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Pneumolysin-Mediated Expression of β-Defensin 2 Is Coordinated by p38 MAP Kinase-MKP1 in Human Airway Cells

Yong-Jae Kim[1], Hee-Sung Shin[1], Jung-Hoon Lee[1], Yong Woo Jung[2], Hyong-Bai Kim[1*], and Un-Hwan Ha[1*]

[1]Department of Biotechnology and Bioinformatics, [2]College of Pharmacy, Korea University, Sejong 339-700, Republic of Korea

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Antimicrobial peptides act as important innate immune defense mediators against invading microbes such as *Streptococcus pneumoniae*. Among a number of antimicrobial peptides, β-defensin 2 (BD2) has strong antimicrobial activity against *S. pneumoniae*. However, little is known about the molecular signaling mechanisms leading to the BD2 expression. Here, we report that BD2 is strongly induced by *S. pneumoniae* in human airway cells including human middle-ear cells. Among diverse pneumococcal virulence factors, pneumolysin is required for inducing BD2 whose expression is under the control of p38 mitogen-activated protein kinase (MAPK). Pneumolysin also selectively regulates the expression of MAPK phosphatase 1 (MKP1), which inhibits the p38 signaling pathway, thereby leading to upregulation of BD2 to mount an effective defense against *S. pneumoniae* infection. These results provide novel insights into the molecular mechanisms underlying the coordinative regulation of BD2 expression via p38-MKP1 in the pathogenesis of airway infectious diseases.

***Keywords*:** hBD2, MKP1, p38, pneumolysin

Introduction

The respiratory track is a major route of entry for airborne microbes such as *Streptococcus pneumoniae*, which is an important human pathogen and the leading cause of high mortality due to community-acquired pneumonia in children worldwide (Welte and Kohnlein, 2009). Thus, airway epithelial cells employ a number of defense mechanisms including the expression of antimicrobial peptides to eliminate invading microbes. The antimicrobial peptides expressed in humans are classified into defensins, cathelicidins and histatins (De Smet and Contreras, 2005). Among them, defensins have a broad range of antimicrobial activity against diverse bacteria by increasing bacterial membrane permeabilization, and they are divided into the α-defensin and β-defensin subfamilies based on their structural characteristics (Schroder, 1999). α-Defensins are predominantly expressed in phagocytic granules, whereas β-defensins are mainly expressed in epithelial cells (Huttner and Bevins, 1999).

The antimicrobial activity of β-defensins has been tested in a number of respiratory microbes including nontypeable *Haemophilus influenza*, *Moraxella catarrhalis*, and *S. pneumoniae*, revealing that β-defensin 2 (BD2) is active against all microbes tested (Lee *et al*., 2004). Increased BD2 expression in patients with respiratory infections supports the importance of this peptide in host defense (Hiratsuka *et al*., 2003; Ishimoto *et al*., 2006). However, little is known about the molecular signaling mechanisms leading to BD2 expression upon *S. pneumoniae* infection.

In this study, we investigated the molecular mechanism by which BD2 expression is induced in response to *S. pneumoniae* in human airway and middle-ear epithelial cells. We further demonstrate that induction of BD2 expression is mediated by pneumolysin, which is a major virulence protein well-conserved among all clinical *S. pneumoniae* isolates. BD2 expression was found to be under the control of p38 mitogen-activated protein kinase (MAPK) whose activation is suppressed by MAPK phosphatase 1 (MKP1). These results bring new insight into signaling mechanisms for the regulation of BD2 against pneumococcal infection.

Materials and Methods

Reagents

The chemical inhibitors SB203580 and Ro31-8220 were purchased from Calbiochem (USA). Polymyxin B was purchased from Sigma-Aldrich (USA).

Bacterial strains and pneumolysin

S. pneumoniae wild-type (wt) strains D39, 6B, 19F, 23F, and D39 isogenic pneumolysin-deficient mutant (Ply mt) were used in this study (Avery *et al*., 1979; Berry *et al*., 1989; Briles *et al*., 1992). D39 was used to treat cells unless specified otherwise. The *S. pneumoniae* culture conditions and pneumolysin purification have been described previously (Yoo *et al*., 2010). Airway cells were treated with either *S. pneumoniae* or pneumolysin for 6 h or 4 h, respectively.

Airway cell culture

Human alveolar epithelial A549 cells were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (GIBCO, USA), penicillin (100 units/ml) and strepto-

*For correspondence. (U.H. Ha) E-mail: haunhwan@korea.ac.kr; Tel.: +82-44-860-1418; Fax: +82-44-860-1411 / (H.B. Kim) E-mail: hyong@korea.ac.kr; Tel.: +82-44-860-1413; Fax: +82-44-860-1411

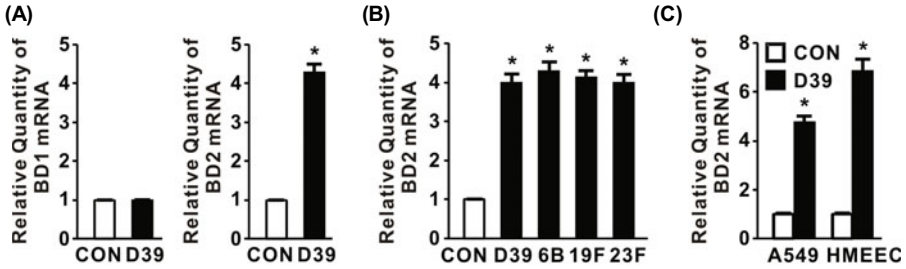

Fig. 1. ***S. pneumoniae*-mediated induction of BD2 expression.** Cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and cultured for 24 h at 37°C. *S. pneumoniae* was then added to each well and incubated for 6 h. BD expression was measured by Q-PCR analysis. Wild-type *S. pneumoniae* strain D39 was added to each well containing A549 cells to measure BD1 and BD2 expression (A). A number of *S. pneumoniae* strains including clinical isolates 6B, 19F, and 23F were added to wells containing A549 cells to measure BD2 expression (B). D39 was added to each well containing either A549 or HMEECs to measure BD2 expression (C). Data in (A–C) are mean±standard deviation (n=3). * $p<0.01$ vs control group.

mycin (0.1 mg/ml). A549 cells were used in all experiments unless specified otherwise. Human middle-ear epithelial cells (HMEECs) were cultivated in bronchial epithelial basal medium (Cambrex, USA) as described previously (Chun *et al.*, 2002). All cells used in this study were incubated at 37°C in an atmosphere of 5% CO_2.

Transfection of plasmids and small interference RNA (siRNA)

p38α dominant-negative (DN), p38β DN, MKP1 wt, and MKP1 DN plasmids were used in this study (Mikami *et al.*, 2005; Ha *et al.*, 2008). siRNA-p38, siRNA-MKP1, and siCONTROL Non-Targeting siRNA Pool were purchased from Dharmacon (USA). Lipofectamine 2000 (Invitrogen, USA) was used to transfer either plasmids or a final concentration of 100 nM siRNA into 40–50% confluent cells following the manufacturer's instructions. Transfections were carried out in duplicate for all experiments.

Real-time quantitative PCR (Q-PCR)

Total RNA was isolated using TRIzol® Reagent (Invitrogen), and cDNA synthesis was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, USA) following the manufacturer's instructions. Primers for BD1, BD2, BD3, and MKP1 are as follows: BD1 5′-AACAGGTGCCTTGAA TTTTGGT-3′ and 5′-TTGCGTCAGCAGTGGAGG-3′; BD2 5′-CCTCTTCATATTCCTGATGCCTCT-3′ and 5′-GGCT CCACTCTTAAGGCAGGT-3′; BD3 5′-TGAGGATCCAT TATCTTCTGTTTGC-3′ and 5′-TGTGTTTATGATTCCT CCATGACC-3′; and MKP1 5′-GCTGTGCAGCAAACAG TCGA-3′ and 5′-CGATTAGTCCTCATAAGGTA-3′. SYBR Green PCR Master Mix (KAPA Biosystems, USA) was used to perform Q-PCR reactions, which were quantified with a CFX96 Real-Time PCR System (Bio-Rad, USA), using the following thermal conditions: stage 1, 50°C for 2 min and 95°C for 10 min; stage 2, 95°C for 15 sec and 60°C for 1 min. Stage 2 was repeated for 40 cycles. Relative mRNA quantities were calculated using the comparative CT method and normalized to human GAPDH (5′-CCCTCCAAAATCAAGTG G-3′ and 5′-CCATCCACAGTCTTCTGG-3′) for the amount of RNA used in each reaction.

Western blot analysis

Phospho-p38 and p38 antibodies were purchased from Cell

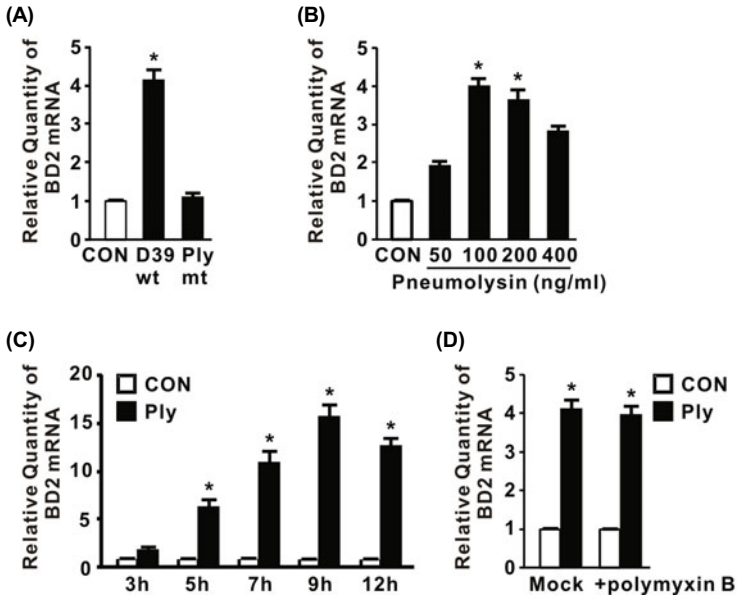

Fig. 2. Pneumococcal pneumolysin-mediated induction of BD2 expression. A549 cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and cultured for 24 h at 37°C. *S. pneumoniae* or purified pneumolysin was added to each well and incubated for 6 or 4 h, respectively. BD2 expression was measured by Q-PCR analysis. D39 and isogenic pneumolysin-deficient mutant (Ply mt) were added to each well to measure expression (A). The indicated pneumolysin doses were added to each well to measure expression (B). Pneumolysin (Ply; 100 ng/ml) was added to each well and incubated for the indicated times to measure expression (C). Polymyxin B-treated Ply was added to each well to measure expression (D). Data in (A–D) are mean±standard deviation (n=3). * $p<0.01$ vs control group.

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

Statistics

All experiments were carried out in triplicate. Results are expressed as mean±standard deviation. Student's *t*-test was used to perform the statistical analysis, and a $P<0.05$ was considered significant.

Results

Induction of BD2 in response to *S. pneumoniae* clinical isolates

As β-defensins (BDs) have been identified as prominent antimicrobial peptides in host airway defense (Hiemstra, 2007), we quantified BD1, BD2, and BD3 expression in A549 cells following treatment with a *S. pneumoniae* lysate containing the cytoplasmic components. As shown in Fig. 1A, D39 potently induced BD2 expression by 6 h post treatment, whereas BD1 expression levels remained unchanged. BD3 expression was not detected under these conditions (data not shown). BD2 expression was commonly induced in response to the lysates of all clinical isolates including 6B, 19F, and 23F (Fig. 1B), and their expression was quantified in HMEECs (Fig. 1C). These results indicate that *S. pneumoniae* generally induces BD2 expression in airway epithelial cells.

Pneumolysin-mediated induction of BD2 expression

We demonstrated that *S. pneumoniae* cytoplasmic components were responsible for BD2 expression (data not shown). Pneumolysin has been identified as a major cytoplasmic protein involved in the pathogenesis of *S. pneumoniae* (Mitchell, 2006). To determine whether pneumolysin plays a role in BD2 expression, we compared the inducing activity of *S. pneumoniae* strain D39 wt to that of Ply mt. As shown in Fig. 2A, BD2 expression increased in response to D39 wt whereas expression was not induced by Ply mt, suggesting that pneumolysin is involved in the induction. We purified and treated cells with pneumolysin from *E. coli* to confirm the effect of pneumolysin. As shown in Figs. 2B and 2C, purified pneumolysin clearly induced BD2 expression in dose-dependent and time-dependent manners. Based on this result, 100 ng/ml pneumolysin was used to treat cells for further study. It has been demonstrated previously that BD2 expression increases following treatment with *Pseudomonas* lipopolysaccharide (LPS) (MacRedmond *et al.*, 2005). Although we applied End-X Endotoxin Removal Affinity Resin (Associates of Cape Code, USA) to remove residual LPS during *E. coli* purification, we pretreated pneumolysin with Polymyxin B, a well-characterized LPS inhibitor (Li *et al.*, 1999, 2000; Yang *et al.*, 2003) to further eliminate the possible effect of accidently contaminated LPS during purification. As shown in Fig. 2D, Polymyxin B pretreatment did not reduce BD2 expression, suggesting that potential LPS contamination was not involved. Collectively, these results indicate that pneumolysin plays an important role in the induction of BD2 expression.

Pneumolysin-induced BD2 expression is mediated by p38 MAPK

MAPKs play an essential role inducing inflammatory responses against infection (Hobbie *et al.*, 1997). To determine the roles of MAPKs in BD2 expression, we pretreated cells with a number of specific chemical inhibitors including SB203580, a specific p38 chemical inhibitor. As shown in Fig. 3A, pretreatment with SB203580 significantly reduced pneumolysin-mediated BD2 expression, indicating that p38 is positively involved in the induction. This result was confirmed by overexpressing p38α DN and p38β DN in cells, as shown in Fig. 3B. p38 knockdown using siRNA-p38 was carried out to further confirm the positive role of p38. As shown in Fig. 3C (*left panel*), BD2 expression decrease by p38 knockdown. The efficiency of siRNA-p38 for reducing the endogenous p38 protein was verified by Western blot analysis (Fig. 3C, *right panel*). Taken together, these data demonstrate that p38 is required for inducing BD2 in response to pneumolysin.

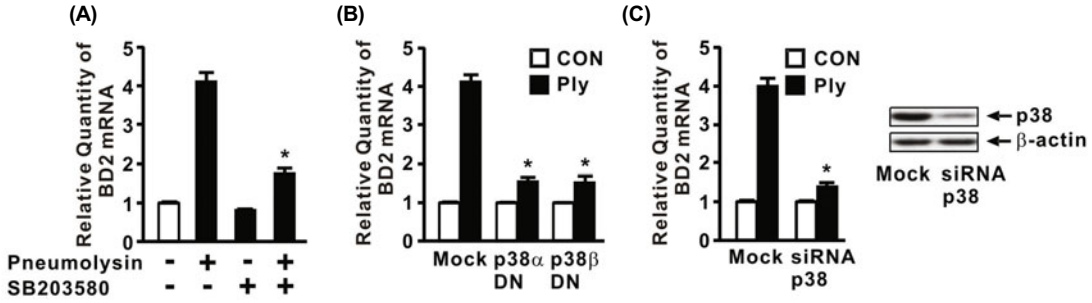

Fig. 3. Effect of p38 MAPK in pneumolysin-mediated expression of BD2. A549 cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and cultured for 24 h at 37°C. Pneumolysin (Ply; 100 ng/ml) was then added to each well and incubated for 4 h. BD2 expression was measured by Q-PCR analysis. Ply was added to cells pretreated with 10 μM SB203580 for 1 h to measure expression (A). Ply was added to cells transfected with overexpressing p38α or p38β DN (B) or siRNA-p38 (C) to measure expression. The efficiency of siRNA-p38 for reducing endogenous p38 protein was confirmed by Western blot analysis (C, *right panel*). Data in (A–C) are mean±standard deviation (n=3). Western data in c 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).

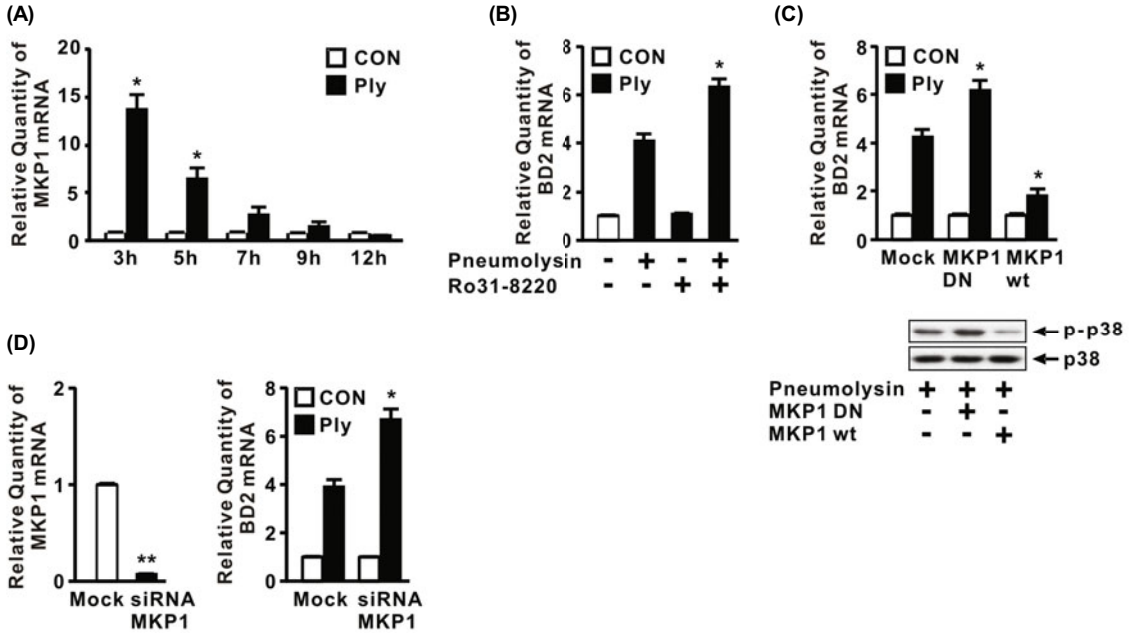

Fig. 4. Effect of MKP1 in p38-mediated expression of BD2 in response to pneumolysin. Cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and cultured for 24 h at 37°C. Pneumolysin (Ply; 100 ng/ml) was then added to each well and incubated for 4 h. MKP1 and BD2 expression was measured by Q-PCR analysis. Ply was added to each well containing HMEECs and incubated for the indicated times to measure MKP1 expression (A). Ply was added to A549 cells pretreated with 2.5 μM Ro31-8220 for 1 h to measure BD2 expression (B). Ply was added to cells transfected with overexpressing MKP1 DN or MKP1 wt (C) or siRNA-MKP1 (D) to measure BD2 expression. The efficiency of DN and wt to control p38 protein activity was confirmed by Western blot analysis (C, *lower panel*). The efficiency of siRNA-MKP1 for reducing endogenous MKP1 expression was confirmed by Q-PCR analysis (D, *left panel*). Data in (A–D) are mean±standard deviation (n=3). Western data in c are representative of three separate experiments. * $p<0.05$ vs in the presence of pneumolysin only (B) and mock in the presence of pneumolysin (C, D). * $p<0.01$ vs control group (A). ** $p<0.01$ vs mock (D).

Effect of MKP1 in p38-mediated BD2 expression in response to pneumolysin

MAPK activities are controlled by the action of dual-specific phosphatase MKPs (Franklin and Kraft, 1997). As p38 was positively involved in the induction of BD2, we were interested in the effects of MKPs in the regulation of BD2 expression in response to pneumolysin. We reported previously that pneumolysin significantly induces MKP1 expression in HeLa and HMEEC cells at 4 h of treatment (Ha *et al*., 2008). Thus, we quantified MKP1 expression in HMEEC cells treated with pneumolysin in a time-dependent manner to confirm expression. As shown in Fig. 4A, MKP1 expression peaked at 3 h after treatment and decreased gradually thereafter, indicating that pneumolysin is capable of inducing MKP1. Then, we pretreated cells with Ro31-8220, a specific chemical inhibitor of MKP1 expression, to determine the effect of MKP1 on pneumolysin-induced BD2 expression. As shown in Fig. 4B, this pretreatment enhanced BD2 expression, suggesting negative involvement of MKP1 in the induction of BD2. Next, we confirmed the negative role of MKP1 using more specific approaches. As shown in Fig. 4C (*upper*), MKP1 DN overexpression enhanced BD2 expression, whereas MKP1 wt overexpression reduced expression. As p38 positively controlled BD2 expression in response to pneumolysin, we assessed the effects of MKP1 DN and wt on pneumolysin-induced p38 phosphorylation. As shown in Fig. 4C (*lower*), MKP1 DN overexpression enhanced p38 activation, whereas MKP1 wt overexpression reduced p38 activation. The negative effect of MKP1 on BD2 expression was further confirmed using siRNA-MKP1. As shown in Fig. 4D (*right panel*), BD2 expression increased following MKP1 knockdown. The efficiency of siRNA-MKP1 for reducing endogenous MKP1 mRNA was verified by Q-PCR analysis (Fig. 4D, *left panel*). Taken together, these data suggest that MKP1 is negatively involved in the induction of BD2 by deactivating p38 signaling in response to pneumolysin.

Discussion

β-Defensins are small cationic antimicrobial peptides that are critical for host defense against respiratory infections (Hiemstra, 2007). Among β-defensins tested in this study, we only detected induced BD2 expression in response to *S. pneumoniae* (Fig. 1A). BD2 induction was further confirmed by applying a number of clinical isolates including 6B, 19F, and 23F (Fig. 1B), which are the most causative serotypes and are used for generating the heptavalent polysaccharide conjugate vaccine (7PCV) (Black *et al*., 2000; Obaro, 2002). These results suggest that the induction of BD2 expression is well conserved among *S. pneumoniae* clinical isolates.

BD2 expression was under the control of p38 MAPK in A549 cells (Fig. 3). In agreement with this observation, it has been reported that NTHi increases BD2 expression in HMEECs via p38 activity (Lee *et al*., 2008). However, BD2 expression is mediated by phosphoinositide 3-kinase (PI3K) in BEAS-2B cells in response to *S. pneumoniae* strain R6x (Scharf *et al*., 2012). Inhibition of PI3K activity did not com-

pletely reduce BD2 expression, suggesting that its expression is not solely dependent on PI3K. Similarly, diverse signaling molecules including PI3K, PKC, p38, JNK, and nuclear factor (NF)-κB are involved in interleukin (IL)-1β-induced BD2 expression in A549 cells (Jang *et al.*, 2004). Indeed, genomic analysis of the BD2 promoter has revealed diverse binding sites for putative transcription factors including activator proteins and NF-κB (Harder *et al.*, 2000). These results suggest that different cell lines may mediate BD2 expression via different signaling cascades initiated by a number of stimuli.

MKPs play a role inactivating MAPKs by dephosphorylation, and MKP1 is most selective for p38 (Franklin and Kraft, 1997). As shown in Fig. 4C, pretreatment with MKP1 DN increased p38 activity, whereas MKP1 wt reduced p38 activity, resulting in regulation of BD2 expression (Fig. 4C). In agreement with this observation, treatment with dexamethasone, a well-known MKP1 inducer, suppresses IL-1β-induced BD2 expression in A549 cells (Jang *et al.*, 2007). However, dexamethasone treatment does not suppress *Pseudomonas aeruginosa*-stimulated BD2 expression in bronchial epithelial cells (Duits *et al.*, 2001), but this treatment up-regulates BD2 expression in corneal epithelial cells (Terai *et al.*, 2004), indicating the presence of diverse signaling cascades in different cell lines.

Pneumolysin, which is a pore forming toxin, appears to be present in virtually all clinical isolates and is released during infections by the action of autolysin (Canvin *et al.*, 1995; Wheeler *et al.*, 1999). In the present study, BD2 expression was mainly dependent on pneumolysin (Fig. 2), and we selected a 100 ng/ml dose of pneumolysin to treat cells. It has been demonstrated previously that the same pneumolysin dose causes about 5% cytotoxicity as measured by the lactate dehydrogenase release assay (Ha *et al.*, 2007), suggesting that cytotoxicity of pneumolysin may not be critically involved in the induction. However, it was recently reported that a *S. pneumoniae* strain R6x isogenic pneumolysin-deficient mutant induces BD2 expression in BEAS-2B cells (Scharf *et al.*, 2012). These results imply that the induction of BD2 may not be solely dependent on pneumolysin and that a number of stimuli may mediate the induction via different signaling cascades in different cell lines.

Hosts have developed a variety of strategies to facilitate pathogen clearance, including release of BD2, as effective host defense responses. In the present study, pneumolysin was identified as a key virulence factor in *S. pneumoniae-induced* BD2 expression via MKP1-dependent activation of p38. The upstream signaling molecules that directly mediate p38 activation are yet to be determined. However, use of these data will make it easier to understand the pathogenesis of this important human pathogen.

Acknowledgements

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