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Molecules and Cells

MKP1 Regulates the Induction of MCP1 by *Streptococcus pneumoniae* Pneumolysin in Human Epithelial Cells

Hee-Sung Shin[3], In-Hwa Yoo[3], Yong-Jae Kim, Ji-Yun Lee[1], Hyong-Bai Kim, Shouguang Jin[2], and Un-Hwan Ha*

Epithelial cells act as the first line of host defense against microbes by producing a range of different molecules for clearance. Chemokines facilitate the clearance of invaders through the recruitment of leukocytes. Thus, upregulation of chemokine expression represents an important innate host defense response against invading microbes such as *Streptococcus pneumoniae*. In this study, we report that the expression of Monocyte Chemotactic Protein 1 (MCP1) was highly induced in response to *S. pneumoniae in vitro* and *in vivo*. Among numerous virulence factors, pneumococcal pneumolysin was found to be the major factor responsible for this induction. Furthermore, MCP1 induction was mediated by the p38 mitogen-activated protein kinase (MAPK) whose activation was controlled by MAPK phosphatase 1 (MKP1). Therefore, this study reveals novel roles of pneumolysin in mediating MKP1 expression for the regulation of MCP1 expression in human epithelial cells.

INTRODUCTION

S. pneumoniae is a significant human pathogen that causes potentially life-threatening diseases with high morbidity and mortality such as pneumonia, septicemia and meningitis (Bluestone et al., 1992). To date, various virulence factors have been identified in *S. pneumoniae*, and they are critically involved in initiating inflammation during infection, which is characterized by the production of inflammatory mediators (Bruyn and van Furth, 1991; Cundell et al., 1995; Tuomanen et al., 1985). Among these factors, numerous cell surface-associated proteins on cell wall are involved in the pathogenesis of *S. pneumoniae* during infection, including autolysin, the pneumococcal surface protein A (PspA), PspC, hyaluronidase, neuraminidase, and the pneumococcal surface antigen A (PsaA) (Mitchell, 2006). In addition, the 53kDa cytoplasmic pneumolysin plays an important role in the induction of inflammation and appears to be released during infections through the action of pneumococcal autolysis (Canvin et al., 1995; Wheeler et al., 1999). However, it has been recently reported that the pneumolysin is also localized to the cell wall compartment (Price and Camilli, 2009).

Epithelial cells act as the first line of host defense against microbes by releasing a range of mediators for clearance. Chemokines assist in the clearance by recruiting leukocytes to the sites of infection. MCP1 is a member of the chemokine superfamily (Gu et al., 1999), and it acts as a potent chemoattractant against monocytes, Natural killer (NK) cells, T cells, eosinophils, and basophils (Dunzendorfer et al., 2001; Heinemann et al., 2000; Jiang et al., 1992; Lee et al., 2008; Loetscher et al., 1996). MCP1 is also known to be involved in a number of inflammatory diseases, such as sepsis and asthma (Bossink et al., 1995; Folkard et al., 1997; Sousa et al., 1994). Thus, an increase in MCP1 levels in humans has been shown to mediate the initial steps of inflammation by recruiting leukocytes (Gu et al., 1998; Reape and Groot, 1999). The expression of MCP1 is induced by a diverse range of inflammatory mediators such as lipopolysaccharide (LPS), interleukin-1β (IL-1β, IL-4, IL-6, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Gau-tam et al., 1995; Kim et al., 2008; Lee et al., 2004; Zhu et al., 1994). Several cell types have been shown to produce MCP1, including mononuclear cells, fibroblasts, endothelial cells, epithelial cells, and smooth muscle cells (Khreiss et al., 2004; Lin et al., 1998; Loghmani et al., 2002; Pype et al., 1999; Sironi et al., 1993).

A number of proinflammatory mediators from airway epithelium play a pivotal role in airway defense against inhaled pathogens such as *S. pneumoniae*. Among the mediators released, MCP1 is a major chemotactic factor for monocytes and natural killer cells (Bokoch, 1995; Luster, 1998). However, how such the induction of MCP1 expression might occur in response to *S. pneumoniae* is still not clearly understood. In the present study, we evaluated the effect of *S. pneumoniae* on the expression of inflammatory mediator MCP1. We found that *S. pneumoniae* is potent in inducing the expression of MCP1. Among the numerous virulence factors encoded by *S. pneu-*

Department of Biotechnology and Bioinformatics, Korea University, Yeongi 339-700, Korea, [1]Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA, [2]Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL 32610, USA, [3]These authors contributed equally to this work.
*Correspondence: haunhwan@korea.ac.kr

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moniae, pneumolysin induced the expression of MCP1 via p38 MAPK whose activation is under the control of MKP1. Thus, this study brings new insights into defense mechanisms against infection of *S. pneumoniae* by the regulation of inflammatory mediators.

MATERIALS AND METHODS

Reagents

PD98059, SB203580 and Ro31-8220 were purchased from Calbiochem. Polymyxin B was purchased from Sigma (USA).

Bacterial strains and culture conditions

Clinical isolates of *S. pneumoniae* wild-type (wt) strains D39, 6B, 3, 19F and 23F were used in this study (Avery et al., 1979; Briles et al., 1992). Unless specified otherwise, the *S. pneumoniae* wt strain D39 was used to treat cells in this study. D39 isogenic pneumolysin-deficient mutant (Ply mt) was developed through insertion-duplication mutagenesis as previously described (Berry et al., 1989). *S. pneumoniae* strains were cultured in a Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) at 37°C in an atmosphere of 5% CO_2. Whole bacterial cells cultured in the broth were harvested at 10,000 × *g* for 20 min at 4°C to obtain the supernatant and pellet after overnight incubation. The bacterial culture supernatant (Sup) was filtered through a 0.22 μm pore size membrane to completely remove bacteria. The bacterial pellet was then suspended in phosphate buffered saline (PBS) for the preparation of live bacteria (Live). 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). Residual intact cells were removed by centrifugation at 12,000 × *g* for 20 min at 4°C. The bacterial lysate (Lysate) was stored at -80°C, and 5 μg/ml lysate was used in all experiments as previously reported (Ha et al., 2007). For the infection with live *S. pneumoniae*, cells were treated with 20 multiplicity of infection (moi) for 6 h unless otherwise specified.

Purification of pneumolysin

His_6 tag-fused pneumolysin was expressed in and purified from an *Escherichia coli* strain, and residual LPS was removed by passage over End-X resin as previously described (Ha et al., 2007; 2008).

Cell culture

All media described below were supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). A549 (human alveolar epithelial) and BEAS-2B (human bronchial epithelial) cells were maintained in RPMI-1640 (Hyclone). Cells were cultured at 37°C in a humidified 5% CO_2 air-jacketed incubator. Unless otherwise specified, A549 cells were mainly used in this study.

Plasmids and transfections

The expression plasmids of p38α dominant-negative (DN), p38β DN, MKP1 wt and MKP1 DN were previously described (Ha et al., 2008; Mikami et al., 2005). All of the transient transfections were carried out in duplicate using the TransIT-LT1 reagent (Mirus, USA) following the manufacturer's instructions. In all transfections, an empty vector was used as the control. Transfected cells were treated with pneumolysin for 6 hrs prior to lysis for real-time quantitative PCR (Q-PCR) analysis.

RNA-mediated Interference

RNA-mediated interference to down-regulate p38α (MAPK14) and MKP1 (DUSP1) expression was performed by transfection with small interference RNA (siRNA) p38 and siRNA MKP1 following the instructions supplied by Invitrogen. siRNAs and siCONTROL Non-Targeting siRNA Pool were purchased from Dharmacon (RNA technologies, USA). The 40-50% confluent cells were transfected with a final concentration of 100 nM siRNA using Lipofectamine 2000 (Invitrogen). Forty hours after the start of transfection, cells were treated with pneumolysin before being lysed for Q-PCR and Western blot analysis.

Q-PCR analysis

Total RNA was isolated using TRIzol® Reagent following Invitrogen's instructions. SYBR Green PCR Master Mix (Applied Biosystems) was employed for Q-PCR. Synthesis of cDNA from total RNA was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems). The primer sequences for human MCP1 and MKP1 were as follows: MCP1 primers, 5′-CAGCCAGATGCAATCAATGCC-3′ and 5′-TGGA ATCCTGAACCCACTTCT-3′; MKP1 primers, 5′-GCTGTGCAG CAAACAGTCGA-3′ and 5′-CGATTAGTCCTCATAAGGTA-3′. The primer sequence for mouse Mcp1 was as follows: 5′-TGTCTGGACCCCATTCCTTC-3′ and 5′-ACCAGCAAGATGA TCCCAAT-3′. Reactions were amplified and quantified using a 7500 Real-Time PCR System and the manufacturer's software (Applied Biosystems). Relative quantities of MCP1 and MKP1 mRNA were calculated using the comparative CT method and normalized by human GAPDH (5′-CCCTCCAAAATCAAGTGG-3′ and 5′-CCATCCACAGTCTTCTGG-3′) or by mouse GAPDH (5′-GGAGAGAACCTGGTCCTCAG-3′ and 5′-ACCCAGAAGA CTGTGGATGG-3′) for the amount of RNA used in each reaction.

Western blot analysis

Antibodies were used to analyze total cell lysates according to the manufacturer's instructions. Phospho-p38 and p38 antibodies were purchased from Cell Signaling Technology (USA). Monoclonal anti-β-actin was purchased from Sigma-Aldrich.

ELISA assay

The culture supernatants were collected and used to determine the levels of secreted MCP1 by using a Human MCP1 Immunoassay (R&D systems). Supernatants were filtered through 0.22 μm filters and used to quantify the amount of MCP1 according to the manufacturer's instructions. The minimum detectable dose of MCP1 was 5.0 pg/ml as reported by the manufacturer of the ELISA kit.

Animal Experiments

The lungs of anesthetized C57BL/6 mice were intratracheally inoculated with *S. pneumoniae* D39 lysate (equivalent of 2.5 × 10^7 CFU). Saline was injected as a control. Mice were sacrificed by overdose injection of sodium pentobarbital 9hr after treatment. Total RNA was extracted from the lung tissues of mice with TRIzol and then subjected to MCP1 mRNA expression analysis by Q-PCR.

RESULTS

Clinical isolates of S. pneumoniae induce MCP1 *in vitro* and *in vivo*

Since MCP1 has been identified as a prominent chemokine, we examined MCP1 expression in response to the clinical isolates of *S. pneumoniae* in human alveolar epithelial A549 cells. Q-PCR was used to quantify the level of mRNA expressions following incubation with clinical isolates of *S. pneumoniae* strains

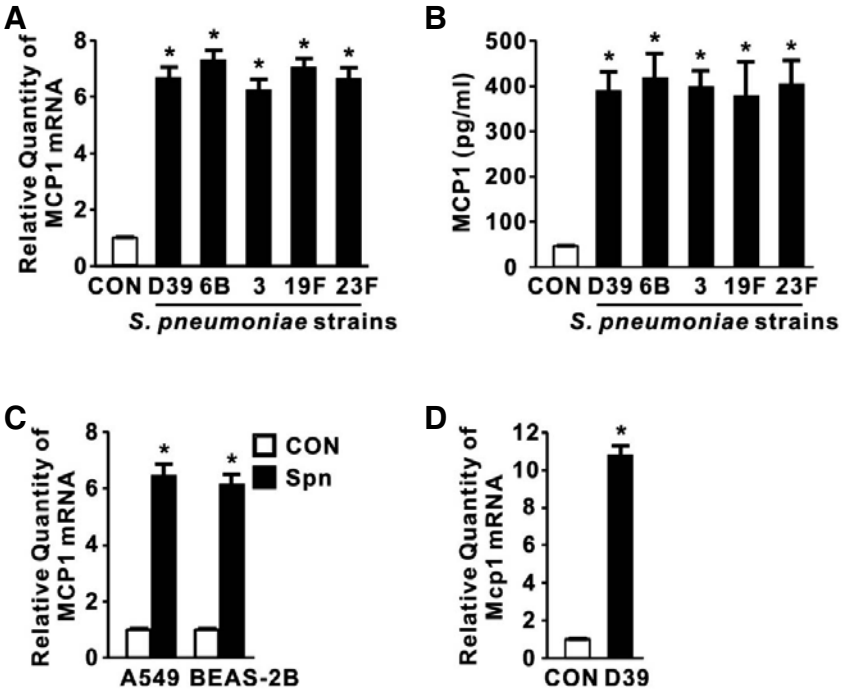

Fig. 1. Clinical isolates of *S. pneumoniae* induce MCP1 *in vitro* and *in vivo* (A) MCP1 mRNA levels in response to lysates from clinical isolates of *S. pneumoniae* strains were measured by Q-PCR analysis. (B) MCP1 protein levels were assayed by ELISA. (C) *S. pneumoniae* (Spn) lysate was used to treat human airway epithelial A549 and BEAS-2B cells. MCP1 mRNA expression was measured 6 hr after treatment by Q-PCR analysis. (D) Mcp1 mRNA levels were upregulated by the *S. pneumoniae* D39 lysate in the lungs of C57BL/6 mice *in vivo*. Data in (A-C) are expressed as mean ± SD (n = 3). Data in (D) are expressed as mean ± SD (n = 4). *, $p < 0.01$ vs control group. CON, PBS.

D39, 6B, 3, 19F and 23F. As shown in Fig. 1A, all clinical isolates tested were able to induce MCP1 expression 6 h post-treatment. Consistent with MCP1 mRNA induction, ELISA revealed increased MCP1 protein production in response to the clinical isolates tested (Fig. 1B). To evaluate the generalizability of these data, MCP1 expression was measured not only in A549 but also in human bronchial epithelial BEAS-2B cells. As shown in Fig. 1C, *S. pneumoniae* induced MCP1 expression in both airway epithelial cells. To confirm whether MCP1 was also induced *in vivo*, the effects of *S. pneumoniae* on Mcp1 expression in mice was determined 9hr post-treatment. As shown in Fig. 1D, the *S. pneumoniae* strain D39 strongly induced Mcp1 mRNA expression in murine upper respiratory tracts. Collectively, these results demonstrate that *S. pneumoniae* was able to potently induce MCP1 transcription.

Pneumolysin is responsible for the induction of MCP1

To identify the pneumococcal factors responsible for MCP1 induction, MCP1-inducing activity of live bacteria, bacterial culture supernatant and bacterial lysate containing soluble cytoplasmic components was compared. As shown in Fig. 2A, MCP1 transcription exhibited the highest increase in response to *S. pneumoniae* lysate versus live bacteria and bacterial culture supernatant. This suggests that *S. pneumoniae* cytoplasmic components likely contain the primary mediators for MCP1 induction. Among the numerous pneumococcal factors contributing to pathogenesis during infection, pneumolysin is a major cytoplasmic protein (Paton et al., 1997). To determine whether pneumolysin was responsible for the increase of MCP1 expression, the ability of the *S. pneumoniae* strain D39 (D39 wt) and its isogenic pneumolysin-deficient mutant (Ply mt) to induce MCP1 expression was compared. As shown in Fig. 2B, D39 wt increased MCP1 expression, whereas Ply mt did not induce expression at all, indicating that pneumolysin is required for expression. By applying purified pneumolysin, it was further confirmed that pneumolysin induces MCP1 expression in a dose-dependent manner and expression was increased up to a level similar to that induced by *S. pneumoniae* (Fig. 2C). Based on this, a pneumolysin dose of 100 ng/ml was used to treat cells for further study. To eliminate the possibility that MCP1 expression was induced by LPS contaminated during purification from *E. coli*, pneumolysin was pretreated with polymyxin B, a well-characterized LPS inhibitor (Li et al., 1999; 2000; Yang et al., 2003). As shown in Fig. 2D, polymyxin B pretreatment did not significantly reduce pneumolysin-induced MCP1 expression, indicating that potential LPS contamination did not occur. Since MCP1 expression was potently induced by pneumolysin, the effect of treatment time on the expression of MCP1 was examined. Pneumolysin minimally induced MCP1 expression 3hr after treatment and maximally induced MCP1 expression 6hr after treatment, which decreased thereafter, indicating a time-dependent induction pattern of MCP1 expression (Fig. 2E). Taken together, these results suggest that pneumolysin was necessary for the induction of MCP1 expression.

p38 MAPK plays a major role in inducing MCP1 expression in response to pneumolysin

Since MAPK plays a key role in the induction of inflammatory mediators (Kaminska, 2005), we investigated to understand the role of p38 and ERK in MCP1 expression after treatment with pneumolysin. As shown in Fig. 3A, pretreatment of SB203580, a specific chemical inhibitor for p38, clearly reduced pneumolysin-induced MCP1 expression, whereas PD98059, a specific chemical inhibitor for ERK, did not, suggesting the involvement of p38 in MCP1 induction. The role of p38 in pneumolysin-induced MCP1 expression was then examined by using more specific approaches. As shown in Fig. 3B, overexpressing of both p38α DN and p38β DN reduced MCP1 expression. To further confirm the requirement of p38, p38 knockdown using siRNA-p38 was carried out. As shown in Fig. 3C (left panel), p38 knockdown reduced MCP1 expression. The efficiency of siRNA-p38 in reducing endogenous p38 protein was confirmed by Western blot analysis (Fig. 3C, right panel). Since p38 is required for the induction of MCP1 expression, activation of p38

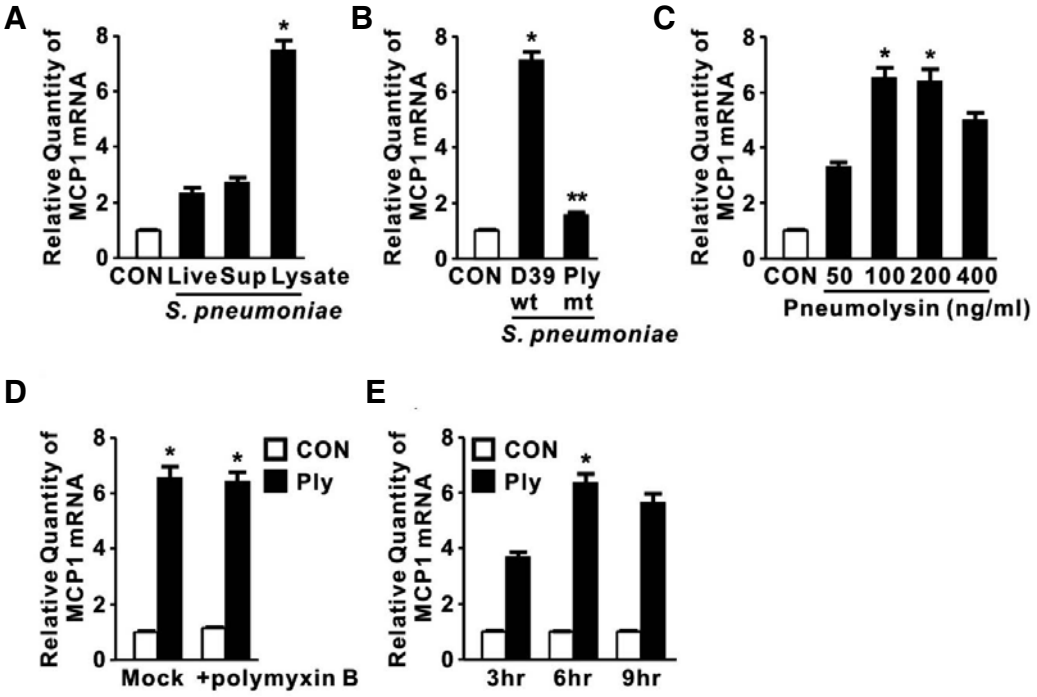

Fig. 2. Pneumolysin is responsible for the induction of MCP1. (A) MCP1 expression was measured by monitoring mRNA levels in response to *S. pneumoniae*. Live, live bacteria; Sup, bacterial culture supernatant; Lysate, bacterial lysate. (B) MCP1 expression after treatment with lysates from S. pneumoniae wild-type strain D39 (D39 wt) and D39 isogenic pneumolysin-deficient mutant (Ply mt) was assessed by Q-PCR analysis. (C) Purified pneumolysin (Ply) induced MCP1 expression at transcriptional levels in a dose-dependent manner. (D) Ply was pretreated with polymyxin B to assess possible contamination of LPS. (E) MCP1 expression 3, 6 and 9 h after treatment with Ply was assessed by Q-PCR analysis. Data in (A-E) are expressed as mean ± SD (n = 3). *, $p < 0.01$ vs control group (A-E). **, $p > 0.05$ vs control group (B). CON, PBS.

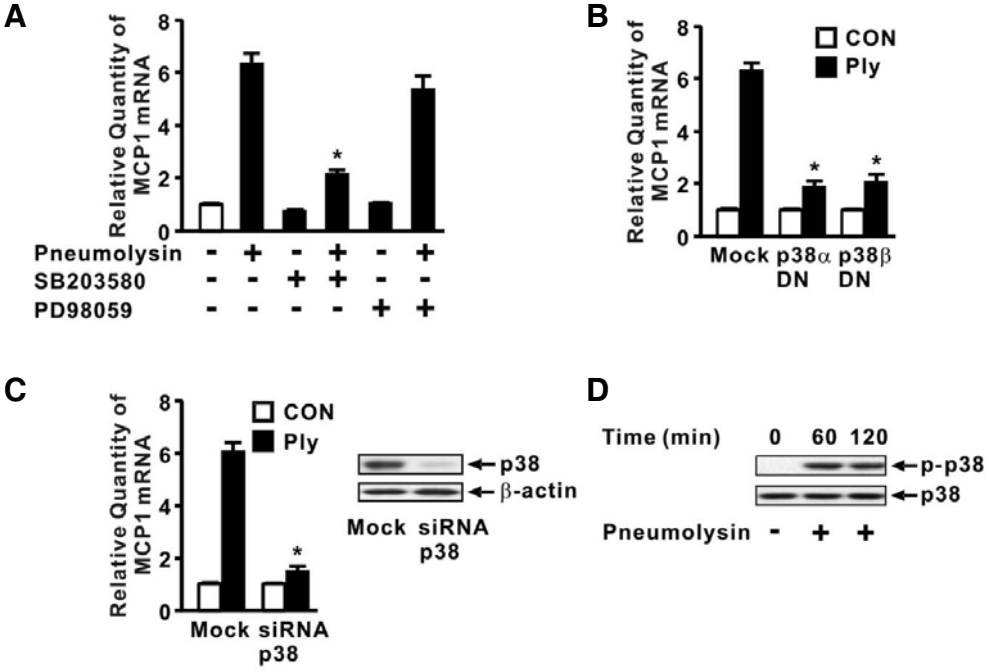

Fig. 3. p38 MAPK plays a major role in inducing MCP1 expression in response to pneumolysin (A) 10 μM SB203580 and 10 μM PD98059 applied to examine pneumolysin-induced MCP1 expression. (B) overexpression of p38α DN and p38β DN reduced Ply-induced MCP1 expression. (C) p38 knockdown using siRNA-p38 (100 nM) inhibited MCP1 expression at mRNA levels by Ply (left panel). The efficiency of siRNA-p38 in reducing endogenous p38 protein was confirmed by Western blot analysis (right panel). (D) Ply induced phosphorylation of p38. Data in (A-C) are expressed as mean ± SD (n = 3). Western data in (C, D) are representative of three separate experiments. *, $p < 0.05$ vs in the presence of Ply only (A) and mock in the presence of Ply (B-C). CON, PBS.

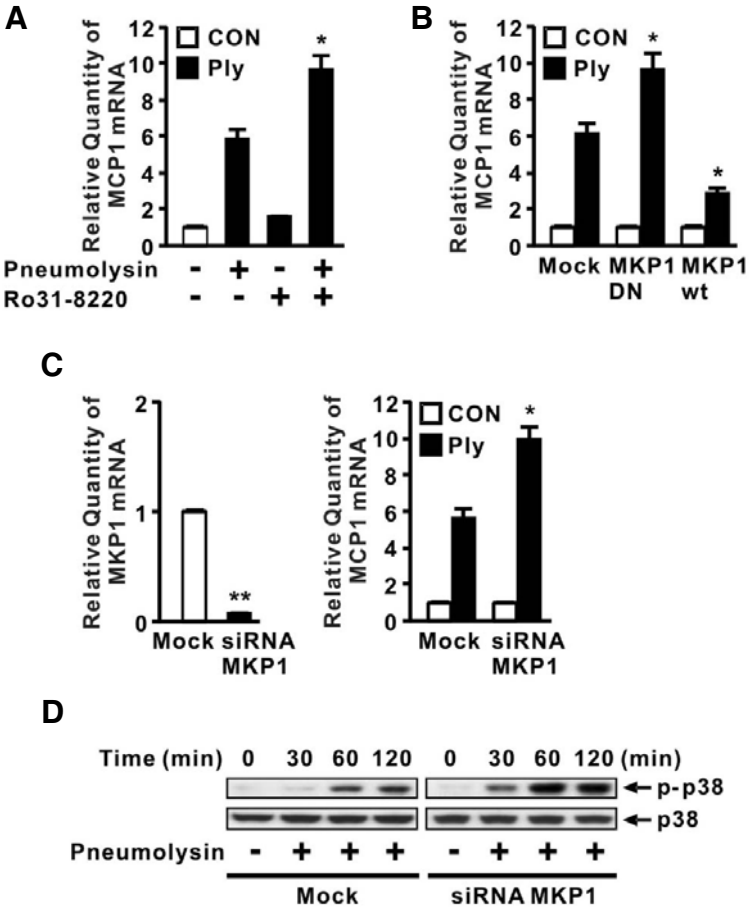

Fig. 4. MKP1 acts as a negative regulator for pneumolysin-induced MCP1 expression. (A) The 2.5 μM Ro31-8220 enhanced Ply-induced MCP1 expression. (B) Overexpression of MKP1 DN enhanced Ply-induced MCP1 expression, whereas overexpression of MKP1 wt reduced expression. (C, D) MKP1 knockdown using siRNA-MKP1 (100 nM) enhanced MCP1 induction (C; right panel) and p38 phosphorylation (D) by Ply. The efficiency of siRNA-MKP1 in reducing endogenous MKP1 mRNA was confirmed by performing Q-PCR analysis (C; left panel). Data in A-C are expressed as mean ± SD (n = 3). Data in D are representative of three separate experiments. *, $p < 0.05$ vs in the presence of Ply only (A) and mock in the presence of Ply (B-C). **, $p < 0.01$ vs mock (C). CON, PBS.

by pneumolysin was observed at the protein level by Western blot analysis (Fig. 3D). From these data, it was concluded that pneumolysin acts as a positive regulator for MCP1 induction.

MKP1 acts as a negative regulator for pneumolysin-induced MCP1 expression

Since p38 MAPK was clearly involved in the induction of MCP1, the involvement of MKP, a critical phosphatase for dephosphorylating MAPK, in regulating MCP1 induction in response to pneumolysin was examined. Previously, it was demonstrated that pneumolysin specifically induced MKP1 expression but not MKP3, 5 and 7 (Ha et al., 2008). Therefore, the effect of Ro31-8220, a specific chemical inhibitor for MKP1 expression, on the induction of MCP1 expression in response to pneumolysin was assessed. As shown in Fig. 4A, pretreatment of Ro31-8220 enhanced pneumolysin-induced MCP1 expression, indicating that MKP1 was negatively involved in MCP1 induction. The role of MKP1 in pneumolysin-induced MCP1 transcription was then examined by using more specific approaches. As presented in Fig. 4B, overexpression of MKP1 DN enhanced MCP1 expression, whereas overexpression of MKP1 wt reduced expression. To further confirm the requirement of MKP1, MKP1 knockdown using siRNA-MKP1 was performed. As shown in Fig. 4C (right panel), MKP1 knockdown enhanced MCP1 expression. The efficiency of siRNA-MKP1 in reducing endogenous MKP1 mRNA was confirmed by performing Q-PCR analysis (Fig. 4C, left panel). The activation of p38 acted as a positive regulator for MCP1 induction in response to pneumolysin, whereas MKP1 acted as a negative regulator for induction. Still unknown was whether MKP1 was negatively involved in MCP1 induction by inhibiting p38 phosphorylation. To test this, the effect of MKP1 knockdown on pneumolysin-induced p38 phosphorylation was examined. As shown in Fig. 4D, MKP1 knockdown increased p38 phosphorylation induced by pneumolysin. Together, these data suggest that MKP1 clearly acts as a negative regulator for MCP1 induction by deactivating p38 signaling in response to pneumolysin.

DISCUSSION

In this study, *S. pneumoniae* was shown to induce the expression of MCP1, which is known as a potent chemoattractant of monocytes. It was further demonstrated that pneumolysin, a key cytoplasmic virulence protein well-conserved among all clinical isolates of *S. pneumoniae*, was involved in the induction of MCP1, whose expression gradually increased and maximal 6hr post-treatment. The expression of MCP1 was mediated by p38, whose activation is under the control of MKP1. Thus, this study reveals novel roles of the pneumolysin in mediating MKP1 expression for the regulation of MCP1 expression in human airway epithelial cells.

S. pneumoniae has more than 90 different serotypes based on the antigenically distinct polysaccharide capsule (Kalin, 1998). Among these, only seven serotypes are covered in the heptavalent polysaccharide conjugate vaccine because those are the most causative serotypes in pneumococcal infection (Black et al., 2000; Obaro, 2002). The seven serotypes include 4, 6B, 9V, 14, 18C, 19F and 23F (Hausdorff et al., 2000a; 2000b;

Spratt and Greenwood, 2000). In this study, the role of 6B, 19F and 23F in the expression of MCP1 was examined. All three serotypes along with 3 and D39 induced expression of MCP1 at both the mRNA and protein levels, indicating that this induction was well conserved among the clinical isolates of *S. pneumoniae* (Figs. 1A and 1B). In addition, this induction was further confirmed in different airway cells as well as in murine upper respiratory tracts (Figs. 1C and 1D).

Cell surface-associated proteins and pneumolysin are thought to play a role in inducing inflammatory responses during pneumococcal infection (Jedrzejas, 2001; Tuomanen et al., 1985). Among them, PspC is a cell surface protein involved in pneumococcal adhesion to cells in nasopharynx (Rosenow et al., 1997) and stimulates the expression of IL-8 from pulmonary epithelial cells (Madsen et al., 2000). In addition, pneumolysin plays an important role in facilitating inflammation by stimulating inflammatory mediators such as IL-1β, TNF-α, nitric oxide, IL-8 and prostaglandins followed by the recruitment of leukocytes to infection sites (Braun et al., 1999; Cockeran et al., 2001; 2002; Houldsworth et al., 1994; Mitchell and Andrew, 1997; Rijneveld et al., 2002). In this study, the fractionation assay demonstrated that a possible factor responsible for the MCP1 induction was localized to the cytoplasm, implying that pneumolysin may be the factor. Indeed, pneumolysin was found to be an essential and sufficient factor for inducing the expression of MCP1 (Figs. 2B and 2C). Pneumolysin is not a secretary protein because it does not have a typical secretion signal, but it can be released by the action of cell-bound autolysin (Canvin et al., 1995). This explains how live bacterial culture and the culture supernatant also displayed a limited induction of MCP1 expression (Fig. 2A). The late exponential-growth phase of *S. pneumoniae* was used to prepare the lysate. After measuring the total protein concentration present in the lysate, 5 μg/ml lysate, which is equivalent to about 10^7 *S. pneumonia*, was used. It was previously reported that 10 pg of pneumolysin is equivalent to about 10^3 *S. pneumoniae* (Houldsworth et al., 1994). In line with these reports, the dose-dependent induction assay shown in Fig. 2C demonstrated that 100~200ng/ml induced MCP1 expression to a comparable extent. However, it was found to be that 100-200ng/ml doses of pneumolysin resulted in a cytotoxicity of 5-25% (Ha et al., 2007). Therefore, 100 ng/ml dose causing lesser cytotoxicity was used in this study.

MKPs are crucial signaling mediators involved in balancing the activity of MAPKs by dephosphorylation. The MKPs have a distinct substrate specificity toward the member of MAPK family (Camps et al., 2000; Keyse, 2000). Pneumolysin is known to specifically induce MKP1 expression 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 the murine model (Ha et al., 2008). MKP1 was identified as a stress-responsive immediate-early gene (Keyse and Emslie, 1992) and plays an important role in the innate immune response (Chi et al., 2006; Salojin et al., 2006). In addition, MKP1 is the most selective for p38 at physiologic levels of expression, although high levels of expression are also associated with JNK and ERK inactivation (Bueno et al., 2001; Franklin and Kraft, 1997; Hutter et al., 2000).

As initial defenses during infection, hosts have developed a variety of strategies to facilitate pathogen clearance, including effective inflammatory responses. In the present study, pneumolysin was identified as a key virulence factor in *S. pneumoniae*-induced MCP1 expression via MKP1-dependent activation of p38. The signaling molecules that directly mediate MKP1 regulation of p38 activation are yet to be determined. However, the information provided in this study will make it easier to understand the pathogenesis of this important human pathogen.

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