Regulation of *Mycoplasma pneumoniae* Lysate-Induced IL-8 Expression in Human Airway Epithelial Cells

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Regulation of \textit{Mycoplasma Pneumoniae} Lysate-Induced IL-8 Expression in Human Airway Epithelial Cells

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ABSTRACT

Regulation of *Mycoplasma Pneumoniae* Lysate-Induced IL-8 Expression in Human Airway Epithelial Cells

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(Directed by Professor Kyu-Earn Kim)

The potential role of *Mycoplasma pneumoniae* infection in the pathogenesis of asthma has been suggested with accumulating evidence. Interleukin (IL)-8 plays a central role in the coordination and persistence
of the inflammatory process in the chronic inflammation of the airways in asthma. I investigated the mechanism whereby *M. pneumoniae* lysate (MPL) triggers IL-8 release from human airway epithelial cells.

Chemical inhibitors were pretreated before addition of MPL for promoter activity and protein synthesis of IL-8. The transcriptional activity of IL-8 promoter was analysed by mutational and deletional analysis and electrophoretic mobility shift assay (EMSA).

Stimulation of H292 cells, human airway epithelial carcinoma cell line, with MPL resulted in a time- and concentration-dependent induction of IL-8 transcription and protein synthesis. IL-8 promoter deletion analysis indicated that position -132 to +41 was essential for MPL-induced IL-8 transcription, and mutants with substitutions in activator protein (AP)-1, nuclear factor (NF)-IL6 and NF-kB binding sites revealed a requirement for NF-kB and NF-IL6, but not AP-1, in MPL-induced activation of the
IL-8 promoter. The DNA-binding activities of NF-κB and NF-IL6 were induced by MPL, as determined by EMSA. The chemical inhibition of extracellular signal regulated kinase (ERK) attenuated MPL-induced transcriptional activity and protein synthesis.

I conclude that MPL-induced IL-8 expression is regulated by transcriptional activation of NF-κB and NF-IL6 coordinating with the ERK pathway in human airway epithelial cells.

Key words: airway epithelial cell, ERK, *Mycoplasma pneumoniae*, IL-8, NF-κB, NF-IL6
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**I. INTRODUCTION**

*Mycoplasma pneumoniae* is a prokaryotic human pathogen belonging to the Mollicute class of organisms. It represents the smallest self-
replicating organism, in both cellular dimensions and genome size, that is capable of cell-free existence. It is characterized by spindle-shape, size of 1 to 2 µm in length and 0.1 to 0.2 µm in width and absence of a cell wall. It is an extracellular pathogen, residing in mucosal surfaces of the respiratory and genital tracts.

In 1898, Nocard and Roux isolated the first *Mycoplasma* organism from cattle with contagious pleuropneumonia. *M. pneumoniae*, originally called the Eaton agent, was eventually first isolated in tissue culture from a patient with primary atypical pneumonia in 1944. The Eaton agent was subsequently cultured on cell-free medium and its taxonomy was proposed to be *M. pneumoniae* in 1963.

*M. pneumoniae* are primarily mucosal pathogen, living a parasitic existence in close association with epithelial cells of, exclusively, humans. There are in short three steps of the pathogenesis of diseases
caused by *M. pneumoniae*. The first is cytadherence, the next step is intracellular localization, and the last is cytotoxicity and inflammation. Cytadherence, attachment of *M. pneumoniae* to the respiratory epithelium, is a prerequisite for later events that develop disease. It needs the attachment organelle which consists of a specialized tip structure with a central core of a dense rod-like central filament surrounded by a lucent space. The tip structure is actually a network of adhesins, interactive proteins, and adherence accessory proteins. Intracellular localization, which fuse with and enter host cells, is different from phagocytosis, and makes possible latent or chronic disease states. Until now, the extent to which *M. pneumoniae* invades and replicates intracellularly is not known. Both cytadherence and intracellular localization can initiate local cytotoxic and inflammatory events. Cytotoxicity represents deterioration of cilia in the respiratory epithelium, both structurally and functionally.
Inflammatory events include chemotactic migration to the site of infection, activation of numerous inflammatory cells, production of immunoglobulins, and release of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), various interleukins (including interleukin-1β [IL-1β], IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-18), vascular endothelial growth factor (VEGF) and nitric oxide (NO) in vivo or in vitro.8-18

It is a usual cause of upper and lower respiratory tract infections that typically manifest as pharyngitis, tracheobronchitis, community-acquired pneumonia, and more recently exacerbation and inception of asthma.19 As many as 25% of persons infected with M. pneumoniae may experience extrapulmonary complications, including skin rashes, arthritis, anemia, thrombocytopenia, myocarditis, encephalitis, Guillain-Barre syndrome, et al., after onset of or even in the absence of respiratory
illness.\textsuperscript{20}

In these pulmonary manifestations, the lungs are often infiltrated by neutrophils and lymphocytes. In mouse model infected with \textit{M. pneumoniae}, airway lumina also represent neutrophilia.\textsuperscript{21, 22} This neutrophilic response, which may contribute to tissue damage, is the main response of respiratory infection with \textit{M. pneumoniae}.

Interleukin (IL)-8 is one of the most important neutrophil chemotactic and activating peptide. Patients with \textit{M. pneumoniae} lung infections have increased IL-8 in their bronchoalveolar lavage fluid (BALF), and the levels of IL-8 correlate with higher bacterial loads and impairment of gas exchange.\textsuperscript{23} Also, we previously demonstrated that both \textit{M. pneumoniae} infection and lysate treatment induce IL-8 production in human airway epithelial cells.\textsuperscript{17, 18}

Stimulus-dependent activation of IL-8 gene is mainly regulated at the
transcriptional level. Several studies have found that a sequence spanning nucleotides -1 to -133 within the 5′ flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of the gene. This promoter element contains a nuclear factor (NF)-kB element that is required for activation in all cell types as well as activator protein (AP)-1 and CCAAT enhancer-binding factor (C/EBP or NF-IL6) sites. Numerous functional studies have proven that activation of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, induces phosphorylation and increases the activity of transcription factors including AP-1, NF-IL6 or NF-kB in the IL-8 promoter.

To the author’s knowledge, no study has addressed the issue to investigate the intracellular signaling pathway of IL-8 expression induced by *M. pneumoniae* lysate (MPL). We therefore aimed to explore whether
the MPL synergistically increases IL-8 in human airway epithelial cells and whether the activities of NF-kB and NF-IL6 regulate IL-8 gene expression by coordinating with the ERK pathway.
II. MATERIALS AND METHODS

1. Cells

The human airway epithelial carcinoma cell line H292 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin (GibcoBRL, Grand Island, MD, USA). H292 cells were grown in sterile T-75 tissue culture flasks and maintained at 37°C in an incubator with 5% CO₂.

2. Preparation of *M. pneumoniae* lysate

To prepare consistent *M. pneumoniae* stock for use in experiments, strain 15531 (American Type Culture Collection; Rockville, MD, USA) was grown in Chanock modified medium.5 *M. pneumoniae* cells in
exponential growth phase were aliquoted and centrifuged at 20,000 g for 30 min. The pellet was washed and resuspended with phosphate-buffered saline solution. A cell extract was prepared by sonication, and the lysate was cleared by centrifugation. It resulted in a negative E-toxate assay for endotoxin. Polymyxin B sulfate (Sigma, St. Louis, MO, USA) at a concentration of 10 μg/mL was used to inhibit endotoxin activity in MPL and did not inhibit significantly IL-8 release in response to MPL. Additionally, endotoxin content was quantitatively measured by limulus amebocyte lysate QCL-1000 (Bio Whittaker, Walkersville, MD, USA), and the endotoxin level was 0.026 endotoxin units per 1 μg/mL of MPL. The minimum detection limit of IL-8 was 15.6 pg/mL.

3. Reagents and antibodies

Specific antibodies (Abs) against phosphorylated (p)-p44/42, p-p38, p-
JNK and control Abs were purchased from Cell Signaling Technology (New England Biolabs, Ipswich, MA, USA). PD98059, U0126, SB202190, JNK inhibitor II and curcumin were purchased from Calbiochem (La Jolla, CA, USA).

4. Measurement of human interleukin-8 protein

Cells grown on six-well plates were stimulated with MPL at 37°C by a series of doses and induction times, as specified. The supernatants were aspirated and stored at -70°C until assayed. IL-8 levels in the culture supernatants were determined by a specific ELISA against human IL-8 according to the manufacturer’s instructions (R&D system, Minneapolis, MN, USA).

5. Reverse transcriptase-polymerase chain reaction
Total RNA was isolated from H292 cells cultured with MPL over specified induction times using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). For synthesis of first-strand cDNA, total RNA (2 µg) was resuspended in 12 µL diethylpyrocarbonate-treated water with random hexamers. The mix was incubated at 65°C for 10 min and cooled on ice. First-strand buffer (100 mM Tris-HCl, pH 8.4, 250 mM KCl), 10 mM dNTP mix, 0.1 M dithiotreitol (DTT) and 25 U Superscript II RT (Gibco) were then added to the samples. The samples were incubated at 42°C for 60 min, and the reverse transcriptase was heat-inactivated at 70°C for 10 min. PCR conditions for human IL-8 PCR assay included an initial denaturation step at 94°C for 5 min; 30 cycles of 94°C (30 s), 55°C (30 s), 72°C (30 s); and a final extension step of 72°C for 5 min. The primer sequences were as follows: human IL-8: 5’-AGA TAT TGC ACG GGA GAA-3’ (sense) and 5’-GAA ATA AAG GAG AAA CCA-3’
antisense); and GAPDH: 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). The final PCR products were run on ethidium bromide-stained 1.5% agarose gels in 0.5 x TAE buffer.

6. Western immunoblot analysis

Cells grown on dishes were stimulated with MPL at 37°C in serum-free RPMI 1640 for specified induction times. Briefly, after treatment, cells were washed with cold 1 x PBS, and lysed for 40 min on ice in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol and 0.5% NP-40) containing 1 mM DTT and 0.1 mM sodium vanadate. Samples were mixed with SDS-PAGE buffer containing β-mercaptoethanol, heated at 95°C for 3 min and ultracentrifuged before loading onto polyacrylamide gels (5% stacking,
10% resolving gel). After electrophoresis, separated proteins were transferred onto a nitrocellulose membrane for 2 h at 200 mA. Membranes were blocked with tetrabromosulphophthalein (TBS) with 5% dry milk for 1 h and incubated with specific Abs in TBS/0.1% Tween and 5% dry milk overnight at 4°C. After washing and incubating with secondary Abs, an ECL detection system was used for protein detection (New England Biolabs).

7. Construction of interleukin-8 promoter fragments and site-directed mutations

The -1322/+42 fragment of full-length human IL-8 promoter was subcloned into a pGL3 luciferase reporter vector (Promega, Madison, WI, USA). Six deletional constructs were made to identify important regulatory elements on the hIL-8 promoter related to MPL-induced gene
expression.

Site-directed mutagenesis of the AP-1-, NF-IL6- or NFkB-binding site in the -132/+42 hIL-8 promoter was introduced by PCR with mutagenic primers to obtain mutAP-1, mutNF-IL6 and mutNF-kB luciferase plasmids using the GeneTailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA, USA). The primer sequences were as follows: mutAP-1: 5’-GAA TTC GCC CTT GTG TGA TAG ATC TGG TTT GCC C-3’ (sense) and 5’-TCA CAC AAG GGC GAA TTC CAG CAC ACT GGC-3’ (antisense); mutNF-IL6: 5’-CTG AGG GGA TGG GCC ATC GAG TGA AAA TCG TGG A-3’ (sense) and 5’-ATG GCC CAT CCC CTC AGG GCA AAC CTG AGT-3’ (antisense); and mutNFkB: 5’-GGC CAT CAG TTG CAA ATC GTT AAC TTT CCT CTG A-3’ (sense) and 5’-ACG ATT TGC AAC TGA TGG CCC ATC CCC TCA-3’ (antisense).
To measure transcription activity in the IL-8 promoter, cells were transfected with the relevant reporter plasmids by a liposome-mediated technique (Invitrogen). Transfection efficiency was assessed by co-transfection with pSVβ-galactosidase. After treatment with MPL, cells were harvested and analysed for luciferase and β-galactosidase activities (luciferase and β-galactosidase enzyme assay systems, Promega) by a luminometer.

8. Electrophoretic mobility shift assays

Nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). Electrophoretic mobility shift assays (EMSAs) were performed using a nuclear extract binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), and biotin-labelled probes incubated at room temperature for 20 min. The sequences
of the sense strands of the oligonucleotides used for EMSA were as follows: NF-IL6: 5′-TGC AGA TTG CGC AAT CTG CA-3′ (Santa Cruz Biotechnology Inc.) and NF-kB: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega). The protein–DNA complexes were electrophoresed on 6% polyacrylamide gels at 4°C in 0.5 x TBE and transferred onto a nylon membrane. The UV cross-linked membrane was then exposed to an X-ray film for detection by the Lightshift chemiluminescent EMSA kit (Pierce).

9. Statistical analysis

Data were expressed as mean ± SEM from three to six independent experiments. Statistical analysis comparing treatment and control groups was assessed by Student’s t-test, and $p < .05$ was considered to be significant.
III. RESULTS

1. *M. pneumoniae* lysate induces interleukin-8 expression in H292 cells

MPL significantly enhanced IL-8 production in a time- and dose-dependent manner in H292 cells. Based on 10 µg/mL MPL treatment, IL-8 production was increased by 7.1 ± 2.6, 326.7 ± 77.2, 1255.0 ± 33.8, 1276.3 ± 34.2 and 1375.6 ± 4.3 pg/mL according to 1, 2, 6, 10 and 24 h, respectively (Fig. 1A). The levels of IL-8 mRNA transcripts were also stimulated by MPL in a time-dependent manner (Fig. 1B).
Figure 1. Effects of *M. pneumoniae* lysate (MPL) on IL-8 protein and mRNA. A: The human epithelial carcinoma line H292 cells were
stimulated with MPL dependent on the times and doses. The IL-8 concentrations of the supernatants from cultures were measured by ELISA. \( ^*p < .01 \) vs. control and 2\( \mu \)g/mL MPL, \( ^\dagger p < .001 \) vs. control, \( ^\ddagger p < .001 \) vs. control and 2\( \mu \)g/mL MPL. All data represent the mean ± SEM of at least three independent experiments performed in duplicate. B: The RNAs from H292 cells treated with MPL for the indicated times were harvested and used for RT-PCR. Similar results were obtained in two other independent experiments.
2. Effect of extracellular signal-regulated kinase on *M. pneumoniae* lysate-induced interleukin-8

To determine whether MAPKs could regulate IL-8 expression, we measured the activation of ERK, p38 and JNK MAPKs by chemical inhibitor treatment and antiphosphorylated ERK (p44/42), p38 and JNK protein Abs. First, chemical inhibition of ERK activation by PD98059 and U0126 significantly decreased MPL-induced IL-8 protein synthesis. However, inhibitions of p38 and JNK by SB202190 and JNK inhibitor II failed to effectively attenuate IL-8 protein abundance in H292 cells. In case of U0126, IL-8 protein was decreased to over 94% and PD98059 also inhibited over 93% compared with MPL alone (Fig. 2A). Second, in the result of Western blot analysis, resting cells before exposure exhibited extremely low levels of phosphorylated p44/42 MAPK. Exposure to MPL resulted in a distinct increase in phosphorylated p44/42
MAPK levels in H292 cells. After a 5-min exposure to MPL, phosphorylation of p44/42 MAPK was detected and continued for a period of 60 min post-exposure. On the other hand, phosphorylations of JNK were not activated by MPL, as can be seen in Fig. 2B.
Figure 2. Requirement of mitogen-activated protein kinase (MAPK) for *M. pneumoniae* lysate (MPL)-induced IL-8 expression. A: Effect of pretreatment with inhibitors of MAPK on MPL-induced IL-8 expression.
Protein of cell supernatants was measured by ELISA. *$p < .0001$ vs. MPL alone. B: Phosphorylation assays of MAPK activities. Cells treated with MPL were harvested at the indicated time points, lysed and equal amounts of cell extracts were resolved on 10% acrylamide gels and then subjected to Western blot analysis. All data represent the mean ± SEM of at least three independent experiments performed in duplicate. ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase.
3. Production of interleukin-8 promoter fragments and promoter activity in a concentration-dependent manner

H292 cells were transiently transfected with IL-8 promoter constructs, -1322, -546, -132, -102, -84, -55 (Fig. 3A), and incubated with MPL (10µg/mL) for 24 h. Their luciferase activities (normalized by β-galactosidase expression) were 2.9, 3.5, 6.6, 0.9 and 0.2 (Fig. 3B). However, the MPL-induced luciferase expression from promoter sequences that included a deletion under -102 was substantially reduced and no longer significant. In summary, the construct -132 was more expressive than -1322, -546, -102 and -55 on MPL-stimulated luciferase production. Through the treatment with MPL in a concentration-dependent manner, the capacity of MPL to activate the transcription of a luciferase reporter gene driven by the IL-8 promoter (-132/+41) was measured. As shown in Fig. 3C, IL-8 promoter activities were
significantly induced by MPL compared with the control.
Figure 3. Deletional analysis and transcriptional activity of IL-8 promoter. A: Schematic diagram of IL-8 promoter fragments. B: Effect of *M. pneumoniae* lysate (MPL) on the transcriptional activities of 5′-upstream deletion fragments of IL-8 promoter. Cells transfected with fragments of an IL-8 promoter subcloned into a luciferase reporter gene were stimulated with MPL, and luciferase activity was measured by a luminometer. *p < .001, †p < .01, ‡p < .005 vs. -1322/+41 IL-8. C: Cells
transfected with -132/+41 IL-8 were stimulated with different MPL concentrations. \*p < .05, \†p < .0001 vs. control. All data represent the mean ± SEM of at least three independent experiments performed in duplicate. AP, activator protein; NF, nuclear factor; JNK, c-Jun NH2-terminal kinase; IRF, interferon regulatory factor; HNF, hepatocyte nuclear factor; GRE, glucocorticoid response element.
4. Implication of the nuclear factor-interleukin 6 and nuclear factor-
kB sites in interleukin-8 promoter activity induced by *M. pneumoniae* lysate

Based on above results, the construct -132/+41 was determined as a wild-type (WT) IL-8 promoter for identification of MPL-activated transcription factors. The IL-8 promoter is widely characterized and involved several binding sites for transcription factors, including the AP-1 (-126/-120), NF-IL6 (-94/-81) and NF-kB (-80/-70) sites. To test the implication of each site related to MPL stimulation, we used IL-8 promoter constructs containing site-specific mutations (Fig. 4A). As shown in Fig. 4B, the NF-IL6 site mutation reduced MPL-induced IL-8 promoter activity by 97%. In case of the NF-kB site mutation, IL-8 promoter activity by MPL was completely abolished. In contrast, the construct with a mutated AP-1 site produced more IL-8 promoter
activation compared with the -132WT. To confirm the role of NF-κB in the protein level, we pretreated NF-κB inhibitor, curcumin (known as a NF-κB and AP-1 inhibitor)\textsuperscript{16} dependent on concentrations before MPL treatment (10 µg/mL). Curcumin significantly inhibited IL-8 protein compared with MPL alone (Fig. 4C).
Figure 4. Implication of activator protein (AP)-1-, nuclear factor (NF)-IL6- and NF-kB-binding sites in IL-8 promoter activity stimulated by M. pneumoniae lysate (MPL). A: Schematic diagram of -132/+41 IL-8 promoter fragments having various mutations in either AP-1, NF-IL6 or NF-kB transcription factor-binding site. B: Contributions of each site in MPL-induced IL-8 promoter activation. Cells transfected with -132/+41 WT, mutated (mut) AP-1, mut NF-IL6 and mut NF-kB promoter
constructs were stimulated with MPL before measurement of luciferase activity. *p < .0001 vs. -132WT. C: Effect of pretreatment with NF-kB (or AP-1) inhibitor, curcumin on MPL-induced IL-8 expression. Protein from cell supernatants was measured by ELISA. *p < .0001 vs. MPL alone. All data represent the mean ± SEM of at least three independent experiments performed in duplicate.
5. Effect of *M. pneumoniae* lysate on nuclear factor-interleukin6- and nuclear factor-kB-binding activity

Besides the previous result of involvement of the NF-IL6 and the NF-kB by chemical inhibitor treatments or their specific mutations, we studied whether NF-kB and NF-IL6 DNA binding was induced by MPL. As shown in Figs 5A and B, exposure of H292 cells to MPL (10 and 50 µg/mL) for 12 h resulted in NF-kB and NF-IL6 DNA-binding activity in a dose-dependent manner. Figure 5C illustrates that MPL-induced NF-kB DNA-binding activity and pretreatment with each U0126 (50 mM) or curcumin (50 mM) interfered with its binding activity. Also, the pretreatment with U0126 (50 mM) partially inhibited its binding activity (Fig. 5D).
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**Figure 5.** Effect of *M. pneumoniae* lysate (MPL) on the activation of nuclear factor (NF)-kB and NF-IL6 DNA-binding activities in a dose-dependent manner (A, B). Electrophoretic mobility shift assay demonstrates MPL-induced binding of nuclear proteins to an oligonucleotide encoding the NF-kB (C) or NF-IL6 (D). Similar results were obtained in two other independent experiments.
IV. Discussion

*M. pneumoniae* is a common cause of respiratory disease, especially in children and young adults, in the infection of which neutrophils have been considered to be an important contributor to the pathogenesis. In an in vivo study of BALB/c mice infected with *M. pneumonia*, the main inflammatory response was by neutrophils.\(^{22}\) Therefore the potent neutrophil chemoattractant, IL-8, has been investigated to play a critical role in the pathogenesis of these infections. It has been suggested that IL-8 is elevated in pleural fluid and BALF from patients with *M. pneumoniae* infection.\(^{14,23}\) Interestingly, these neutrophilic inflammation has been proposed to be associated with airway hyperresponsiveness (AHR).\(^{22}\) Recent studies show that *M. pneumoniae*-infected BALB/c mice developed significant airway obstruction and manifested
significantly elevated AHR as well.\(^{21, 28}\) On the other hand, there is accumulating evidence linking \textit{M. pneumoniae} infection and the inception, chronicity of asthma, and especially exacerbation as well as single wheezing episode.\(^{29, 30}\) \textit{M. pneumoniae}-induced asthma exacerbations are known to be mainly characterized by neutrophilic inflammation, chiefly involving IL-8.\(^{31}\) Besides, it has been paid attention because patients with noneosinophilic asthma have increased number of neutrophils and IL-8 levels in the airways.\(^{32}\) These phenotypes of asthma are common and might be poorly treated by conventional therapies.\(^{33}\) Accordingly, macrolide antibiotics, which have been widely used in the treatment of infections caused by bacteria such as \textit{M. pneumoniae}, has been proposed as a treatment option for asthma, especially for acute asthma exacerbation and refractory asthma.\(^{34, 35}\) For these reasons, the author focused on IL-8 expression induced by MPL.
The present study shows that MPL can increase IL-8 protein depending on the times and concentrations in human airway epithelial cells. In addition, the author found that MPL induced a time-dependent expression of IL-8 mRNA. Those correspond well with the previous study.\textsuperscript{17} In the present study, the author concentrated on the regulation of IL-8 expression induced by MPL inside the airway epithelial cells. IL-8 gene expression in airway epithelial cells requires at least one or two MAPK pathways.\textsuperscript{36} Because MAPK pathways have been shown to regulate various cytokine expressions in airway epithelial cells,\textsuperscript{17,37,38} the author investigated the requirements of ERK, p38 and JNK for transcription from IL-8 promoter and IL-8 protein expression induced by MPL. In this study, it has been shown that inhibition of ERK by pretreatment with chemical inhibitors PD98059 and U0126 almost abolished MPL-induced transcription and protein abundance of IL-8. On
the other hand, inhibitions of both p38 and JNK by specific chemical inhibitor for each failed to reduce the transcription and protein expression of IL-8 by MPL. Furthermore, only ERK signaling intermediates (with the exception of p38 and JNK) are phosphorylated and detected by anti-phosphorylated p44/42. These results suggest that the ERK pathway more strongly contributes to IL-8 expression by MPL than p38 or JNK.

The next step was the transcriptional regulation in the nucleus. Based on a well-characterized IL-8 promoter sequence, analysis of promoter deletions indicated that the region from -132 to +41 was an essential part for IL-8 expression and the region under -102 did not affect the promoter activity of IL-8. This promoter element from -132 to -102 contains AP-1, NF-IL6 and NF-kB, and IL-8 gene expression is mainly regulated by these elements. For mutation of different binding sites for transcription factors, the author used the fragment from -132 to +41 of IL-8 promoter
as a wild type. The results of site-directed mutational analysis show that NF-IL6 and NF-kB binding sites are essential for MPL-induced activation of the IL-8 promoter. However, mutation of AP-1 binding site reduced the IL-8 transcriptional activity without statistical significance. In short, mutation of the IL-8 promoter AP-1 site showed the trend to reduce the transcriptional activity, whereas mutation of the IL-8 promoter NF-kB and NF-IL6 sites blocked the response to MPL by 100% and 97%. Therefore, this deletional and mutational analysis demonstrates that NF-kB and NF-IL6 are necessary for the induction of IL-8 transactivation in response to MPL. These results correspond well with those of the earlier study which reported that maximal activation of IL-8 has been shown to be regulated by the binding of both NF-kB and NF-IL6 transcription factors. NF-IL6 has been shown to bind weakly to the IL-8 promoter, but when NF-kB is bound to the adjacent site, NF-IL6 shows strong
NF-κB plays a critical role in immune and inflammatory responses and many of the stimuli that increased inflammation in asthmatic airways resulted in the activation of NF-κB, particularly proinflammatory cytokines. However, considering the result of deletional analysis that -121/+41 (deleted AP-1-binding site) totally lost IL-8 promoter activity, the function of AP-1 could not be excluded in MPL-induced IL-8 promoter activation. These findings support the previous results that AP-1 appears to act as a basal-level enhancer element of IL-8 transcription in airway epithelial cells, which can be increased by powerful stimuli such as TNF-α via NF-κB. Also, the author examined whether chemical inhibitors of NF-κB blocked IL-8 protein synthesis by MPL. Pretreatment with curcumin completely abrogated protein abundance of MPL-induced IL-8. Additionally, current data showed that MPL stimulation led to the activation of both NF-IL6
and NF-kB DNA binding, as shown by EMSA. NF-IL6 binding to the IL-8 promoter is not affected by inhibitor treatments of NF-kB, and instead is slightly disturbed by U0126. Although the author was unable to conduct an inhibition test of NF-IL6 with the few existing inhibitors available commercially, the author believes that NF-IL6 also has a key role in mediating MPL-stimulated IL-8 expression by gel shift assay and mutational analysis. In contrast, the binding activity of NF-kB to DNA was totally abolished by U0126 and curcumin. These data demonstrate that the activation of NF-kB and NF-IL6 induces IL-8 promoter activation.

The author has to point out in the reporter gene assay that the region from -132 to +41 was more expressive than -1322/+41 and -546/+41. This result can make a speculation that the promoter region from -1322 to -132 may play an inhibitory role. Further studies on the inhibitory or
regulatory action of this region are clearly needed.

The limitation of the present study is the use of MPL instead of live *M. pneumoniae*, because it is possible that there are some differences between them. The author demonstrated in the previous study that *M. pneumoniae* live infection induces IL-8 expression depending on times and independent of *M. pneumoniae* loads.\textsuperscript{18} In case of MPL, it induced IL-8 expression depending on both times and the doses. The first difference might be whether treatment doses of *M. pneumoniae* itself or MPL affect IL-8 expression. And then, it has been reported that IL-1β induction is strongly inhibited when airway epithelial cells are infected with *M. pneumoniae*, preincubated with anti-P1 antibodies, which inhibit cytadherence of *M. pneumoniae*.\textsuperscript{16} On the other hand, it has been shown that IL-8 production is not inhibited when incubation of airway epithelial cells with anti-P1 antibodies before MPL treatment is carried out.\textsuperscript{17} The
second difference might be whether anti-P1 antibodies affect IL-8 expression. Cytadherence of *M. pneumoniae* to the respiratory epithelium is regarded as an essential primary step in tissue colonization and subsequent disease pathogenesis. The tip structure, which is essential for cytadherence, includes P1 adhesin, P30, P90, P65, HMW1, HMW2, HMW3, HMW4, HMW5, and so on. Among these proteins, P1 adhesin has been known to play an important role in this cytadherence. It has been suggested that P1 blockade does affect *M. pneumoniae* infection-induced IL-8 expression, but not MPL-induced IL-8 expression. Cytadherence takes place extracellularly. The difference caused by treatment dose also occurs outside cells. The author concentrated on the intracellular mechanisms of the regulation of *M. pneumoniae*-induced IL-8 expression in this study. It is possible that there are some differences in the intracellular signaling between live infection and lysate treatment.
Further studies are underway to investigate the regulation of *M. pneumoniae*-induced IL-8 expression on the cell surface associated with toll like receptors, cytadherences, and so on.

Results of the present study are similar to those of previous studies regarding other stimulants in different type of cells. It has been shown that an exposure to the respiratory syncytial virus (RSV) increases IL-8 expression by modulating NF-kB and activating ERK kinase in airway epithelial cells.\(^{43, 44}\) However, these studies have not proven any in the study involvement of or interactions with other transcription factors. Another study also has reported that human intrahepatic biliary epithelial cells in innate immunity produces IL-8 induced by lipopolysaccharide (LPS) via the NF-kB and MAPK signaling pathways.\(^{45}\) This study too has not shown any association with other transcription factors and it also has not clarified which MAPK is involved. One recent study is in need
for introduction here, the authors of which investigated that German cockroach extract with protease activity-induced IL-8 expression is controlled by NF-kB and NF-IL6 joining with the ERK pathway in airway epithelial cells. These results are in close agreement with those of the present study, except for the different type of stimulant. Therefore, these studies may provisionally suggest that IL-8 expression induced by different stimulants is modulated by NF-kB and MAPK pathway.

In conclusion, the author demonstrated that the mechanism by which MPL induces IL-8 expression is regulated by the transcriptional activation of NF-kB and NF-IL6. Also, the present data have shown that their MPL-induced transcriptional regulations are mediated by the ERK MAPK pathway (Fig. 6).
Figure 6. Model of nuclear factor (NF)-IL6 and NF-kB transactivation in *M. pneumoniae* lysate (MPL)-induced IL-8 expression. The activity of transcriptional factors, NF-IL6 and NF-kB is dependent on ERK of MAP kinases.
V. CONCLUSIONS

The author demonstrated that MPL increases IL-8 in human airway epithelial cells and these MPL-induced IL-8 expression requires activation of ERK. Also, our data have shown that ERK increases NF-IL6 and NF-kB activity to regulate IL-8 promoter activity in human airway epithelial cells.

Following researches could be needed to better understand MPL-induced IL-8 regulation, which toll-like receptor is mediated. Also, further studies are underway to confirm whether *M. pneumonias* infection does require the same signaling pathway as MPL does.
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ABSTRACT (IN KOREAN)

인체 기관지 상피세포에서 Mycoplasma pneumoniae에 의한
IL-8의 유전자 발현과 신호전달체계의 규명

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목적: Mycoplasma pneumoniae 는 기도 점막의 상피세포에서
증식하면서 호흡기 질환을 일으키며 기관지 천식의 발생이나
악화에 관여한다고 알려져 있다. IL-8 은 기도에서
염증부위로의 세포이동을 매개하는 chemokine 으로 호중구와 호산구에 대한 화학주성을 가지며 기관지 천식의 알레르기 염증 반응에 밀접한 관련을 가진다. 본 연구에서는 기관지 상피세포에 M. pneumoniae 항원을 자극했을 때 IL-8 유전자 발현과 신호전달경로를 밝히고자 하였다.

방법: M. pneumoniae 추출물을 기관지 상피 세포주인 H292 세포에 노출시켜 시간과 농도에 따른 IL-8 단백질 생성과 mRNA 발현을 측정하였다. IL-8 발현을 위한 transcriptional activity 는 IL-8 promoter contracts 를 제작하고 deletional analysis 와 mutational analysis 를 통해 각각 조사하였다. Mitogen-activated protein (MAP) kinase 의 연관성 여부와 활성화되는 전사인자들을 확인하기 위해 chemical inhibitor 를 처리하거나 electrophoretic mobility shift assay (EMSA) 방법을 이용하였다.
결과: H292 세포에 M. pneumoniae 항원을 노출시켰을 때 시간과 농도가 증가함에 따라 IL-8 단백질 생성과 mRNA 발현이 증가됨을 관찰할 수 있었다. 또한 ERK inhibitor 인 PD98059, U0126 을 전처치했을 때 IL-8 생성이 감소하였고, MAPK 활성 분석에서도 p44/42 MAPK 의 활성도가 증가되었다. 또한 luciferase assay 를 이용하여 deletional analysis 를 실시한 결과 IL-8 promoter 의 -132/+41 에 해당하는 부위가 IL-8 발현에 밀접한 연관이 있음을 알 수 있었으며, mutational analysis 와 chemical inhibitor 전처치를 통해 NF-IL6 와 NF-kB 의 translocation 을 확인하였으며 ERK inhibitor 전처치를 한 경우 이들이 translocation 이 감소함을 알 수 있었다.

결론: M. pneumoniae 항원은 기관지 상피 세포에서 ERK pathway 를 통해 IL-8 promoter 내 NF-IL6 와 NF-kB 를
활성화시키고 이들을 통해 IL-8 유전자 발현을 조절하는 것으로 사료된다.

핵심되는 말: *Mycoplasma pneumoniae*, 기관지 상피세포, IL-8, ERK, 전사인자