**Research Article**

**MKP1 regulates the induction of inflammatory response by pneumococcal pneumolysin in human epithelial cells**

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**Abstract**

The expression of proinflammatory cytokines represents an important host innate response during infections. The reduction of cytokine expression thus mediates impaired host defenses. We previously reported that pneumococcal pneumolysin is less potent in inducing inflammatory responses in human epithelial cells at the early stage of treatment. How this might occur in response to pneumolysin is still not clearly understood. Here, we show the expression of tumor necrosis factor-α (TNF-α) was reduced by MAPK phosphatase 1 (MKP1), expression of which was significantly increased in response to pneumolysin at the early stage of treatment. TNF-α expression was mediated in a time-dependent manner by p38 mitogen-activated protein kinase, activation of which is under the control of MKP1. Thus, this study reveals novel roles of pneumolysin in mediating MKP1 expression for the regulation of proinflammatory cytokine expression in a time-dependent manner.

**Introduction**

Cytokines are involved in host inflammatory responses and are synthesized by nearly all nucleated cells, including macrophages, mast cells and epithelial cells (Henderson et al., 1996). However, excessive inflammatory responses are harmful to the host as they cause severe tissue damage (Hersh et al., 1998). Tight control of inflammation is thus critical for host immune defenses and this can be achieved by balancing the expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and anti-inflammatory cytokines such as IL-4 and IL-10 (Dinarello, 2000; Yoo et al., 2010). The release of proinflammatory cytokines can be triggered by various bacterial products such as lipopolysaccharide and peptidoglycan (Henderson et al., 1996).

*Streptococcus pneumoniae* is a gram-positive bacterial pathogen that causes infections in humans, especially among young children, with high morbidity and mortality (Bluestone et al., 1992). To date, numerous virulence factors have been identified in *S. pneumoniae*, and a 53-kDa cytoplasmic protein pneumolysin has been shown to be a key virulence factor released during infection by the action of pneumococcal autolysin (Berry et al., 1989; Benton et al., 1995; Wheeler et al., 1999). Virtually all clinical isolates of *S. pneumoniae* encode the functional pneumolysin, which is a multifunctional protein that causes cytolysis and induces complement activation and the production of nitric oxide (Mitchell et al., 1991; Paton, 1996; Braun et al., 1999).

We previously reported that pneumolysin was less potent in inducing inflammatory responses in human epithelial cells at the early stage of treatment (Yoo et al., 2010). How such reduced induction might occur in response to pneumolysin is still not clearly understood. In the present study, we examine novel roles for pneumolysin in mediating TNF-α expression through the regulation of MAPK phosphatase 1 (MKP1). We found that pneumolysin induced the expression of TNF-α via the p38 mitogen-activated protein kinase (MAPK) pathway. Pneumolysin was also identified as the major factor involved in the reduction of TNF-α via enhanced MKP1 expression, suggesting a major role of MKP1 in inactivating p38 by dephosphorylation. These studies thus bring new insight into the signaling mechanism for the regulation of proinflammatory cytokines at the early stage of infection.
Materials and methods

Reagents

PD98059, SB203580 and Ro31-8220 were purchased from Calbiochem (Gibbstown, NJ). Polymyxin B was purchased from Sigma-Aldrich (St. Louis, MO).

Bacterial strains and culture conditions

Clinical isolates of S. pneumoniae wild-type (WT) strains D39, 6B, 19F and 23F were used in this study (Avery et al., 1979; Briles et al., 1992). D39 isogenic pneumolysin-deficient mutant (Ply mt) was developed through insertion–duplication mutagenesis as described previously (Berry et al., 1989). Bacteria were grown on chocolate agar plates at 37 °C in an atmosphere of 5% CO₂. Streptococcus pneumoniae strains were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract. Whole bacterial cells cultured in broth were harvested at 10 000 g for 20 min at 4 °C to obtain the pellet after overnight incubation. The bacterial pellet was suspended in phosphate-buffered saline, and the bacterial cell suspension was sonicated on ice three times at 150 W for 3 min at 5-min intervals as previously reported (Ha et al., 2007; Yoo et al., 2010). Residual intact cells were removed by centrifugation at 12 000 g for 20 min at 4 °C. The bacterial lysate was stored at −80 °C, and 5 μg mL⁻¹ lysate was used in all experiments as previously reported (Shin et al., 2010; Yoo et al., 2010).

Purification of pneumolysin

His₆ tag-fused pneumolysin was expressed and purified from an Escherichia coli strain, and residual lipopolysaccharide was removed by passage over End-X resin as previously described (Shin et al., 2010; Yoo et al., 2010). Unless specified, 100 ng mL⁻¹ dose of pneumolysin was used to treat cells in this study.

Cell culture

All media described below were supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), penicillin (100 U mL⁻¹) and streptomycin (0.1 mg mL⁻¹) (Yoo et al., 2010). A549 (human alveolar epithelial) and BEAS-2B (human bronchial epithelial) cells were maintained in RPMI-1640 (Hyclone, Waltham, MA). HeLa (human cervix epithelial) and 16HBE (human bronchial epithelial) cells were maintained in MEM (Hyclone). NHBE cells were purchased from Cambrex (East Rutherford, NJ) and maintained in bronchial epithelial growth medium (Cambrex) supplemented with defined growth factors and retinoic acid as suggested by the supplier. Only NHBE cells at passage 4 were used for experiments. Unless specified, HeLa cells were commonly used in this study. Cells were cultured at 37 °C in a humidified 5% CO₂ air-jacketed incubator.

Plasmids and transfections

The expression plasmids of p38ζ dominant-negative (DN), p38β DN, MKP1 WT and MKP1 DN were previously described (Shin et al., 2010). All transient transfections were carried out in duplicate using TransIT-LT1 reagent (Mirus, Madison, WI) following the manufacturer’s instructions. In all transfections, an empty vector was used as a control. Transfected cells were treated with pneumolysin before lysis for quantitative PCR (Q-PCR) and Western blot analysis.

RNA-mediated interference

RNA-mediated interference for downregulating p38ζ (MAPK14) and MKP1 (DUSP1) expression was conducted by the transfection of small interfering (si)RNA-p38ζ and siRNA-MKP1 (Shin et al., 2010). siRNAs and siCONTROL Non-Targeting siRNA Pool were purchased from Dharmacon (RNA Technologies, Lafayette, CO). Forty percent to 50% confluent HeLa cells were transfected with a final concentration of 100 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty hours after the start of transfection, cells were treated with pneumolysin before being lysed for Q-PCR and Western blot analysis.

Real-time Q-PCR analysis

Total RNA was isolated using TRIzol® reagent following the instructions provided by Invitrogen (Yoo et al., 2010). SYBR Green PCR Master Mix (ABI, Carlsbad, CA) was used for Q-PCR. Synthesis of cDNA from total RNA was performed using TaqMan Reverse Transcription Reagents (ABI). The primer sequence information for human TNF-α and MKP1 was as follows: TNF-α primers, 5'-CAGAGGGAAGAGTTCCCGAG-3' and 5'-CCTTGCTCCTGTAGAGACG-3'; MKP1 primers, 5'-GCTGTCACGACACAGTGCA-3' and 5'-CGATTAGTCCCTCATAGGTA-3'. Reactions were amplified and quantified using a 7500 Real-Time PCR System and the manufacturer’s software (ABI). Relative quantities of TNF-α and MKP1 mRNA were calculated using the comparative CT method and were normalized based on human GAPDH (5'-CCCTCCCAAATCAAGTGG-3' and 5'-CCATCCACAGTCTCTGG-3') for the amount of RNA used in each reaction.

Western blot analysis

Antibodies were used to analyze total cell lysates (Shin et al., 2010). Phospho-p38 and p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-β-actin was purchased from Sigma-Aldrich.
Pneumolysin is responsible for the induction of TNF-α

We previously reported that clinical isolates of *S. pneumoniae* strains induced the expression of TNF-α in a variety of human epithelial cells (Yoo et al., 2010). To determine whether pneumolysin plays a major role in the induction, the ability of *S. pneumoniae* strain D39 (D39 WT) and its isogenic Ply mt to induce TNF-α expression was compared. As shown in Fig. 1a, D39 WT increased TNF-α expression whereas Ply mt did not induce expression at all, indicating that pneumolysin is required for the expression. By applying purified pneumolysin, we further confirmed that pneumolysin increased TNF-α expression to a level similar to that induced by *S. pneumoniae* (Fig. 1b). To eliminate the possibility that the TNF-α expression was induced by lipopolysaccharide contaminated during purification from *E. coli*, we pretreated pneumolysin with Polymyxin B, a well-characterized lipopolysaccharide inhibitor (Li et al., 1999, 2000; Yang et al., 2003). As shown in Fig. 1c, Polymyxin B pretreatment did not significantly reduce pneumolysin-induced TNF-α expression, indicating that potential lipopolysaccharide contamination was not involved. Because TNF-α expression was less potently induced by *S. pneumoniae* at the early stage of treatment (Yoo et al., 2010), we examined the effect of treatment time on the expression of TNF-α. As shown in Fig. 1d, pneumolysin minimally induced TNF-α expression at 3 h after treatment, gradually increased at 5 h, maximally induced expression at 7 h and declined thereafter, indicating a time-dependent induction pattern of TNF-α expression. Together, these results suggest that pneumolysin was required for the induction of TNF-α expression to a limited level and was able to potently induce the expression at the later stage of treatment.

p38 MAPK plays a major role in inducing TNF-α expression in response to pneumolysin

As MAPK plays a role in the expression of proinflammatory cytokines, we investigated the role of p38 and ERK for TNF-α expression in response to pneumolysin. As shown in Fig. 2a, pretreatment of SB203580, a specific chemical inhibitor for p38, significantly reduced pneumolysin-induced TNF-α expression whereas PD98059, a specific chemical inhibitor for ERK, did not, indicating the positive involvement of p38 in TNF-α induction. We next confirmed the role of p38 in pneumolysin-induced TNF-α expression using more specific approaches. As shown in Fig. 2b, overexpressing both p38α DN and p38β DN reduced TNF-α expression. To further confirm the requirement of p38, p38 knockdown using siRNA-p38 was carried out. As shown in Fig. 2c (left panel), p38 knockdown reduced TNF-α expression. The efficiency of siRNA-p38 in reducing endogenous p38 protein was confirmed by Western blot analysis (Fig. 2c, right panel). As phospho-p38 is required for the induction of TNF-α expression, induction of p38 by pneumolysin was observed at the protein level by Western blot analysis (Fig. 2d). Collectively, we conclude from these data that pneumolysin activated p38, which acted as a positive regulator for TNF-α induction.

MKP1 acts as a negative regulator for pneumolysin-induced TNF-α expression

Given that p38 was clearly involved in the induction of TNF-α, we investigated whether MKP, a critical phosphatase for dephosphorylating MAPK, was involved in regulating TNF-α induction in response to pneumolysin. Previously, it was demonstrated that pneumolysin specifically induced
expression of MKP1 but not MKP3, 5 and 7 (Ha et al., 2008). Therefore, we first investigated whether pretreatment with Ro31-8220, a specific chemical inhibitor for MKP1 expression, increased induction of TNF-α in response to pneumolysin. As shown in Fig. 3a, pretreatment of Ro31-8220 enhanced pneumolysin-induced TNF-α expression, indicating the negative involvement of MKP1 in TNF-α induction. We next confirmed the role of MKP1 in pneumolysin-induced TNF-α transcription using more specific approaches. As shown in Fig. 3b, overexpressing MKP1 DN enhanced TNF-α expression, whereas overexpressing MKP1 WT reduced the expression. To further confirm the requirement of MKP1, MKP1 knockdown using siRNA-MKP1 was carried out. As shown in Fig. 3c (right panel), MKP1 knockdown enhanced TNF-α expression. The efficiency of siRNA-MKP1 in reducing endogenous MKP1 mRNA was confirmed by performing Q-PCR analysis (Fig. 3c, left panel). These data suggest that MKP1 acted as a negative regulator for TNF-α induction in response to pneumolysin.

**MKP1 negatively acting upstream of p38 is highly induced by pneumolysin at the early stage of treatment**

We previously reported that D39 WT increased MKP1 expression whereas Ply mt did not induce expression at all, indicating that pneumolysin was required for the expression of MKP1 (Ha et al., 2008). As pneumolysin is known to be encoded in virtually all clinical isolates of *S. pneumoniae*, we examined whether MKP1 expression was induced by diverse

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**Fig. 2.** p38 MAPK plays a major role in inducing TNF-α expression in response to pneumolysin (a) SB203580 and PD98059 (both at 10 μM) were applied to examine pneumolysin-induced TNF-α expression 5 h after treatment. (b) Overexpressing p38α DN and p38βj DN reduced pneumolysin-induced TNF-α expression. (c) p38 knockdown using siRNA-p38 (100 nM) inhibited TNF-α expression at mRNA levels by pneumolysin (left panel). The efficiency of siRNA-p38 in reducing endogenous p38 protein was confirmed by Western blot analysis (right panel). (d) Pneumolysin induced phosphorylation of p38. Data in (a)–(c) are expressed as mean ± SD (n = 3). Western blot data in (c) and (d) are representative of three separate experiments. *P < 0.05 vs. in the presence of pneumolysin only (a) and mock in the presence of pneumolysin (b, c).

**Fig. 3.** MKP1 acts as a negative regulator for pneumolysin-induced TNF-α expression. (a) One hour of pretreatment with 2.5 μM Ro31-8220 enhanced pneumolysin-induced TNF-α expression. (b) Overexpressing MKP1 DN enhanced pneumolysin-induced TNF-α expression, whereas overexpressing MKP1 WT reduced the expression. (c) MKP1 knockdown using siRNA-MKP1 (100 nM) enhanced TNF-α induction by pneumolysin (right panel). The efficiency of siRNA-MKP1 in reducing endogenous MKP1 mRNA was confirmed by performing Q-PCR analysis (Fig. 3c, left panel). Data in (a)–(c) are expressed as mean ± SD (n = 3). *P < 0.05 vs. in the presence of pneumolysin only (a) and mock in the presence of pneumolysin (b, c). **P < 0.01 vs. mock (c).
As shown in Fig. 4a, all clinical isolates tested were able to induce MKP1 expression. To evaluate the generalizability of these data, we measured MKP1 expression in a variety of human epithelial cell lines, including HeLa, A549, BEAS-2B, 16HBE and primary bronchial epithelial NHBE cells. As shown in Fig. 4b, pneumolysin induced MKP1 expression in all human epithelial cells tested, indicating that MKP1 induction by pneumolysin may be generalizable to most human epithelial cells. The expression of TNF-α was low in response to pneumolysin 3 h after treatment, gradually increased at 5 h, was maximally induced at 7 h and declined thereafter, demonstrating a time-dependent induction pattern of TNF-α expression (Fig. 1d). As MKP1 acts as a negative regulator for TNF-α induction by pneumolysin, we quantified the expression level of MKP1 by pneumolysin. As shown in Fig. 4c, pneumolysin maximally induced MKP1 expression at 1 h after treatment, gradually decreased and was maximally reduced at 7 h, indicating a time-dependent reduction pattern of MKP1 expression. These results indicate that pneumolysin was able to potently induce MKP1 expression at the early stage of infection, and that expression gradually declined thereafter.

The activation of p38 acted as a positive regulator for TNF-α induction in response to pneumolysin, whereas MKP1 acted as a negative regulator for the induction. Additionally, the expression level of MKP1 was maximally induced at early stages but was reduced later. To determine whether MKP1 was negatively involved in TNF-α induction by inhibiting p38 phosphorylation, we assessed whether overexpressing MKP1 DN enhanced p38 phosphorylation, whereas overexpressing MKP1 WT reduced the phosphorylation. As shown in Fig. 4d, pneumolysin-induced p38 phosphorylation was enhanced by overexpressing MKP1 DN but reduced by overexpressing MKP1 WT. Together, these data suggest that MKP1 acted clearly as a negative regulator for TNF-α induction by deactivating p38 signaling in response to pneumolysin at the early stage of treatment.

**Discussion**

Proinflammatory cytokines TNF-α and IL-1β play an important role in host inflammatory responses such as leukocyte infiltration (Murphy, 2006). Leukocyte infiltration is a noted characteristic of pneumonia, otitis media and chronic obstructive pulmonary disease associated with *S. pneumoniae* (Murphy, 2006). However, histologic features induced by infection of *S. pneumoniae* in a murine model revealed little leukocyte infiltration compared with non-typeable *Haemophilus influenzae* (NTHi) infection (Lim et al., 2007a, b). In addition, this observation was highly relevant to that of pneumococcal lobar pneumonia in human patients during early stages of the disease (Lagoa et al., 2005; Ware et al., 2005). At the early stage of infection, infected lungs are not filled with many polymorphonuclear neutrophils but are fully filled with red blood cells, suggesting that the expression of proinflammatory cytokines is probably reduced in response to *S. pneumoniae*. In agreement with this, we previously reported that pneumolysin was less potent in inducing inflammatory responses compared with NTHi at the early stage of treatment (Yoo et al., 2010).

Pneumolysin is a multifunctional protein possessing both pore-forming cytolytic and immunomodulatory properties...
(Mitchell & Andrew, 1997). It is thought to be present at relatively low concentrations early in infection, when it mediates both immunomodulatory effects and low sublytic activities (Mitchell et al., 1991; Cockeran et al., 2001). Later in infection, pneumolysin increases to lytic concentrations where it mainly forms membrane pores that cause direct cellular and tissue damage, promoting widespread pneumococcal dissemination (Gilbert et al., 1999). In the present study, we found that 50–100 ng mL⁻¹ doses of pneumolysin induce TNF-α expression via activation of p38. Based on a previous report, this concentration causes about 5% cytotoxicity as measured by an lactate dehydrogenase release assay (Ha et al., 2007).

MKPs are important signaling mediators involved in balancing the activity of MAPks by dephosphorylation. The MKPs have distinct substrate specificity toward the MAPK family members (Camps et al., 2000; Keyse, 2000). Pneumolysin is known to specifically induce the expression of MKP1 not only at the mRNA level but also at the protein level, and the induction of MKP1 in response to S. pneumoniae was observed in a murine model (Ha et al., 2008). MKP1, also termed DUSP1, was identified as a stress-responsive immediate-early gene (Keyse & Emslie, 1992) and was shown to play an important role in the innate immune response (Chi et al., 2006; Salojin et al., 2006). MKP1 is most selective for p38 MAPK at physiologic levels of expression, although high levels of expression are also associated with JNK and even mild ERK1/2 inactivation (Franklin & Kraft, 1997; Hutter et al., 2000; Bueno et al., 2001). Indeed, at physiologic expression levels MKP1 binds directly to p38 but not ERK or JNK (Franklin & Kraft, 1997; Hutter et al., 2000). Our kinetic studies showed that MKP1 expression peaked at 1 h after treatment with pneumolysin and declined gradually thereafter (Fig. 4c). The expression was generalizable to a range of human epithelial cells such as HeLa, A549, BEAS-2B, 16HBE and NHBE (Fig. 4b). Interestingly, the induction of TNF-α began to increase at 3 h after treatment with pneumolysin and peaked at 7 h (Fig. 1d), suggesting that high expression of MKP1 at early time points leads to less activation of p38, resulting in less induction of TNF-α. At later time points, decreased expression of MKP1 increased the activated form of p38. This may explain the increased induction of TNF-α at later time points.

The tumor suppressor cylindromatosis (CYLD), known as a deubiquitinase, has been identified as a key negative regulator for nuclear factor-κB signaling and many other cell signaling events, including the regulation of acute lung injury in lethal S. pneumoniae infections (Trompouki et al., 2003; Reiley et al., 2006; Jin et al., 2007; Lim et al., 2007b). Previously, it was reported that CYLD interacts with and deubiquitinates TAK1 and in turn negatively regulates activation of its downstream signaling molecule p38 in response to pneumolysin (Koga et al., 2008). Therefore, it is likely that the regulation of TNF-α expression is highly coordinated by the activity of both MKP1 and CYLD in response to pneumolysin during infection.

To facilitate pathogen clearance, hosts have developed a variety of strategies, including effective inflammatory responses, to form the initial defense system at the early stage of infection. During evolution, bacteria may develop an evasion mechanism against inflammation to avoid the initial formation of a defense system. Pneumolysin is known to play a diverse role in the pathogenesis of pneumococcal infections by evading the immune system, such as inhibiting lymphocyte function and interfering with the complement pathway (Paton & Ferrante, 1983; Ferrante et al., 1984; Paton et al., 1984). The reduced induction of cytokine expression in response to pneumolysin could be another diverse role to evade the immune system. The information provided by these studies will make it easier to understand the pathogenesis of this important human pathogen.

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Authors’ contribution

H.-S.S. and I.-H.Y. contributed equally to this study.

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