling [23] and TGF-β signaling [31,32]. Considering that *P. aeruginosa* and NTHi commonly trigger TLR2 signaling [23,33,34], the enhancer activity of the distal NF-κB binding motif maybe associated with TLR2 signaling, but further studies are needed to unveil receptor signaling specific to activity of the distal NF-κB binding site.

Taken together, we have demonstrated that NTHi up-regulates DEFB4 through NF-κB activation mediated by an IκKα/β–IκBα signaling pathway. Moreover, our study shows that the distal NF-κB binding site of DEFB4 functions as an enhancer of DEFB4 regulation responding to NTHi. This study may provide the scientific basis for development of a therapeutic inducer regulating antimicrobial peptides such as DEFB4 for the management of infectious diseases.

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## References

- [1] J. Harder, J. Bartels, E. Christophers, J.M. Schroder, A peptide antibiotic from human skin, *Nature* 387 (1997) 861.
- [2] A.M. McDermott, R.L. Redfern, B. Zhang, Human beta-defensin 2 is up-regulated during re-epithelialization of the cornea, *Curr. Eye Res.* 22 (2001) 64–67.
- [3] S.K. Moon, H.Y. Lee, J.D. Li, M. Nagura, S.H. Kang, Y.M. Chun, F.H. Linthicum, T. Ganz, A. Andalibi, D.J. Lim, Activation of a Src-dependent Raf-MEK1/2-ERK signaling pathway is required for IL-1α-induced upregulation of beta-defensin 2 in human middle ear epithelial cells, *Biochim. Biophys. Acta* 1590 (2002) 41–51.
- [4] E. Martin, T. Ganz, R.L. Lehrer, Defensins and other endogenous peptide antibiotics of vertebrates, *J. Leukocyte Biol.* 58 (1995) 128–136.
- [5] Y. Tsutsumi-Ishii, I. Nagaoka, Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production, *J. Immunol.* 170 (2003) 4226–4236.
- [6] C.Y. Kao, Y. Chen, P. Thai, S. Wachi, F. Huang, C. Kim, R.W. Harper, R. Wu, IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-κB signaling pathways, *J. Immunol.* 173 (2004) 3482–3491.
- [7] S.K. Moon, H.Y. Lee, H. Pan, T. Takeshita, R. Park, K. Cha, A. Andalibi, D.J. Lim, Synergistic effect of interleukin 1 alpha on nontypeable *Haemophilus influenzae*-induced up-regulation of human beta-defensin 2 in middle ear epithelial cells, *BMC Infect. Dis.* 6 (2006) 12.
- [8] J. Wehkamp, J. Harder, K. Wehkamp, B. Wehkamp-von Meissner, M. Schlee, C. Enders, U. Sonnenborn, S. Nuding, S. Bengmark, K. Fellermann, J.M. Schroder, E.F. Stange, NF-κB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium, *Infect. Immun.* 72 (2004) 5750–5758.
- [9] K. Ogushi, A. Wada, T. Niidome, N. Mori, K. Oishi, T. Nagatake, A. Takahashi, H. Asakura, S. Makino, H. Hojo, Y. Nakahara, M. Ohsaki, T. Hatakeyama, H. Aoyagi, H. Kurazono, J. Moss, T. Hirayama, Salmonella enteritidis FliC (flagella filament protein) induces human beta-defensin-2 mRNA production by Caco-2 cells, *J. Biol. Chem.* 276 (2001) 30521–30526.
- [10] A.M. McDermott, R.L. Redfern, B. Zhang, Y. Pei, L. Huang, R.J. Proske, Defensin expression by the cornea: multiple signalling pathways mediate IL-1β

- stimulation of hBD-2 expression by human corneal epithelial cells, *Invest. Ophthalmol. Vis. Sci.* 44 (2003) 1859–1865.
- [11] W.O. Chung, S.R. Hansen, D. Rao, B.A. Dale, Protease-activated receptor signaling increases epithelial antimicrobial peptide expression, *J. Immunol.* 173 (2004) 5165–5170.
  - [12] X. Wang, Z. Zhang, J.P. Louboutin, C. Moser, D.J. Weiner, J.M. Wilson, Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2, *FASEB J.* 17 (2003) 1727–1729.
  - [13] S. Krisanaprakornkit, J.R. Kimball, B.A. Dale, Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family, *J. Immunol.* 168 (2002) 316–324.
  - [14] A. Wada, K. Ogushi, T. Kimura, H. Hojo, N. Mori, S. Suzuki, A. Kumatori, M. Se, Y. Nakahara, M. Nakamura, J. Moss, T. Hirayama, *Helicobacter pylori*-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB, *Cell Microbiol.* 3 (2001) 115–123.
  - [15] K. Wehkamp, L. Schwichtenberg, J.M. Schroder, J. Harder, *Pseudomonas aeruginosa*- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is controlled by NF-kappaB and AP-1, *J. Invest. Dermatol.* 126 (2006) 121–127.
  - [16] Y. Tsutsumi-Ishii, I. Nagaoka, NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation, *J. Leukocyte Biol.* 71 (2002) 154–162.
  - [17] S. Maxson, T. Yamauchi, Acute otitis media, *Pediatr. Rev.* 17 (1996) 191–195, quiz 196.
  - [18] B.W. Gibson, W. Melaugh, N.J. Phillips, M.A. Apicella, A.A. Campagnari, J.M. Griffiss, Investigation of the structural heterogeneity of lipooligosaccharides from pathogenic *Haemophilus* and *Neisseria* species and of R-type lipopolysaccharides from *Salmonella typhimurium* by electrospray mass spectrometry, *J. Bacteriol.* 175 (1993) 2702–2712.
  - [19] A.R. Foxwell, J.M. Kyd, A.W. Cripps, Nontypeable *Haemophilus influenzae*: pathogenesis and prevention, *Microbiol. Mol. Biol. Rev.* 62 (1998) 294–308.
  - [20] T.F. Murphy, Bacterial otitis media: pathogenetic considerations, *Pediatr. Infect. Dis. J.* 19 (2000) S9–S15, discussion S15–S16.
  - [21] T. Shuto, H. Xu, B. Wang, J. Han, H. Kai, X.X. Gu, T.F. Murphy, D.J. Lim, J.D. Li, Activation of NF-kappa B by nontypeable *Haemophilus influenzae* is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha /beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells, *Proc. Natl. Acad. Sci. USA* 98 (2001) 8774–8779.
  - [22] J.C. Chow, D.W. Young, D.T. Golenbock, W.J. Christ, F. Gusovsky, Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction, *J. Biol. Chem.* 274 (1999) 10689–10692.
  - [23] H.Y. Lee, T. Takeshita, J. Shimada, A. Akopyan, J.I. Woo, H. Pan, S.K. Moon, A. Andalibi, R.K. Park, S.H. Kang, S.S. Kang, R. Gellibolian, D.J. Lim, Induction of beta defensin 2 by NTHi requires TLR2 mediated MyD88 and IRAK-TRAF6-p38MAPK signaling pathway in human middle ear epithelial cells, *BMC Infect. Dis.* 8 (2008) 87.
  - [24] H.Y. Lee, A. Andalibi, P. Webster, S.K. Moon, K. Teufert, S.H. Kang, J.D. Li, M. Nagura, T. Ganz, D.J. Lim, Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*, *BMC Infect. Dis.* 4 (2004) 12.
  - [25] S.K. Moon, J.I. Woo, H.Y. Lee, R. Park, J. Shimada, H. Pan, R. Gellibolian, D.J. Lim, Toll-like receptor 2-dependent NF-kappaB activation is involved in nontypeable *Haemophilus influenzae*-induced monocyte chemotactic protein 1 up-regulation in the spiral ligament fibrocytes of the inner ear, *Infect. Immun.* 75 (2007) 3361–3372.
  - [26] S.J. Barenkamp, E. Leininger, Cloning, expression, and DNA sequence analysis of genes encoding nontypeable *Haemophilus influenzae* high-molecular-weight surface-exposed proteins related to filamentous hemagglutinin of *Bordetella pertussis*, *Infect. Immun.* 60 (1992) 1302–1313.
  - [27] G. Diamond, V. Kaiser, J. Rhodes, J.P. Russell, C.L. Bevins, Transcriptional regulation of beta-defensin gene expression in tracheal epithelial cells, *Infect. Immun.* 68 (2000) 113–119.
  - [28] M.A. Suico, Z. Lu, T. Shuto, T. Koga, T. Uchikawa, H. Yoshida, K. Matsuzaki, M. Nakao, J.D. Li, H. Kai, The regulation of human beta-defensin 2 by the ETS transcription factor MEF (myeloid Elf-1-like factor) is enhanced by promyelocytic leukemia protein, *J. Pharmacol. Sci.* 95 (2004) 466–470.
  - [29] L.O. Bakaletz, S.J. Barenkamp, Localization of high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* by immunoelectron microscopy, *Infect. Immun.* 62 (1994) 4460–4468.
  - [30] C.S. Berenson, T.F. Murphy, C.T. Wrona, S. Sethi, Outer membrane protein P6 of nontypeable *Haemophilus influenzae* is a potent and selective inducer of human macrophage proinflammatory cytokines, *Infect. Immun.* 73 (2005) 2728–2735.
  - [31] H. Ishinaga, H. Jono, J.H. Lim, S.M. Kweon, H. Xu, U.H. Ha, H. Xu, T. Koga, C. Yan, X.H. Feng, L.F. Chen, J.D. Li, TGF-beta induces p65 acetylation to enhance bacteria-induced NF-kappaB activation, *EMBO J.* 26 (2007) 1150–1162.
  - [32] N. Miyamoto, L.O. Bakaletz, Selective adherence of non-typeable *Haemophilus influenzae* (NTHi) to mucus or epithelial cells in the chinchilla eustachian tube and middle ear, *Microb. Pathog.* 21 (1996) 343–356.
  - [33] T.H. Flo, L. Ryan, E. Latz, O. Takeuchi, B.G. Monks, E. Lien, O. Halaas, S. Akira, G. Skjak-Braek, D.T. Golenbock, T. Espevik, Involvement of toll-like receptor (TLR) 2 and TLR4 in cell activation by mannuronic acid polymers, *J. Biol. Chem.* 277 (2002) 35489–35495.
  - [34] R. Adamo, S. Sokol, G. Soong, M.I. Gomez, A. Prince, *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5, *Am. J. Respir. Cell Mol. Biol.* 30 (2004) 627–634.