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Cooperation of TLR2 with MyD88, PI3K, and Rac1 in Lipoteichoic Acid–Induced cPLA$_2$/COX-2–Dependent Airway Inflammatory Responses

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Lipoteichoic acid (LTA) plays a role in the pathogenesis of severe inflammatory responses induced by Gram-positive bacterial infection. Cytosolic phospholipase A$_2$ (cPLA$_2$), cyclooxygenase-2 (COX-2), prostaglandin E$_2$ (PGE$_2$), and interleukin (IL)-6 have been demonstrated to engage in airway inflammation. In this study, LTA-induced cPLA$_2$ and COX-2 expression and PGE$_2$ or IL-6 synthesis were attenuated by transfection with siRNAs of TLR2, MyD88, Akt, p42, p38, JNK2, and p65 or pretreatment with the inhibitors of PI3K (LY294002), p38 (SB202190), MEK1/2 (U0126), JNK1/2 (SP600125), and NF-κB (helenalin) in human tracheal smooth muscle cells (HTSMCs). LTA also induced cPLA$_2$ and COX-2 expression and leukocyte count in bronchoalveolar lavage fluid in mice. LTA-regulated PGE$_2$ or IL-6 production was inhibited by pretreatment with the inhibitors of cPLA$_2$ (AACOCF$_3$) and COX-2 (NS-398) or transfection with cPLA$_2$ siRNA or COX-2 siRNA, respectively. LTA-stimulated NF-κB translocation or cPLA$_2$ phosphorylation was attenuated by pretreatment with LY294002, SB202190, U0126, or SP600125. Furthermore, LTA could stimulate TLR2, MyD88, PI3K, and Rac1 complex formation. We also demonstrated that *Staphylococcus aureus* could trigger these responses through a similar signaling cascade in HTSMCs. It was found that PGE$_2$ could directly stimulate IL-6 production in HTSMCs or leukocyte count in bronchoalveolar lavage fluid in mice. These results demonstrate that LTA-induced MAPKs activation is mediated through the TLR2/MyD88/PI3K/Rac1/Akt pathway, which in turn initiates the activation of NF-κB, and ultimately induces cPLA$_2$/COX-2–dependent PGE$_2$ and IL-6 generation. ***(Am J Pathol 2010, 176:1671–1684; DOI: 10.2353/ajpath.2010.090714)***

Airway smooth muscle is considered as an end-response effector regulating regional differences in ventilation by contraction in response to various neurotransmitters, proinflammatory mediators, and exogenous substances released under homeostatic or pathological conditions, such as asthma.[1]

Lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria, is an amphiphilic negatively-charged glycolipid.[2] LTA functions as an adhesion molecule to facilitate the binding of bacteria to cells, colonization, and invasion.[3] Recently, several reports have indicated that LTA shares many inflammatory properties of lipopolysaccharide (LPS) and plays a role in the pathogenesis of septic shock or severe inflammatory responses induced by Gram-positive bacterial infection.[4] Toll-like receptors (TLRs) recognize microbial components and evoke diverse responses in immune and other respiratory cells through distinct signaling cascades, in which MyD88 and tumor necrosis factor (TNF) receptor–associated factor (TRAF)6 are key adaptor proteins.[5] TLR2 is involved in the recognition of Gram-positive bacteria.[6] Moreover, recognition of LTA by TLR2 leads to induction of innate immune responses through activation of many protein kinases, transcription factors, inflammatory cytokines, and chemokines.[7] Therefore, the roles of Gram-positive bacteria and LTA associated with airway inflammation are determined.

Metabolites of arachidonic acid (AA) and lysophosphatides have been identified *in situ* in airway secretion of asthmatics.[8,9] AA is further converted to prostaglandins

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(PGs) through cyclooxygenases (COX), including the constitutive enzyme COX-1 and the inducible COX-2.[10] It has been well established that the production of eicosanoids (ie, PGE_2) is also controlled by the availability of free AA, which is released from membrane phospholipid by the action of phospholipase A_2 (PLA_2). The PLA_2 superfamily is composed of three main types of lipolytic enzymes including secretory PLA_2, the 85-kDa cytosolic group IV PLA_2 ($cPLA_2$), and a calcium-independent group VI PLA_2 in mammalian cells.[11] The members from each of these families of PLA_2 can contribute to the release of AA.[11] However, $cPLA_2$ is the only one that exhibits specificity for AA, and its role in mediating agonist-induced AA release for eicosanoid production is well studied in various cell types.[12] $cPLA_2$ has been shown to be implicated in acute lung injury induced by sepsis and bronchial reactivity associated with anaphylaxis.[13,14] COX-2 is recognized to mediate inflammatory responses and is highly restricted under basal conditions, but is rapidly induced by proinflammatory cytokines, LPS, or LTA.[15–17] Furthermore, increased synthesis of PGE_2 is dependent on an increase in $cPLA_2$ activity in various cell types.[18,19] These results demonstrate that $cPLA_2$ and COX-2 play important roles in mediating AA release for production of PGE_2 by inflammatory cells.

LTA binds CD14 and TLR2 to trigger several intracellular signaling pathways.[5] However, the mechanisms of LTA-induced $cPLA_2$ or COX-2 expression in human tracheal smooth muscle cells (HTSMCs) are not completely determined. Several studies have indicated that LTA-induced inflammatory responses are mediated through TLR2-dependent multiple signaling molecules, including MyD88, TRAF6, PI3K/Akt, MAPKs, and NF-κB.[7,17,20,21] MyD88 plays a key role in TLR signaling.[5] Our previous study has shown that LTA induces the association of TLR2/MyD88 in HTSMCs.[7] Akt, a serine/threonine kinase, is a direct downstream effector of PI3K.[22] Akt can be regulated by multiple intracellular signaling pathways and acts as a transducer for many pathways initiated by growth factor receptor–activated PI3K.[23] Rac1, a Rho family GTPase, participates in regulation of various cellular functions, such as cytoskeletal reorganization, cellular growth, and apoptosis.[22] Rac1 is involved in different aspects of host defenses against bacterial infection, including leukocyte chemotaxis and pathogen phagocytosis.[24,25] It has also been demonstrated that LTA is a potent stimulator of MAPKs pathways, including ERK1/2, p38 MAPK, and JNK1/2 in several cell types.[17,26,27] NF-κB is a key regulator of immune responses and inflammation through the induction of numerous genes, including those coding for cytokines, chemokines, and adhesion molecules.[28] Rac1 has been shown to regulate peptidoglycan-induced NF-κB activation and COX-2 expression by activating the PI3K/Akt pathway.[22] In addition, NF-κB translocation and subsequent inflammatory responses have been shown to be mediated via TLR2.[29] However, whether these signaling molecules participating in $cPLA_2$ and COX-2 expression induced by LTA in HTSMCs are still unknown. Thus, we investigated the roles of TLR2, MyD88, PI3K/Akt, Rac1, MAPKs, and NF-κB in regulating LTA-induced $cPLA_2$ and COX-2–dependent airway inflammation in *in vitro* and *in vivo*. We report here for the first time that in HTSMCs, TLR2, but not TLR4, is involved in the induction of $cPLA_2$ and COX-2–dependent airway inflammatory responses mediated via MyD88, PI3K/Akt, Rac1, MAPKs, and NF-κB in response to LTA.

Materials and Methods

Materials

LY294002, U0126, SP600125, SB202190, AH 6809, SC-19220, GW627368X, $AACOCF_3$, NS-398, and helenalin were from Biomol (Plymouth Meeting, PA). Metafectene transfection reagent was from Biontex (Munich, Germany). Luciferase assay kit was from Promega (Madison, WI). Anti-TLR2 and anti-TLR4 antibodies (Abs) were from Invivogen (San Diego, CA). Anti-COX-2, anti-$cPLA_2$, anti-MyD88, anti-p85α, anti-p110, anti-Rac1, anti-p65, anti-p38α, anti-p38β, anti-p38δ, anti-p38γ, anti-p42, anti-JNK2, anti–β-actin, and anti-Akt Abs were from Santa Cruz (Santa Cruz, CA). Anti–phospho-Akt, anti–phospho-p65, anti–phospho-p38 MAPK, anti–phospho-p42/p44 MAPK, anti–phospho-JNK1/2, and anti–phospho-$cPLA_2$ Abs were from Cell Signaling (Danver, MA). GAPDH was from Biogenesis (Boumemouth, UK). LTA from *Staphylococcus aureus* (L2515) and other chemicals were from Sigma (St. Louis, MO).

Cell Culture

HTSMCs were purchased from ScienCell Research Lab (San Diego, CA) and grown as previously described.[26] Experiments were performed with cells from passages 4 to 7.

Preparation of Staphylococcus aureus

Staphylococcus aureus (*S. aureus*; strain 8325; a gift of Dr. J.C. Shu, Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Taiwan) was maintained in brain heart infusion broth (BHI; Sigma). Before performing experiments, bacteria were inoculated in 10 ml of BHI and incubated at 37°C overnight. Bacteria were centrifuged at 800*g* and resuspended in prewarmed PBS. Aliquots of the bacterial suspension were serially diluted and plated onto agar to quantify the cell density. Heat-killed bacteria were prepared in the same manner, but after suspension in PBS, they were heated to 80°C for 45 minutes and were assessed for nonviability by plating on BHI agar plates. Suspensions were adjusted to 10^7 colony-forming units (CFU)/ml, and then frozen with 20% glycerol in aliquots before use in cell culture experiments.

Animal Care and Experimental Procedures

Male imprinting control region mice aged 6 weeks were purchased from the National Laboratory Animal Centre (Taipei, Taiwan) and handled according to the guidelines of Animal Care Committee of Chang Gung University and National Institutes of Health Guides for the Care and Use of Laboratory Animals. Imprinting control region mice

were anesthetized with ethyl ether and placed individually on a board in a near vertical position, and the tongues were withdrawn with a lined forceps. LTA (4 mg/kg body weight) was placed posterior in the throat and aspirated into lungs. Control mice were administrated sterile H_2O. Mice regained consciousness after 15 minutes. Mice were i.p. given one dose of LY294002, U0126, SB202190, SP600125, or helenalin (2 mg/kg) for 1 hour before LTA treatment, and sacrificed after 24 hours.

Isolation of Bronchoalveolar Lavage Fluid

Mice were intratracheally administered with LTA or *S. aureus* at a dose of 4 mg/kg or 10^7 CFU/mouse, respectively and sacrificed 24 hours later. Bronchoalveolar lavage (BAL) fluid was performed through a tracheal cannula using 1 ml aliquots of ice-cold PBS medium. BAL fluid was centrifuged at 500*g* at 4°C, and cell pellets were washed and re-suspended in PBS. Leukocyte count was determined by a hemocytometer.

Measurement of IL-6 Generation

Interleukin (IL)-6 released into the medium of HTSMCs cultures was detected using an ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

Transient Transfection with siRNAs

SMARTpool RNA duplexes corresponding to human TLR2, MyD88, Akt, p38α, p38β, p38δ, p38γ, p42, JNK2, p65, $cPLA_2$, COX-2, and scrambled #2 siRNA were from Dharmacon Research Inc (Lafayette, CO). Transient transfection of siRNAs was performed using Metafectene transfection reagent. siRNA (100 nmol/L) was formulated with Metafectene transfection reagent according to the manufacturer's instruction. The transfection efficiency (approximate 60%) was determined by transfection with EGFP.

Western Blot Analysis

Growth-arrested HTSMCs were incubated with 50 μg/ml LTA at 37°C for the indicated times. The cells were washed, scraped, collected, and centrifuged at 45,000*g* at 4°C for 1 hour to yield the whole cell extract, as previously described.[26] Samples were denatured, subjected to SDS-PAGE using a 12% running gel, and transferred to nitrocellulose membrane. Membranes were incubated with anti-$cPLA_2$ or anti–COX-2 Ab for 24 hours, and then membranes were incubated with anti-mouse or anti-rabbit horseradish peroxidase Ab for 1 hour. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

RT-PCR Analysis

Total RNA was isolated with Trizol according to the protocol of the manufacturer. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification as previously described.[30] The primers used were as follows: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (sense) and 5′-CTAGAAGCATTTGCGGTGGACGATG-3′ (anti-sense) for β-actin; 5′-CTCACACCACAGAAAGTTA-AAAGAT-3′ (sense) and 5′-GCTACCACAGGCACAT-CACG-3′ (anti-sense) for $cPLA_2$; 5′-TTCAAATGAGATTGT-GGGAAAATTGCT-3′ (sense) and 5′-AGATCATCTCT-GCCTGAGTATCTT-3′ (anti-sense) for COX-2.

Isolation of Cell Fraction

Cells were harvested, sonicated for 5 seconds at output 1.5 with a sonicator (Ultrasonics Inc. NY, USA), and centrifuged at 6800*g* for 15 minutes at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 20,000*g* for 60 minutes at 4°C to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

Measurement of $cPLA_2$ and COX-2 Luciferase Activity

For construction of the $cPLA_2$-luc or COX-2-luc plasmid, human $cPLA_2$ or COX-2 promoter, a region spanning -2375 to $+75$ bp or -459 to $+9$ bp, respectively, was cloned into pGL3-basic vector (Promega, Madison, WI). $cPLA_2$-luc or COX-2-luc activity was determined as previously described using a luciferase assay system (Promega, Madison, WI).[30] Firefly luciferase activities were standardized for β-gal activity.

Measurement of PGE_2 Generation

HTSMCs were cultured in six-well culture plates. After reaching confluence, cells were treated with LTA (50 μg/ml) for the indicated times at 37°C. After treatment, the medium were collected and stored at -80°C until being assayed. PGE_2 was assayed using the PGE_2 enzyme immunoassay kit (Cayman) according to the manufacturer's instructions.

Co-Immunoprecipitation Assay

Cell lysates containing 1 mg of protein were incubated with 2 μg of anti-TLR2 or anti-MyD88 Ab at 4°C for 24 hours, and then 10 μl of 50% protein A-agarose beads was added and mixed at 4°C for 24 hours. The immunoprecipitates were collected and washed three times with lysis buffer without Triton X-100. 5X Laemmli buffer was added, subjected to electrophoresis on 12% SDS-PAGE, and then blotted using an anti-TLR2, anti-MyD88, anti-p85α, anti-p110, or anti-Rac1 Ab.

Immunofluorescence Staining

HTSMCs were plated on six-well culture plates with coverslips. Cells were further cultured in DMEM/F-12 containing 1% FBS for 24 hours, and then incubated with 50 μg/ml LTA for the indicated times. Cells were fixed, permeabilized, and

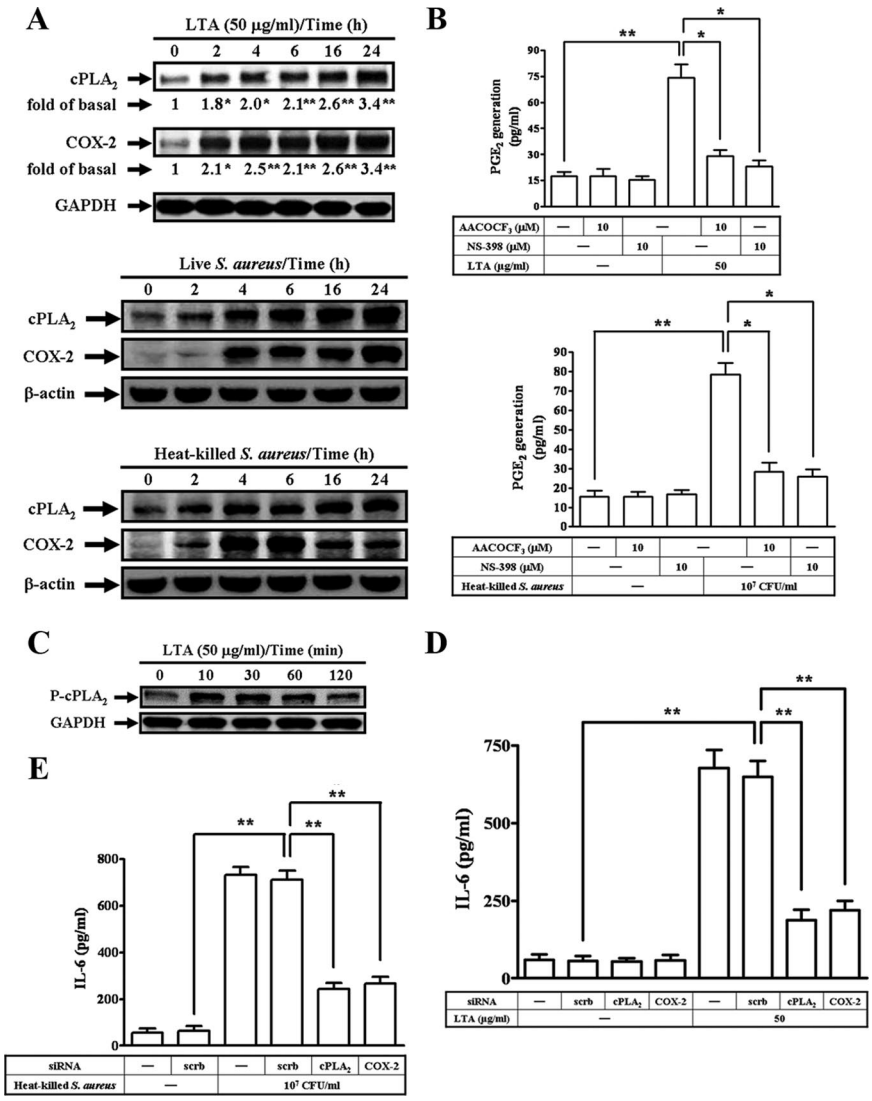

Figure 1. LTA and *S. aureus* induce PGE_2 and IL-6 generation in a $cPLA_2$/COX-2–dependent manner. **A:** HTSMCs were incubated with 50 μg/ml LTA or 10^7 CFU/ml live or heat-killed *S. aureus* for the indicated time intervals. The expression of $cPLA_2$ and COX-2 were determined by Western blot. **B:** HTSMCs were pretreated with the inhibitor of $cPLA_2$ ($AACOCF_3$) or COX-2 (NS-398) for 1 hour, and then incubated with 50 μg/ml LTA or 10^7 CFU/ml heat-killed *S. aureus* for 24 hours. The generation of PGE_2 was determined. **C:** HTSMCs were stimulated with 50 μg/ml LTA for the indicated time intervals. The cell lysates were subjected to Western blot using an anti–phospho-$cPLA_2$ or anti-GAPDH Ab. HTSMCs were transfected with scrambled siRNA, $cPLA_2$ siRNA, or COX-2 siRNA, and then challenged with LTA (**D**) or heat-killed *S. aureus* (**E**) for 24 hours. The generation of IL-6 was determined. Data are expressed as mean ± SEM of at least three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ as compared with the basal level (**A**). Significant differences between the compared groups are indicated: $^*P < 0.05$; $^{**}P < 0.01$ (**B**, **D**, **E**).

stained using an anti-p65 Ab as described previously.[30] The images observed under a fluorescence microscope (Zeiss, Axiovert 200M).

Analysis of Data

Concentration-effect curves were fitted and EC_{50} values were estimated using the GraphPad Prism Program (GraphPad, San Diego, CA). Data were expressed as the mean ± SEM and analyzed by one-way analysis of variance followed with Tukey *post hoc* test. $P < 0.05$ was considered significant.

Results

LTA and S. aureus *Induce $cPLA_2$/COX-2–Dependent PGE_2 and IL-6 Generation in HTSMCs*

LTA plays a role in the pathogenesis of severe inflammatory responses induced by Gram-positive bacterial infection, such as *S. aureus*.[2] To determine the effects of LTA and *S. aureus* on $cPLA_2$ and COX-2 expression, HTSMCs were incubated with LTA (50 μg/ml) or *S. aureus* (10^7 CFU/ml) for the indicated time intervals. We found that LTA and *S. aureus* markedly induced $cPLA_2$ and COX-2 protein expression in a time-dependent manner (Figure 1A). Moreover, LTA also enhanced $cPLA_2$ and COX-2 mRNA accumulation in a time-dependent manner (Supplemental Figure S1 at *http://ajp.amjpathol.org*). In addition, incubation with LTA or *S. aureus* stimulated $cPLA_2$ and COX-2 promoter activities or PGE_2 synthesis (Supplemental Figure S1 at *http://ajp.amjpathol.org*). Pretreatment with the inhibitors of $cPLA_2$ ($AACOCF_3$) or COX-2 (NS-398) attenuated PGE_2 synthesis induced by LTA or *S. aureus* (Figure 1B). Increased PGE_2 synthesis has been shown to be dependent on an increase in $cPLA_2$ activity in various cell types.[18,19] We further investigated the effect of LTA on the phosphorylation of $cPLA_2$ in HTSMCs. As shown in Figure 1C, LTA stimulated $cPLA_2$ phosphorylation in a time-dependent manner. These data suggested that PGE_2 generation induced by LTA or *S. aureus* was dependent on the up-regulation of $cPLA_2$ and

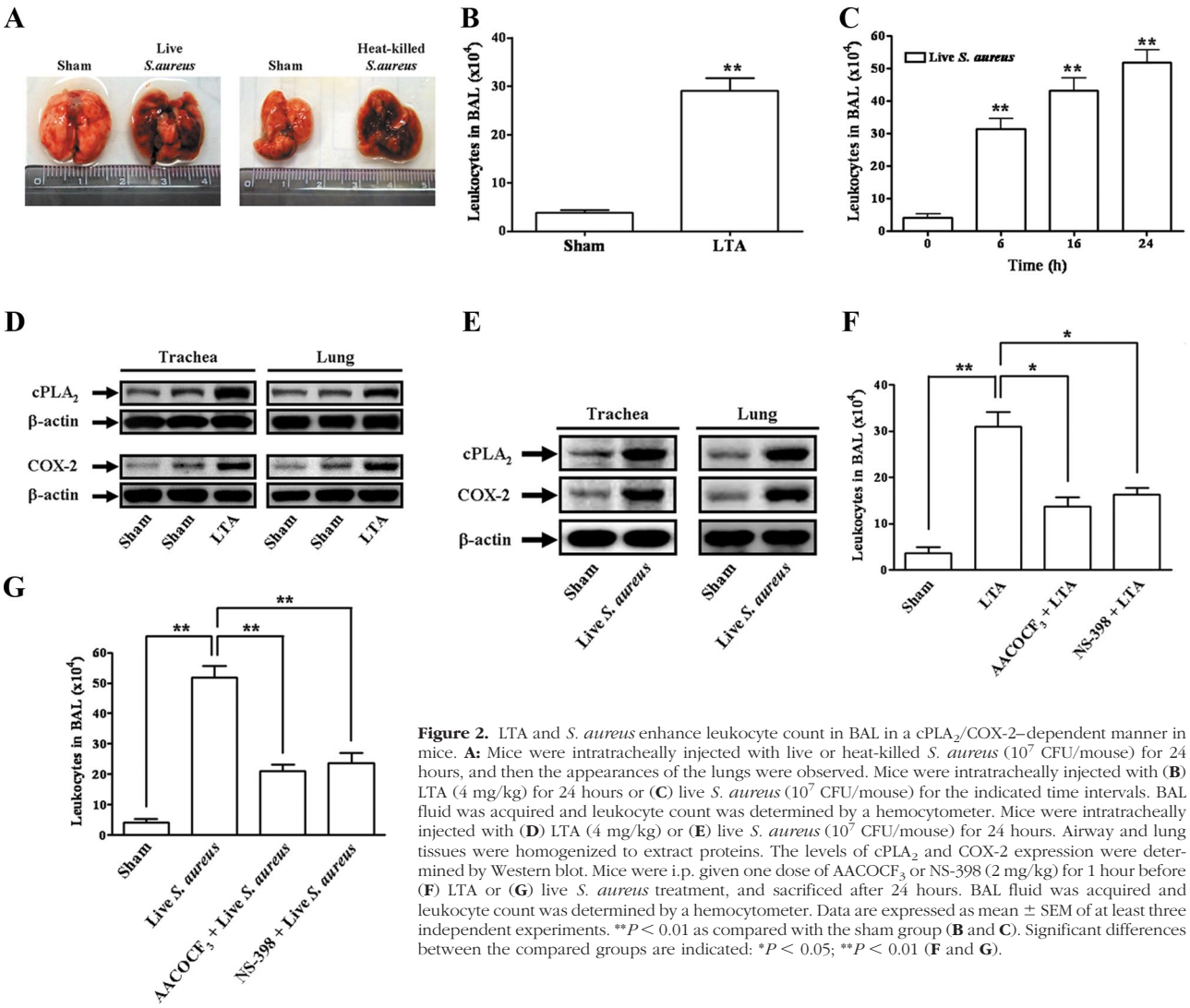

Figure 2. LTA and *S. aureus* enhance leukocyte count in BAL in a $cPLA_2$/COX-2–dependent manner in mice. **A:** Mice were intratracheally injected with live or heat-killed *S. aureus* (10^7 CFU/mouse) for 24 hours, and then the appearances of the lungs were observed. Mice were intratracheally injected with (**B**) LTA (4 mg/kg) for 24 hours or (**C**) live *S. aureus* (10^7 CFU/mouse) for the indicated time intervals. BAL fluid was acquired and leukocyte count was determined by a hemocytometer. Mice were intratracheally injected with (**D**) LTA (4 mg/kg) or (**E**) live *S. aureus* (10^7 CFU/mouse) for 24 hours. Airway and lung tissues were homogenized to extract proteins. The levels of $cPLA_2$ and COX-2 expression were determined by Western blot. Mice were i.p. given one dose of $AACOCF_3$ or NS-398 (2 mg/kg) for 1 hour before (**F**) LTA or (**G**) live *S. aureus* treatment, and sacrificed after 24 hours. BAL fluid was acquired and leukocyte count was determined by a hemocytometer. Data are expressed as mean ± SEM of at least three independent experiments. $^{**}P < 0.01$ as compared with the sham group (**B** and **C**). Significant differences between the compared groups are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$ (**F** and **G**).

COX-2 in HTSMCs. LTA or LPS has been shown to stimulate the expression of various proinflammatory mediators, including IL-6, TNF-α, or IL-1β.[4] Moreover, we also found that LTA and *S. aureus* induced IL-6 secretion in HTSMCs (Supplemental Figure S1 at *http://ajp.amjpathol.org*). To determine whether LTA and *S. aureus* could induce IL-6 generation via a $cPLA_2$/COX-2-dependent signaling, as shown in Figure 1, D and E, transfection of HTSMCs with $cPLA_2$ siRNA or COX-2 siRNA attenuated IL-6 production induced by LTA and *S. aureus*, suggesting that LTA and *S. aureus* induced PGE_2 and IL-6 secretion in a $cPLA_2$/COX-2–dependent manner in these cells.

LTA and S. aureus *Regulate $cPLA_2$ and COX-2 Expression and Leukocyte Count in BAL Fluid* in Vivo *in Mice*

In an *in vivo* study, mice were intratracheally administered with LTA or *S. aureus*. As shown in Figure 2A, *S. aureus* markedly caused pulmonary hematoma. In addition, BAL fluid was acquired and airway or lung tissues were homogenized to extract proteins. As shown in Figure 2, B–E, LTA and *S. aureus* significantly enhanced leukocyte (eosinophils and neutrophils) count in BAL fluid and $cPLA_2$ and COX-2 expression in mice. LTA and *S. aureus* also increased the numbers of macrophages and lymphocytes in BAL fluid (data not shown). Moreover, mice were i.p. administered with $AACOCF_3$ or NS-398 for 1 hour, and then followed with LTA or *S. aureus*. As shown in Figure 2, F and G, administration with $AACOCF_3$ or NS-398 significantly inhibited LTA- and *S. aureus*-induced leukocyte count in BAL fluid in mice. These data demonstrated that LTA and *S. aureus* induced airway inflammation in a $cPLA_2$/COX-2-dependent manner associated with leukocyte recruitment in the airways.

Requirement of TLR2 and MyD88 for LTA-Induced $cPLA_2$ and COX-2 Expression in HTSMCs

TLRs play an important role in detection and recognition of the pathogens to initiate a rapid defensive mechanism

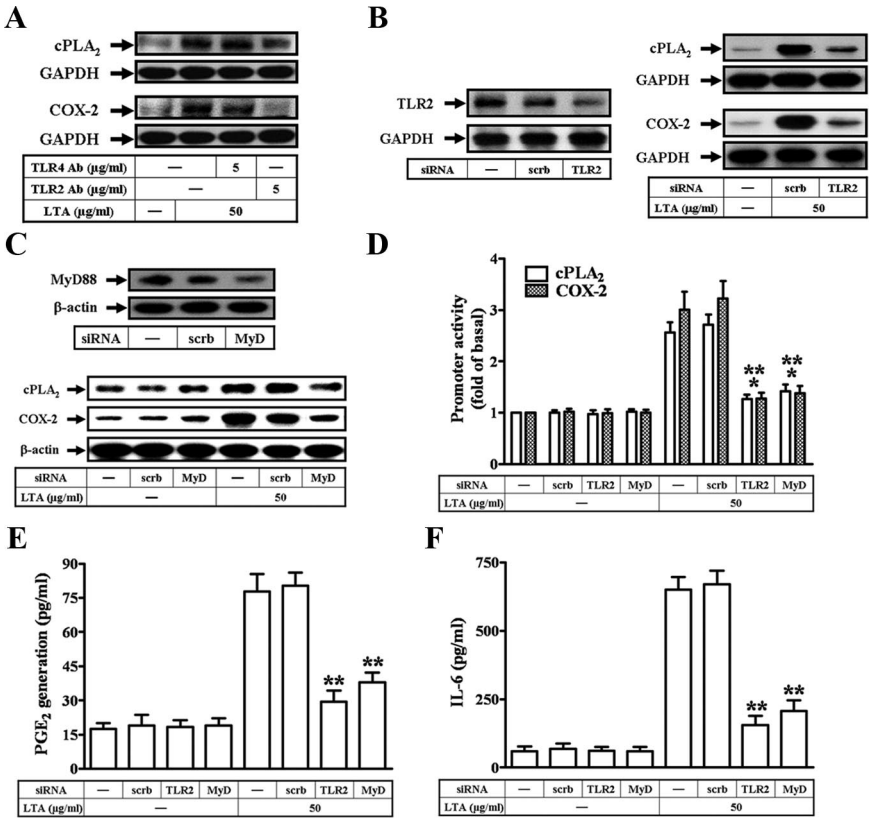

Figure 3. LTA induces $cPLA_2$ and COX-2 expression through a MyD88-dependent TLR2 signaling pathway. **A:** HTSMCs were pretreated with an anti-TLR2 or anti-TLR4 Ab for 1 hour, and then incubated with 50 μg/ml LTA for 24 hours. The expression of $cPLA_2$ and COX-2 were determined by Western blot. **B:** HTSMCs were transfected with scrambled siRNA or TLR2 siRNA, and then challenged with LTA for 24 hours. The protein levels of TLR2, $cPLA_2$, and COX-2 were determined by Western blot. **C:** HTSMCs were transfected with scrambled siRNA or MyD88 siRNA, and then challenged with LTA for 24 hours. The protein levels of MyD88, $cPLA_2$, and COX-2 were determined by Western blot. **D:** HTSMCs were co-transfected with TLR2 siRNA, MyD88 siRNA, or scrambled siRNA and $cPLA_2$-luc or COX-2-luc reporter gene, and then treated with 50 μg/ml LTA for 4 hours. The $cPLA_2$ and COX-2 promoter activities were determined in the cell lysates. **E** and **F:** HTSMCs were transfected with scrambled siRNA, TLR2 siRNA, or MyD88 siRNA, and then challenged with LTA for 24 hours. The media were collected and analyzed for PGE_2 and IL-6 production. Data are expressed as mean ± SEM of at least three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ as compared with those of the scrambled siRNA-transfected cells exposed to LTA.

in the host.[31] We have previously demonstrated that both TLR2 and TLR4 are expressed on HTSMCs.[26] To investigate whether the LTA-induced $cPLA_2$ and COX-2 expression were mediated through these TLRs, as illustrated in Figure 3A, LTA-induced $cPLA_2$ and COX-2 expression were inhibited by pretreatment with an anti-TLR2 Ab, but not an anti-TLR4 Ab, indicating that LTA induced these responses via TLR2 in HTSMCs. To confirm these results, HTSMCs were transfected with TLR2 siRNA, and then incubated with LTA for 24 hours. We found that transfection of HTSMCs with TLR2 siRNA down-regulated the expression of total TLR2 protein and also attenuated LTA-induced $cPLA_2$ and COX-2 expression (Figure 3B). MyD88 is an adaptor protein that is shared by all TLR pathways.[31] To determine whether the induction of $cPLA_2$ and COX-2 expression occurred through MyD88, as shown in Figure 3C, transfection of HTSMCs with MyD88 siRNA down-regulated the expression of total MyD88 protein and markedly inhibited $cPLA_2$ and COX-2 expression induced by LTA. The down-regulation of $cPLA_2$ and COX-2 protein expression were further confirmed by the reduction of $cPLA_2$ and COX-2 promoter activities induced by LTA in HTSMCs transfected with TLR2 siRNA or MyD88 siRNA (Figure 3D). Moreover, transfection with TLR2 siRNA or MyD88 siRNA also inhibited PGE_2 or IL-6 release induced by LTA (Figure 3, E and F). These results suggested that LTA-induced $cPLA_2$ and COX-2 expression were mediated through a MyD88-dependent TLR2 signaling pathway in HTSMCs.

LTA Induces $cPLA_2$ and COX-2 Expression via PI3K/Akt

PI3K represents a key signaling molecule that transduces extracellular signals to gene expression in different cell types.[32] Thus, we investigated the role of PI3K in LTA-induced $cPLA_2$ and COX-2 expression and PGE_2 or IL-6 generation. As shown in Figure 4A, pretreatment of HTSMCs with a PI3K inhibitor (LY294002) attenuated LTA-induced $cPLA_2$ and COX-2 expression in a concentration-dependent manner. Moreover, pretreatment of HTSMCs with LY294002 also inhibited $cPLA_2$ and COX-2 mRNA expression and promoter activities and PGE_2 or IL-6 release induced by LTA (Supplemental Figure S2 at *http://ajp.amjpathol.org*). These results suggested that PI3K is involved in LTA-induced $cPLA_2$ and COX-2 expression in HTSMCs. Akt is a serine/threonine kinase that is implicated in mediating a variety of biological responses, including cell growth, proliferation, and survival.[23] Akt is activated by phosphorylation on Ser^{473}.[23] Thus, we further examined Akt phosphorylation at Ser^{473} stimulated by LTA in HTSMCs using an anti–phospho-Akt Ab at Ser^{473}. As shown in Figure 4B, LTA stimulated a time-dependent Ser^{473} phosphorylation of Akt with a maximal response within 5 to 10 minutes. Moreover, pretreatment of HTSMCs with LY294002 (30 μmol/L) significantly attenuated Akt phosphorylation in response to LTA during the period of observation. To further ensure that LTA-induced $cPLA_2$ and COX-2 expression were mediated via Akt, as shown in Figure 4C, transfection

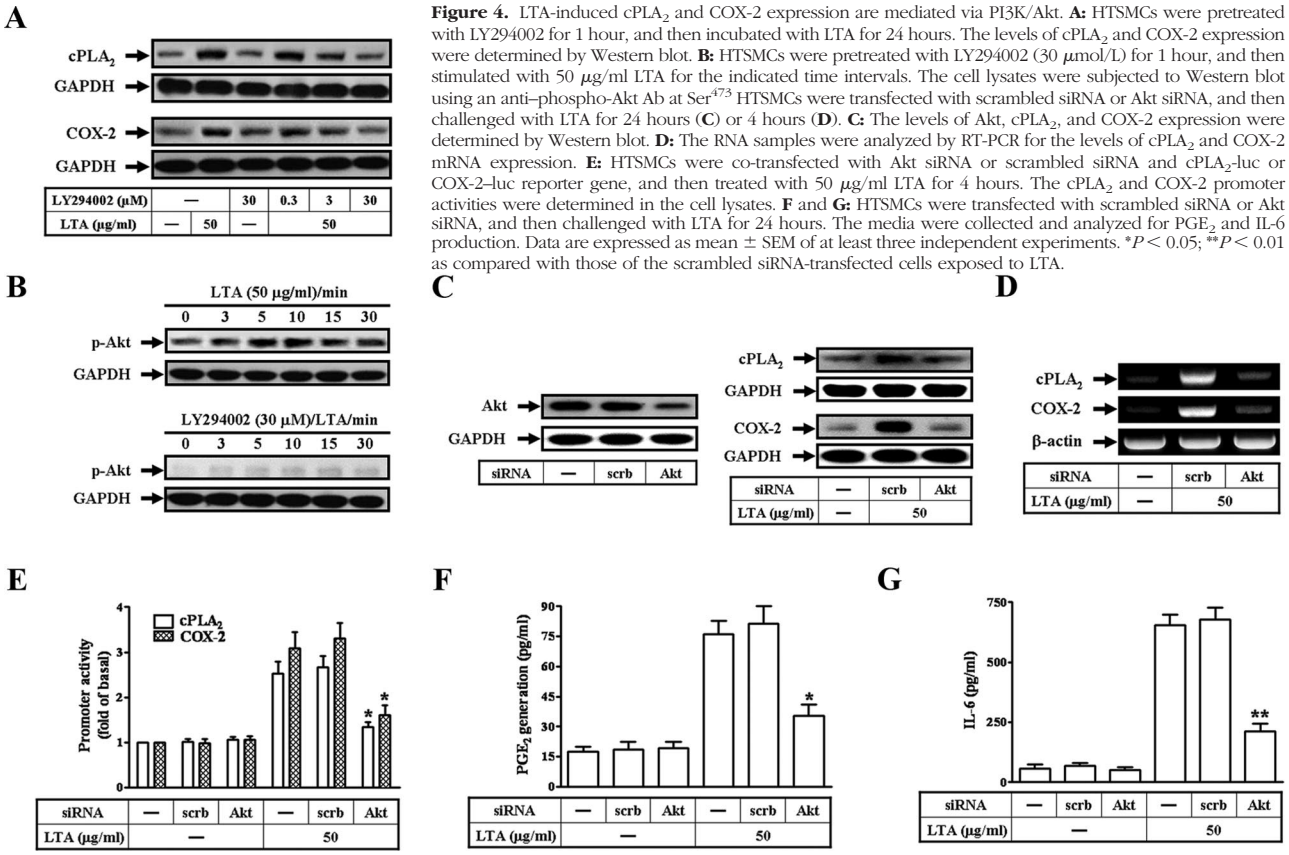

Figure 4. LTA-induced $cPLA_2$ and COX-2 expression are mediated via PI3K/Akt. **A:** HTSMCs were pretreated with LY294002 for 1 hour, and then incubated with LTA for 24 hours. The levels of $cPLA_2$ and COX-2 expression were determined by Western blot. **B:** HTSMCs were pretreated with LY294002 (30 μmol/L) for 1 hour, and then stimulated with 50 μg/ml LTA for the indicated time intervals. The cell lysates were subjected to Western blot using an anti–phospho-Akt Ab at Ser^{473} HTSMCs were transfected with scrambled siRNA or Akt siRNA, and then challenged with LTA for 24 hours (**C**) or 4 hours (**D**). **C:** The levels of Akt, $cPLA_2$, and COX-2 expression were determined by Western blot. **D:** The RNA samples were analyzed by RT-PCR for the levels of $cPLA_2$ and COX-2 mRNA expression. **E:** HTSMCs were co-transfected with Akt siRNA or scrambled siRNA and $cPLA_2$-luc or COX-2–luc reporter gene, and then treated with 50 μg/ml LTA for 4 hours. The $cPLA_2$ and COX-2 promoter activities were determined in the cell lysates. **F** and **G:** HTSMCs were transfected with scrambled siRNA or Akt siRNA, and then challenged with LTA for 24 hours. The media were collected and analyzed for PGE_2 and IL-6 production. Data are expressed as mean ± SEM of at least three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ as compared with those of the scrambled siRNA-transfected cells exposed to LTA.

with Akt siRNA significantly down-regulated the expression of total Akt protein and subsequently led to a decrease of $cPLA_2$ and COX-2 protein expression in response to LTA. This down-regulation of $cPLA_2$ and COX-2 protein expression resulted from the reduction of $cPLA_2$ and COX-2 mRNA levels (Figure 4D) and promoter activities (Figure 4E) in HTSMCs transfected with Akt siRNA. In addition, transfection with Akt siRNA also reduced LTA-stimulated PGE_2 and IL-6 release (Figure 4, F and G). Taken together, these data suggested that LTA induces $cPLA_2$ and COX-2 expression via a PI3K/Akt signaling in HTSMCs.

LTA- and S. aureus*—Induced $cPLA_2$ and COX-2 Expression Are Mediated via the Formation of a TLR2/MyD88/PI3K/Rac1 Complex*

MyD88 plays a key role in TLRs signaling.[31] Our previous study has shown that LTA induces the association of TLR2/MyD88 in HTSMCs.[7] In addition, LTA has been shown to induce the formation of a TLR2/PI3K complex.[6] PI3K has been shown to activate a downstream component Rac1 in SH-SY5Y cells.[33] Thus, we further investigated the physical association among TLR2, MyD88, PI3K, and Rac1 in LTA- and *S. aureus*-induced $cPLA_2$ and COX-2 expression. HTSMCs were stimulated with LTA (50 μg/ml) or *S. aureus* (10^7 CFU/ml) for the indicated time intervals. The cell lysates were subjected to immunoprecipitation using an anti-TLR2 or anti-MyD88 Ab, and then the immunoprecipitates were analyzed by Western blot using an anti-MyD88, anti-p85α, anti-p110, anti-Rac1, or anti-TLR2 Ab. As shown in Figure 5A, the protein levels of MyD88, p85α, p110, and Rac1 were time-dependently increased in a TLR2-immunoprecipitated complex. Moreover, the protein levels of TLR2, p85α, p110, and Rac1 were also up-regulated in MyD88-immunoprecipitated complex (Figure 5B). These results suggested

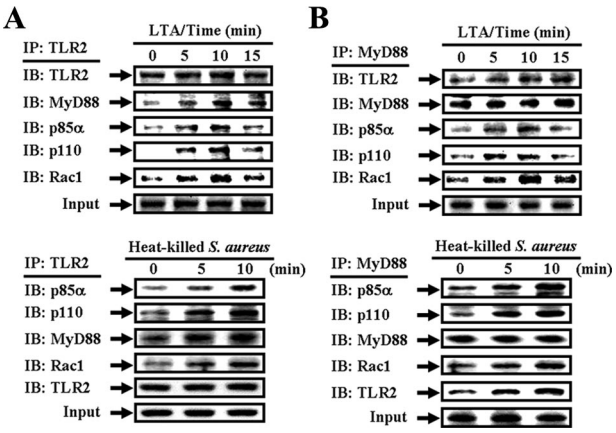

Figure 5. LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression are mediated through the formation of a TLR2/MyD88/PI3K/Rac1 complex. HTSMCs were stimulated with 50 μg/ml LTA or 10^7 CFU/ml heat-killed *S. aureus* for the indicated time intervals. The cell lysates were subjected to immunoprecipitation with anti-TLR2 (**A**) or anti-MyD88 Ab (**B**), and then the immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using an anti-TLR2, anti-MyD88, anti-p85α, anti-p110, or anti-Rac1 Ab.

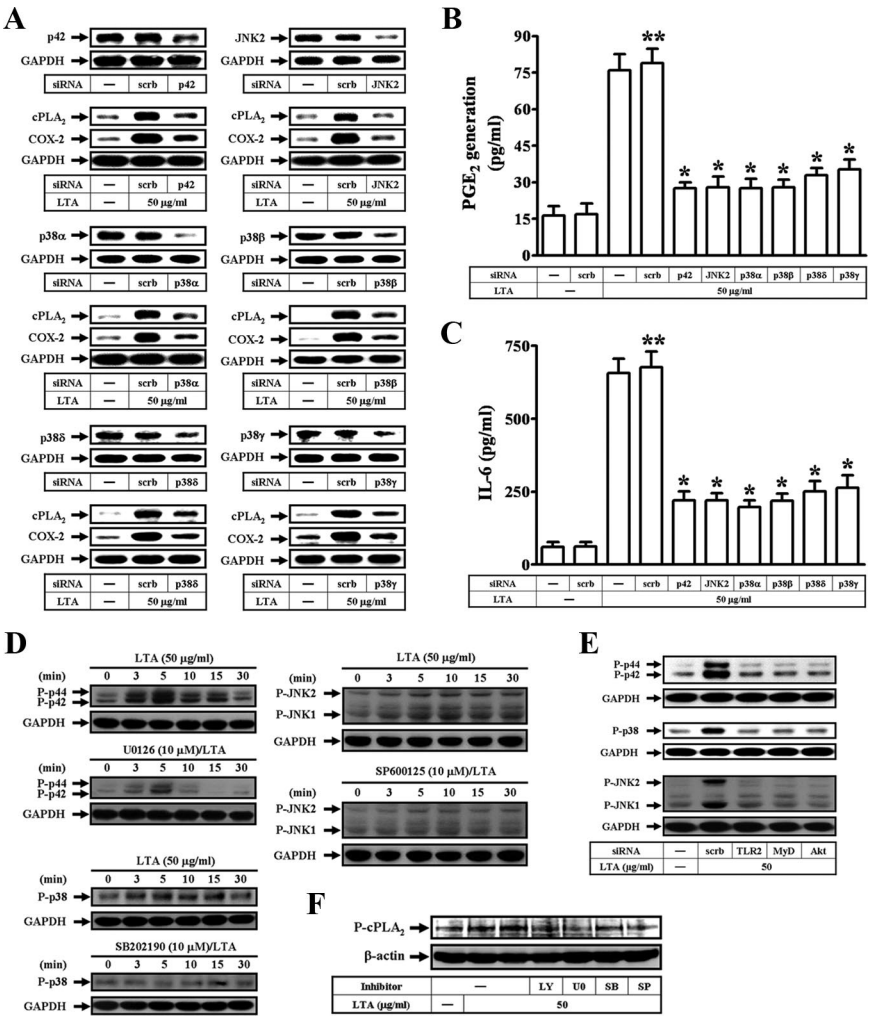

Figure 6. Involvement of TLR2-dependent MAPKs phosphorylation in LTA-mediated $cPLA_2$ and COX-2 expression in HTSMCs. Cells were transfected with the siRNA of scrambled, p42, p38α, p38β, p38δ, p38γ, or JNK2, and then challenged with LTA for 24 hours. **A:** The levels of p42, p38α, p38β, p38δ, p38γ, JNK2, $cPLA_2$, and COX-2 expression were determined by Western blot. **B** and **C:** The media were collected and analyzed for PGE_2 and IL-6 release, respectively. **D:** HTSMCs were pretreated without or with 10 μmol/L U0126, 10 μmol/L SB202190, or 10 μmol/L SP600125 for 1 hour, and then stimulated with 50 μg/ml LTA for the indicated time intervals. The cell lysates were subjected to Western blot using an anti–phospho-p42/p44 MAPK, anti-phospho-p38, anti-phospho-JNK1/2, or anti-GAPDH Ab. **E:** HTSMCs were transfected with the siRNA of scrambled, TLR2, MyD88, or Akt, and then incubated with 50 μg/ml LTA for 5 minutes. The cell lysates were subjected to Western blot using an anti–phospho-p42/p44 MAPK, anti–phospho-p38, anti–phospho-JNK1/2, or anti-GAPDH Ab. **F:** HTSMCs were pretreated with 30 μmol/L LY294002, 10 μmol/L U0126, 10 μmol/L SB202190, or 10 μmol/L SP600125 for 1 hour, and then stimulated with LTA for 10 minutes. The cell lysates were subjected to Western blot using an anti–phospho-$cPLA_2$ or anti–β-actin Ab. Data are expressed as mean ± SEM of at least three independent experiments. $^{**}P < 0.01$ as compared with those of the scrambled siRNA-transfected cells. $^{*}P < 0.05$ as compared with those of the scrambled siRNA-transfected cells exposed to LTA.

that LTA and *S. aureus* trigger the association among TLR2, MyD88, PI3K, and Rac1 leading to $cPLA_2$ and COX-2 expression in HTSMCs.

Involvement of ERK1/2, p38 MAPK, and JNK1/2 in LTA-Induced $cPLA_2$ and COX-2 Expression

Recently, MAPK family members have been shown to regulate $cPLA_2$ and COX-2 expression in various cell types.[34,35] In this study, we investigated the roles of ERK1/2, p38 MAPK, and JNK1/2 in LTA-mediated $cPLA_2$ and COX-2 expression in HTSMCs. It was found that LTA-enhanced $cPLA_2$ and COX-2 protein and mRNA expression, promoter activities, and PGE_2 or IL-6 generation were inhibited by pretreatment with the inhibitors of MEK1/2 (U0126), p38 MAPK (SB202190), or JNK1/2 (SP600125; Supplemental Figure S3 at *http://ajp.amjpathol.org*). To further ensure that LTA-induced $cPLA_2$ and COX-2 expression were mediated via these MAPKs, transfection of HTSMCs with siRNA of p42, p38α, p38β, p38γ, p38δ, or JNK2 knocked down the expression of respective protein and subsequently attenuated LTA-enhanced $cPLA_2$ and COX-2 expression and PGE_2 and IL-6 release (Figure 6, A–C). In addition, LTA also significantly stimulated ERK1/2, p38 MAPK, and JNK1/2 phosphorylation, which were inhibited by pretreatment with U0126, SB202190, and SP600125, respectively (Figure 6D) or transfection with siRNA of TLR2, MyD88, or Akt (Figure 6E). MAPKs have been shown to mediate the phosphorylation of $cPLA_2$.[36] Thus, we next investigated whether the involvement of PI3K/Akt, ERK1/2, p38 MAPK, or JNK1/2 in LTA-stimulated $cPLA_2$ phosphorylation. We found that pretreatment with LY294002, U0126, SB202190, or SP600125 inhibited LTA-stimulated $cPLA_2$ phosphorylation in HTSMCs (Figure 6F). These results suggested that LTA-stimulated $cPLA_2$ activation via MAPKs phosphorylation in a TLR2/MyD88/PI3K/Akt-dependent manner in HTSMCs.

LTA-Induced $cPLA_2$ and COX-2 Expression Are Mediated via a TLR2-Dependent NF-κB Signaling

Inflammatory responses after stimulation with various insults, such as LPS and LTA, are highly dependent on NF-κB activation.[15,20] In addition, NF-κB has been shown to regulate $cPLA_2$ and COX-2 expression in various cell types.[15,22,34] Moreover, in this study, we found that LTA-

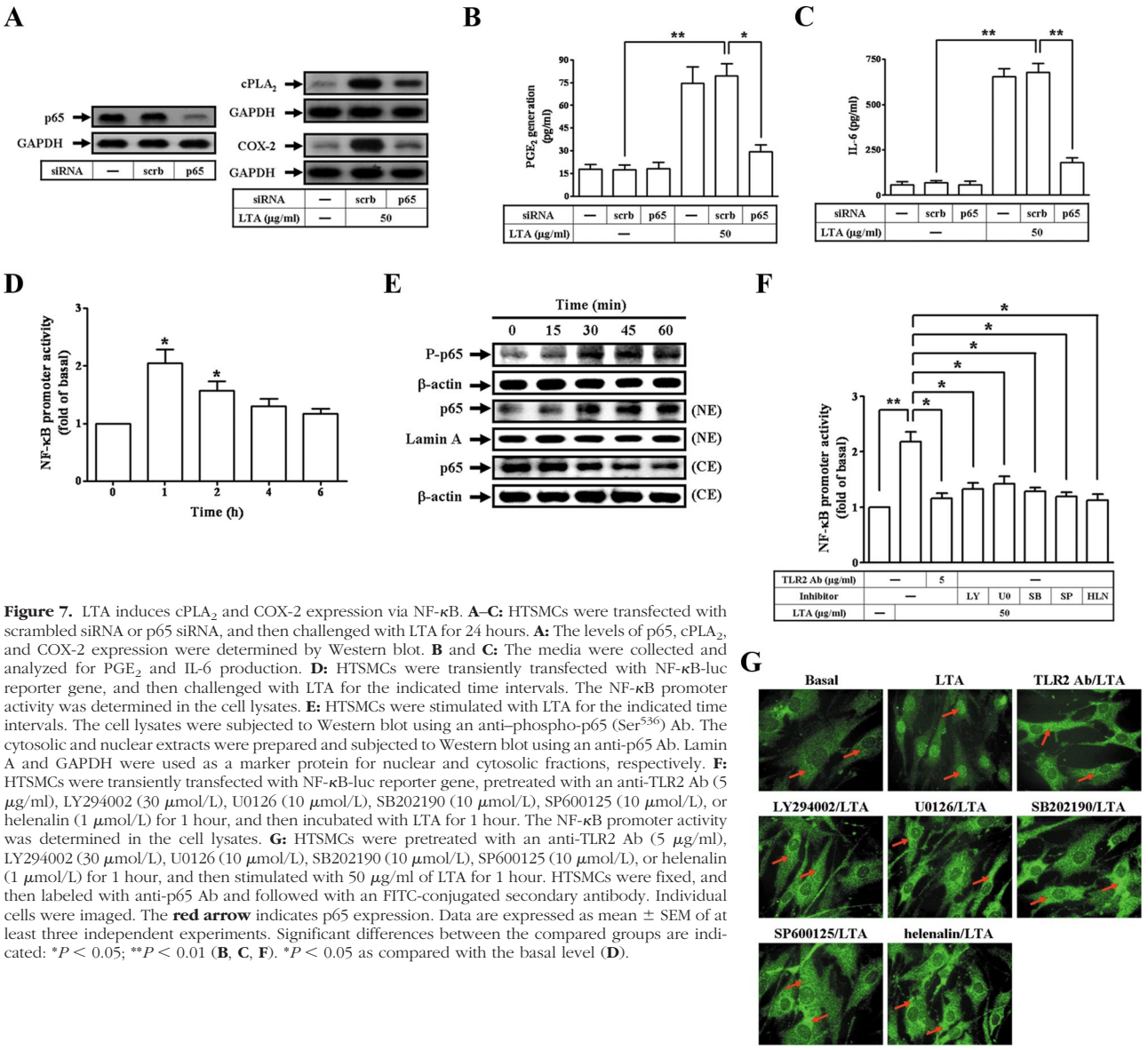

Figure 7. LTA induces $cPLA_2$ and COX-2 expression via NF-κB. **A–C:** HTSMCs were transfected with scrambled siRNA or p65 siRNA, and then challenged with LTA for 24 hours. **A:** The levels of p65, $cPLA_2$, and COX-2 expression were determined by Western blot. **B** and **C:** The media were collected and analyzed for PGE_2 and IL-6 production. **D:** HTSMCs were transiently transfected with NF-κB-luc reporter gene, and then challenged with LTA for the indicated time intervals. The NF-κB promoter activity was determined in the cell lysates. **E:** HTSMCs were stimulated with LTA for the indicated time intervals. The cell lysates were subjected to Western blot using an anti–phospho-p65 (Ser^{536}) Ab. The cytosolic and nuclear extracts were prepared and subjected to Western blot using an anti-p65 Ab. Lamin A and GAPDH were used as a marker protein for nuclear and cytosolic fractions, respectively. **F:** HTSMCs were transiently transfected with NF-κB-luc reporter gene, pretreated with an anti-TLR2 Ab (5 μg/ml), LY294002 (30 μmol/L), U0126 (10 μmol/L), SB202190 (10 μmol/L), SP600125 (10 μmol/L), or helenalin (1 μmol/L) for 1 hour, and then incubated with LTA for 1 hour. The NF-κB promoter activity was determined in the cell lysates. **G:** HTSMCs were pretreated with an anti-TLR2 Ab (5 μg/ml), LY294002 (30 μmol/L), U0126 (10 μmol/L), SB202190 (10 μmol/L), SP600125 (10 μmol/L), or helenalin (1 μmol/L) for 1 hour, and then stimulated with 50 μg/ml of LTA for 1 hour. HTSMCs were fixed, and then labeled with anti-p65 Ab and followed with an FITC-conjugated secondary antibody. Individual cells were imaged. The **red arrow** indicates p65 expression. Data are expressed as mean ± SEM of at least three independent experiments. Significant differences between the compared groups are indicated: $^*P < 0.05$; $^{**}P < 0.01$ (**B**, **C**, **F**). $^*P < 0.05$ as compared with the basal level (**D**).

enhanced $cPLA_2$ and COX-2 protein and mRNA expression, promoter activities, and PGE_2 or IL-6 generation were inhibited by pretreatment with helenalin (an inhibitor of NF-κB; Supplemental Figure S4 at *http://ajp.amjpathol.org*). To confirm these results, as shown in Figure 7, A–C, transfection of HTSMCs with p65 siRNA down-regulated the expression of total p65 protein and markedly inhibited LTA-induced $cPLA_2$ and COX-2 expression and PGE_2 or IL-6 generation. LTA also stimulated an increase in NF-κB promoter activity (Figure 7D) and the phosphorylation and translocation of NF-κB (p65) (Figure 7E). We next investigated the roles of TLR2, PI3K/Akt, and MAPKs in LTA-mediated NF-κB promoter activity and translocation. Our results showed that LTA-induced NF-κB promoter activity and nuclear translocation were inhibited by pretreatment with an anti-TLR2 Ab, LY294002, U0126, SB202190, SP600125, or helenalin, determined by promoter assay and immunofluorescence staining, respectively (Figure 7, F and G). These results suggested that LTA-induced $cPLA_2$/COX-2–dependent PGE_2 and IL-6 generation are mediated through a TLR2-dependent NF-κB signaling in HTSMCs.

LTA- and S. aureus *Induce cPLA$_2$ and COX-2 Expression via PI3K/Akt, MAPKs, and NF-κB* in Vivo *in Mice and* in Vitro *in HTSMCs*

To further determine whether LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression were mediated via PI3K/Akt, MAPKs, and NF-κB *in vivo*, mice were i.p. administered with LY294002, U0126, SB202190, SP600125, or helenalin for 1 hour, and then followed with LTA or *S. aureus*. As shown in Figure 8, A and B, administration with these inhibitors significantly attenuated LTA- and *S. aureus*–induced leukocyte count in BAL fluid in mice. In addition, LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression were also attenuated by administration with these inhibitors (Figure 8, C and D). On the other hand,

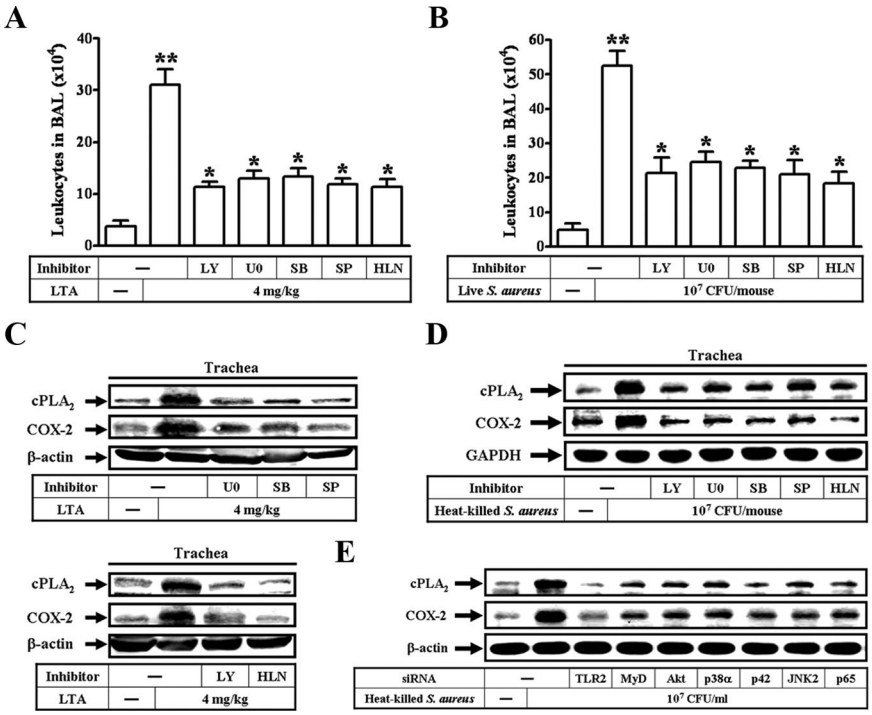

Figure 8. LTA and *S. aureus* induce leukocytes in BAL and $cPLA_2$ and COX-2 expression in mice via PI3K/Akt, MAPKs, and NF-κB. Mice were given one dose of LY294002, U0126, SB202190, SP600125, or helenalin (2 mg/kg) for 1 hour before LTA (**A**) or live *S. aureus* treatment (**B**), and sacrificed after 24 hours. BAL fluid was acquired and leukocyte count was determined by a hemocytometer. Mice were i.p. given one dose of LY294002, U0126, SB202190, SP600125, or helenalin (2 mg/kg) for 1 hour before LTA (**C**) or heat-killed *S. aureus* treatment (**D**), and sacrificed after 24 hours. Airway tissues were homogenized to extract protein. The levels of $cPLA_2$ and COX-2 expression were determined by Western blot. **E:** HTSMCs were transfected with siRNAs of TLR2, MyD88, Akt, p38α, p42, JNK2, or p65, and then incubated with heat-killed *S. aureus* for 24 hours. The levels of $cPLA_2$ and COX-2 expression were determined by Western blot. Data are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$ as compared with the sham group. $^{*}P < 0.05$ as compared with the mice exposed to LTA (**A**) or live *S. aureus* (**B**) alone.

transfection with the siRNA of TLR2, MyD88, Akt, p38α, p42, JNK2, or p65 could reduce *S. aureus*–induced $cPLA_2$ and COX-2 expression in HTSMCs (Figure 8E). These data suggested that LTA induces $cPLA_2$ and COX-2 expression via PI3K/Akt, MAPKs, and NF-κB in the airways. In addition, *S. aureus* could trigger $cPLA_2$ and COX-2 expression via a similar signaling cascade in HTSMCs.

Arachidonic Acid Enhances $cPLA_2$ Phosphorylation and $cPLA_2$/COX-2 Expression in HTSMCs

Metabolites of AA and lysophosphatides have been identified *in situ* in airway secretion of asthmatics.[8,9] AA has also been shown to induce COX-2 protein expression in PC-3 human prostate cancer cells.[37] Thus, we further investigated the effects of AA on $cPLA_2$ phosphorylation or $cPLA_2$ and COX-2 expression in HTSMCs. Cells were incubated with 30 μmol/L AA for the indicated time intervals. We found that AA markedly enhanced $cPLA_2$ phosphorylation (Figure 9A) or $cPLA_2$ and COX-2 expression (Figure 9B) in a time-dependent manner. These data suggested that AA could cause a positive feedback response, and then amplify the airway inflammatory responses through the induction of $cPLA_2$ and COX-2 expression.

PGE_2 Regulates IL-6 Release in Vitro *in HTSMCs and Leukocyte Count in BAL Fluid* in Vivo *in Mice*

PGE_2 has been shown to regulate IL-6 production in RAW 264.7 macrophages.[38] In addition, PGE_2, one of the major PGs products, exerts its biological activities by binding to specific cell surface receptors, designated PGE_2 receptors (EPs).[39] Thus, we investigated whether LTA or *S. aureus* could induce IL-6 production via PGE_2 in HTSMCs. Cells were pretreated with AH 6809 (an EP1 and EP2 receptor antagonist), SC-19220 (an EP1 receptor antagonist), or GW627368X (an EP4 receptor antag-

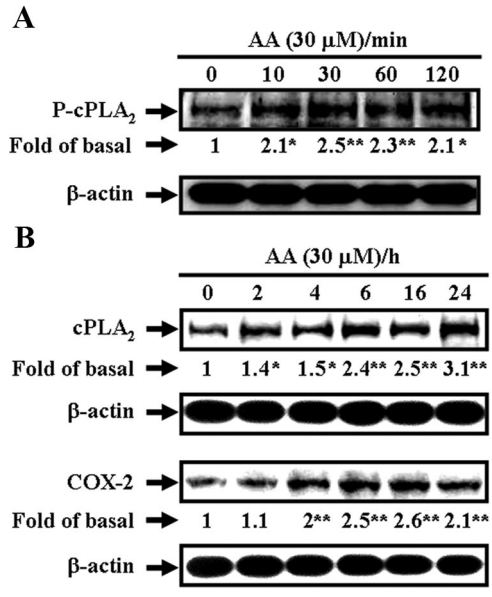

Figure 9. Arachidonic acid induces $cPLA_2$ phosphorylation and $cPLA_2$ and COX-2 expression in HTSMCs. Cells were incubated with 30 μmol/L arachidonic acid (AA) for the indicated times. **A:** The cell lysates were subjected to Western blot using an anti–phospho-$cPLA_2$ or anti–β-actin Ab. **B:** The levels of $cPLA_2$ and COX-2 expression were determined by Western blot analysis. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with the basal level.

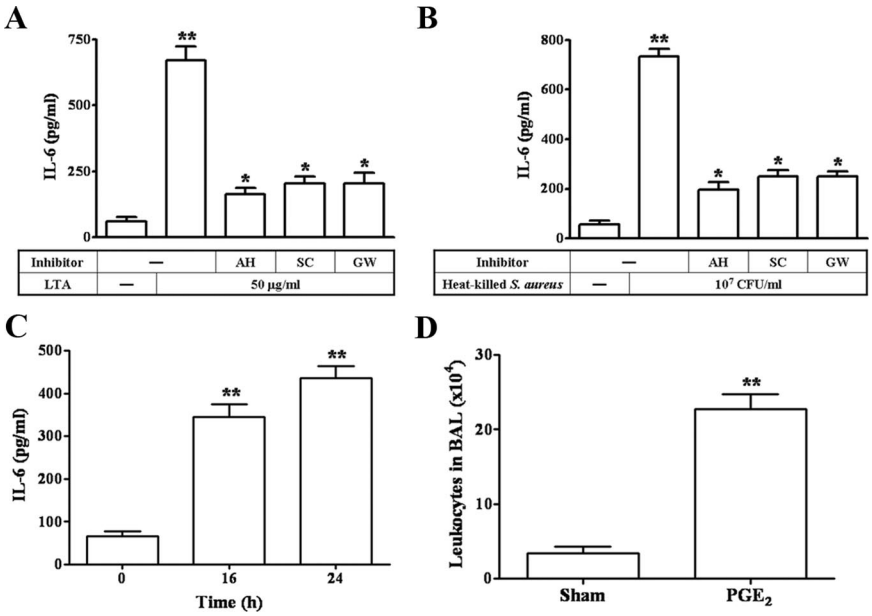

Figure 10. PGE_2 induces IL-6 production in HTSMCs and leukocytes in BAL in mice. HTSMCs were pretreated with 10 μmol/L AH 6809 (an EP1 and EP2 receptor antagonist), 10 μmol/L SC-19220 (an EP1 receptor antagonist), or 10 μmol/L GW627368X (an EP4 receptor antagonist) for 1 hour, and then incubated with LTA (**A**) or heat-killed *S. aureus* (**B**) for 24 hours. The media were collected and analyzed for IL-6 production. **C:** HTSMCs were incubated with 30 μmol/L PGE_2 for 16 hours and 24 hours, and then IL-6 generation was measured. **D:** Mice were intratracheally injected with PGE_2 (2 mg/kg) for 24 hours, and then BAL fluid was acquired and leukocyte count was determined by a hemocytometer. Data are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$ as compared with the basal level (**A**–**C**) or the sham group (**D**). $^{*}P < 0.05$ as compared with the cells exposed to LTA (**A**) or heat-killed *S. aureus* (**B**) alone.

onist) for 1 hour, and then incubated with LTA or *S. aureus*. As shown in Figure 10, A and B, pretreatment with these PGE_2 receptor antagonists markedly inhibited IL-6 secretion induced by LTA or *S. aureus*. Moreover, we also found that treatment with PGE_2 directly stimulated IL-6 generation in HTSMCs (Figure 10C). On the other hand, in an *in vivo* study, administration with PGE_2 increased leukocyte count in BAL fluid in mice (Figure 10D). Taken together, these data showed that LTA and *S. aureus* enhanced airway inflammation, at least in part, through the induction of PGE_2/IL-6 secretion.

Discussion

$cPLA_2$ and COX-2 play a pivotal role in the metabolism of AA and production of proinflammatory eicosanoids.[14,39] Moreover, $cPLA_2$ and COX-2 are thought to be important mediators in airway inflammatory responses.[15,34] Several lines of evidence suggest that high levels of PGE_2, synthesized by COX-2, are also involved in inflammatory responses.[39] LTA, a component of cell wall released from Gram-positive bacteria, has been shown to be a key player in the expression of inflammatory genes.[40] Accumulating evidence has demonstrated that LTA is a potent inducer for COX-2 or PGE_2 production, which contributes to the development of inflammation.[17,21] However, little was known about the molecular mechanisms by LTA- and *S. aureus*–induced $cPLA_2$/COX-2–dependent airway inflammation. Here, we showed, for the first time, that $cPLA_2$ and COX-2 expression and PGE_2 release were increased on stimulation with LTA and *S. aureus*. By Western blot analysis, RT-PCR, and promoter assay coupling with pharmacological inhibitors and transfection with siRNAs revealed that LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression and PGE_2 release were mediated through a TLR2/MyD88/PI3K/Rac1/Akt/MAPKs/NF-κB signaling pathway in HTSMCs. Moreover, we demonstrated that the induction of PGE_2 secretion was involved in LTA- and *S. aureus*–triggered airway inflammatory responses.

TLRs are transmembrane proteins that detect invading pathogens by binding conserved, microbially derived molecules and that induce signaling cascades for proinflammatory gene expression.[31] Moreover, TLR2 acts as the receptor for LTA.[7,26] A recent study has clearly shown that repurified LTA from *S. aureus* induces TNF-α secretion through a TLR2-signaling pathway.[41] Here, we found that LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression were inhibited by transfection with TLR2 siRNA, consistent with the results indicating that LTA activated the signaling transduction pathways mediated through TLR2 in various cell types.[6,7] The TLR-regulated intracellular signalings are initiated by TIR-domain-dependent heterophilic interactions with TIR-domain-containing cytosolic adapters, such as MyD88.[31] On activation of TLR, MyD88 is recruited to TLR domains and links TLR with the downstream intracellular signaling components.[42] This is confirmed by our observation that LTA- and *S. aureus*–enhanced $cPLA_2$ and COX-2 expression were attenuated by transfection with MyD88 siRNA. On the other hand, LTA or LPS has been shown to stimulate the expression of various proinflammatory mediators, including IL-6, TNF-α, or IL-1β.[4] We further ascertained that LTA and *S. aureus* induced PGE_2 and IL-6 release in a $cPLA_2$/COX-2-dependent manner by pretreatment with $AACOCF_3$ and NS-398 and transfection with $cPLA_2$ siRNA and COX-2 siRNA, respectively. These data confirmed that LTA and *S. aureus* induced $cPLA_2$/COX-2–dependent PGE_2 and IL-6 generation through a TLR2/MyD88 signaling pathway in HTSMCs.

The PI3Ks are a conserved family of signal transduction enzymes that are involved in regulation of cellular function, inflammatory responses, chemotaxis, and apoptosis.[43] Akt is a serine/threonine kinase that is implicated in mediating a variety of biological responses, including cell growth, proliferation, and survival.[23] More-

over, we also showed that LTA- or *S. aureus*–induced $cPLA_2$/COX-2 expression and PGE_2/IL-6 secretion were mediated via PI3K/Akt by pretreatment with LY294002 or transfection with Akt siRNA. Akt is activated by phosphorylation on two critical residues, namely Thr^{308} and Ser^{473}.[23] This is confirmed by our observation that LTA stimulated a time-dependent Ser^{473} phosphorylation of Akt in HTSMCs. TLRs recognize microbial components and evoke diverse responses in immune and other respiratory cells through distinct signaling cascades, in which MyD88 and TRAF6 are key adaptor proteins.[5] Our previous study has found that LTA induces the association of TLR2/MyD88/TRAF6/c-Src.[7] In addition, LTA has been shown to induce the formation of a TLR2/PI3K complex.[6] PI3K has been shown to activate a downstream component Rac1 in SH-SY5Y cells.[33] Rac1 is involved in different aspects of host defenses against bacteria, including leukocyte chemotaxis and pathogen phagocytosis.[24,25] We also showed that the physical association of TLR2, MyD88, PI3K, and Rac1 was involved in LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression. Although the detail protein–protein interactions among TLR2, MyD88, PI3K, and Rac1 are not known, our results are the first time to show a novel role of TLR2/MyD88/PI3K/Rac1 complex formation in LTA- and *S. aureus*–induced $cPLA_2$/COX-2–dependent PGE_2 generation in HTSMCs. In the future, we will further determine which domains of TLR2, MyD88, PI3K, and Rac1 are involved in protein–protein interactions caused by LTA and *S. aureus*.

The MAPKs family consists of three major members: ERK (including ERK1/2), p38 MAPK (including p38α, p38β, p38γ, and p38δ), and JNK (including JNK1, JNK2, and JNK3). MAPKs are important for intracellular signal transduction and play critical roles in regulating inflammatory responses.[44] Moreover, in this study, we also found that LTA and *S. aureus* induced $cPLA_2$ and COX-2 expression via these three MAPKs by transfection with siRNAs of p42, p38α, p38β, p38γ, p38δ, and JNK2. On the other hand, each p38 isoform displays a particular pattern of tissue-specific expression, and produces different responses in a cell-type–specific manner.[45] p38α and p38β are ubiquitously expressed, whereas p38δ and p38γ display a more restricted pattern of expression.[45] p38γ is expressed in skeletal muscle, heart, lung, thymus, and testis, whereas p38δ is expressed in lung, pancreas, small intestine, kidney, testis, and epidermis.[45] Interestingly, we found that all these four p38 isoforms are expressed in HTSMCs. In addition, transfection with siRNAs of TLR2, MyD88, and Akt inhibited LTA-stimulated MAPKs activation. Although in various cell types, such as macrophages, an elevation in phospho-Akt levels is often associated with a decrease in phosho-p38 levels, our results are the first time to show a novel role of Akt in LTA-induced p38 phosphorylation in HTSMCs.

The transcription factor NF-κB is a key regulator of immune responses and inflammation through the induction of numerous genes, including those coding for cytokines, chemokines, and adhesion molecules.[28] Moreover, NF-κB is one of the major mediators of the intracellular functions of LTA.[17,20] This is confirmed by our observation that LTA- and *S. aureus*–induced $cPLA_2$ and COX-2 expression were attenuated by pretreatment with a selective NF-κB inhibitor or transfection with p65 siRNA. NF-κB dimers containing RelA or c-Rel are retained in the cytoplasm through interaction with the inhibitors of NF-κB (IκBs). In response to a variety of stimuli, IκBs are phosphorylated by the activated IκB kinase (IKK) complex, followed by rapid ubiquitin-dependent degradation by the 26S proteosome.[28] This allows NF-κB dimers to translocate to the nucleus, where they stimulate expression of target genes. In our study, it was also found that LTA markedly stimulated p65 phosphorylation and translocation and NF-κB promoter activity through TLR2,

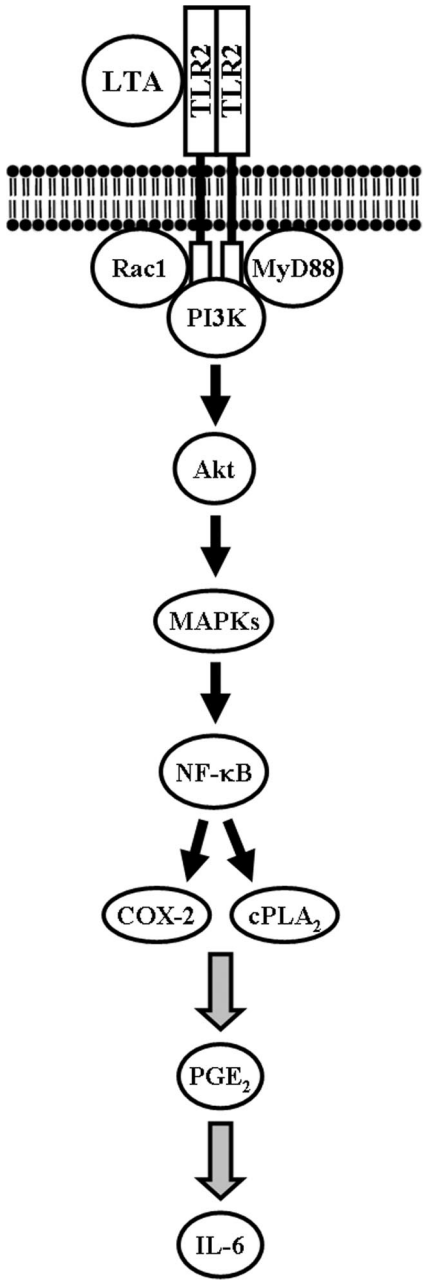

Figure 11. Schematic diagram illustrating the proposed signaling pathway involved in LTA-induced $cPLA_2$/COX-2–dependent airway inflammation. LTA activates the TLR2/MyD88/PI3K/Rac1/Akt pathway to enhance ERK1/2, p38 MAPK, and JNK1/2 phosphorylation, which in turn initiates the activation of NF-κB and ultimately induces $cPLA_2$/COX-2–dependent PGE_2 and IL-6 generation in HTSMCs.

PI3K/Akt, and MAPKs in HTSMCs. A major challenge for future research is to completely elucidate the mechanisms that are involved in regulation of NF-κB activation and the physiological relevance of NF-κB–targeted genes in different cells. This may lead to the birth of therapeutic inhibitors that selectively block NF-κB–regulated inflammatory responses.

Metabolites of AA and lysophosphatides have been identified *in situ* in airway secretion of asthmatics.[8,9] AA has also been shown to induce COX-2 protein expression in PC-3 human prostate cancer cells.[37] Moreover, we also showed that AA enhanced $cPLA_2$ phosphorylation or $cPLA_2$ and COX-2 expression, suggesting that AA could cause a positive feedback response, and then amplify the airway inflammatory responses through the induction of $cPLA_2$ and COX-2 expression. On the other hand, IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. IL-6 is the predominant mediator of the acute phase response, an innate immune mechanism that is triggered by infection and inflammation.[38] PGE_2, one of the major PGs produced, exerts its biological effects by binding to specific cell surface receptors, designated PGE_2 receptors (EPs).[39] There are four different EPs that have been identified, named EP1 to EP4, and several splice variants of EP3 are known.[39] PGE_2, synthesized by many cells and tissues throughout the body, has long been considered the principal prostaglandin in acute inflammation, as well as in respiratory diseases, such as COPD.[46] Previous studies have shown a positive association between endogenous PGE_2 production and IL-6 synthesis both *in vitro* and *in vivo*.[39] This is confirmed by our observation that pretreatment with AH 6809 (an EP1 and EP2 receptor antagonist), SC-19220 (an EP1 receptor antagonist), or GW627368X (an EP4 receptor antagonist) markedly inhibited IL-6 secretion induced by LTA or *S. aureus*. In addition, administration with PGE_2 increased IL-6 secretion in HTSMCs and leukocyte count in BAL fluid in mice, suggesting that LTA and *S. aureus* could enhance airway inflammation through the induction of PGE_2/IL-6 secretion.

In summary, as depicted in Figure 11, our results demonstrate that in HTSMCs, LTA induces MAPKs activation through TLR2/MyD88/PI3K/Rac1/Akt, in turn initiates the activation of NF-κB. Activated NF-κB is recruited to the promoter region of $cPLA_2$ or COX-2 leading to an increase of $cPLA_2$ or COX-2 expression. Moreover, $cPLA_2$ and COX-2 contribute to the generation of PGE_2/IL-6. These results indicated a role for HTSMCs, in addition to their contractile function, as inflammatory cells involved in the production of chemical mediators that may contribute to the inflammatory response seen in asthma.

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References

1. Hirst SJ, Martin JG, Bonacci JV, Chan V, Fixman ED, Hamid QA, Herszberg B, Lavoie JP, McVicker CG, Moir LM, Nguyen TT, Peng Q, Ramos-Barbon D, Stewart AG: Proliferative aspects of airway smooth muscle. J Allergy Clin Immunol 2004, 114:S2–S17
2. Sutcliffe IC, Shaw N: Atypical lipoteichoic acids of gram-positive bacteria. J Bacteriol 1991, 173:7065–7069
3. Alkan ML, Beachey EH: Excretion of lipoteichoic acid by group A streptococci. Influence of penicillin on excretion and loss of ability to adhere to human oral mucosal cells J Clin Invest 1978, 61:671–677
4. Su SC, Hua KF, Lee H, Chao LK, Tan SK, Lee H, Yang SF, Hsu HY: LTA and LPS mediated activation of protein kinases in the regulation of inflammatory cytokines expression in macrophages. Clin Chim Acta 2006, 374:106–115
5. Suzuki N, Suzuki S, Yeh WC: IRAK-4 as the central TIR signaling mediator in innate immunity. Trends Immunol 2002, 23:503–506
6. Liljeroos M, Vuolteenaho R, Morath S, Hartung T, Hallman M, Ojaniemi M: Bruton's tyrosine kinase together with PI 3-kinase are part of Toll-like receptor 2 multiprotein complex and mediate LTA induced Toll-like receptor 2 responses in macrophages. Cell Signal 2007, 19:625–633
7. Lee IT, Wang SW, Lee CW, Chang CC, Lin CC, Luo SF, Yang CM: Lipoteichoic acid induces HO-1 expression via the TLR2/MyD88/c-Src/NADPH oxidase pathway and Nrf2 in human tracheal smooth muscle cells. J Immunol 2008, 181:5098–5110
8. Barnes PJ: New concepts in the pathogenesis of bronchial hyperresponsiveness and asthma. J Allergy Clin Immunol 1989, 83:1013–1026
9. Smith LJ: Bioactive mediators in asthma. Chest 1992, 101:381S–384S
10. DeWitt DL: Cox-2-selective inhibitors: the new super aspirins. Mol Pharmacol 1999, 55:625–631
11. Six DA, Dennis EA: The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim Biophys Acta 2000, 1488:1–19
12. Leslie CC: Properties and regulation of cytosolic phospholipase A_2. J Biol Chem 1997, 272:16709–16712
13. Nagase T, Uozumi N, Ishii S, Kume K, Izumi T, Ouchi Y, Shimizu T: Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A_2. Nat Immunol 2000, 1:42–46
14. Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, Komagata Y, Maki K, Ikuta K, Ouchi Y, Miyazaki J, Shimizu T: Role of cytosolic phospholipase A_2 in allergic response and parturition. Nature 1997, 390:618–622
15. Luo SF, Wang CC, Chien CS, Hsiao LD, Yang CM: Induction of cyclooxygenase-2 by lipopolysaccharide in canine tracheal smooth muscle cells: involvement of p42/p44 and p38 mitogen-activated protein kinases and nuclear factor-κB pathways. Cell Signal 2003, 15:497–509
16. Yang CM, Chien CS, Hsiao LD, Luo SF, Wang CC: Interleukin-1β-induced cyclooxygenase-2 expression is mediated through activation of p42/44 and p38 MAPKS, and NF-κB pathways in canine tracheal smooth muscle cells. Cell Signal 2002, 14:899–911
17. Chang YC, Li PC, Chen BC, Chang MS, Wang JL, Chiu WT, Lin CH: Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E_2, protein kinase A, p38 MAPK, and nuclear factor-κB pathways. Cell Signal 2006, 18:1235–1243
18. Dieter P, Kolada A, Kamionka S, Schadow A, Kaszkin M: Lipopolysaccharide-induced release of arachidonic acid and prostaglandins in liver macrophages: regulation by Group IV cytosolic phospholipase A_2, but not by Group V and Group IIA secretory phospholipase A_2. Cell Signal 2002, 14:199–204
19. Gilroy DW, Newson J, Sawmynaden P, Willoughby DA, Croxtall JD: A novel role for phospholipase A_2 isoforms in the checkpoint control of acute inflammation. FASEB J 2004, 18:489–498
20. Kao SJ, Lei HC, Kuo CT, Chang MS, Chen BC, Chang YC, Chiu WT, Lin CH: Lipoteichoic acid induces nuclear factor-κB activation and nitric oxide synthase expression via phosphatidylinositol 3-kinase, Akt, and p38 MAPK in RAW 264.7 macrophages. Immunology 2005, 115:366–374
21. Wu HH, Hsieh WS, Yang YY, Tsai MC: Lipoteichoic acid induces prostaglandin E_2 release and cyclooxygenase-2 synthesis in rat cortical neuronal cells: involvement of PKCε and ERK activation. Life Sci 2006, 79:272–280

22. Chen BC, Kang JC, Lu YT, Hsu MJ, Liao CC, Chiu WT, Yeh FL, Lin CH: Rac1 regulates peptidoglycan-induced nuclear factor-κB activation and cyclooxygenase-2 expression in RAW 264.7 macrophages by activating the phosphatidylinositol 3-kinase/Akt pathway. Mol Immunol 2009, 46:1179–1188
23. Cicenas J: The potential role of Akt phosphorylation in human cancers. Int J Biol Markers 2008, 23:1–9
24. Lee DJ, Cox D, Li J, Greenberg S: Rac1 and Cdc42 are required for phagocytosis, but not NF-κB-dependent gene expression, in macrophages challenged with Pseudomonas aeruginosa. J Biol Chem 2000, 275:141–146
25. Van AL, Souza-Schorey C: Rho GTPases and signaling networks. Genes Dev 1997, 11:2295–2322
26. Lee CW, Chien CS, Yang CM: Lipoteichoic acid-stimulated p42/p44 MAPK activation via Toll-like receptor 2 in tracheal smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2004, 286:L921–L930
27. You L, Kruse FE, Bacher S, Schmitz ML: Lipoteichoic acid selectively induces the ERK signaling pathway in the cornea. Invest Ophthalmol Vis Sci 2002, 43:2272–2277
28. Lin A, Karin M: NF-κB in cancer: a marked target. Semin Cancer Biol 2003, 13:107–114
29. Fischer J, Suire C, Hale-Donze H: Toll-like receptor 2 recognition of the microsporidia Encephalitozoon spp. induces nuclear translocation of NF-κB and subsequent inflammatory responses. Infect Immun 2008, 76:4737–4744
30. Lee CW, Lin WN, Lin CC, Luo SF, Wang JS, Pouyssegur J, Yang CM: Transcriptional regulation of VCAM-1 expression by tumor necrosis factor-α in human tracheal smooth muscle cells: involvement of MAPKs. NF-κB, p300, and histone acetylation. J Cell Physiol 2006, 207:174–186
31. Takeda K, Akira S: Toll-like receptors in innate immunity. Int Immunol 2005, 17:1–14
32. Silfani TN, Freeman EJ: Phosphatidylinositide 3-kinase regulates angiotensin II-induced cytosolic phospholipase A_2 activity and growth in vascular smooth muscle cells. Arch Biochem Biophys 2002, 402:84–93
33. Pan J, Kao YL, Joshi S, Jeetendran S, Dipette D, Singh US: Activation of Rac1 by phosphatidylinositol 3-kinase in vivo: role in activation of mitogen-activated protein kinase (MAPK) pathways and retinoic acid-induced neuronal differentiation of SH-SY5Y cells. J Neurochem 2005, 93:571–583
34. Luo SF, Lin WN, Yang CM, Lee CW, Liao CH, Leu YL, Hsiao LD: Induction of cytosolic phospholipase A_2 by lipopolysaccharide in canine tracheal smooth muscle cells: involvement of MAPKs and NF-κB pathways. Cell Signal 2006, 18:1201–1211
35. Scott T, Owens MD: Thrombocytes respond to lipopolysaccharide through Toll-like receptor-4, and MAP kinase and NF-κB pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E_2. Mol Immunol 2008, 45:1001–1008
36. Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ: $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell 1993, 72:269–278
37. Hughes-Fulford M, Li CF, Boonyaratanakornkit J, Sayyah S: Arachidonic acid activates phosphatidylinositol 3-kinase signaling and induces gene expression in prostate cancer. Cancer Res 2006, 66:1427–1433
38. Chen BC, Liao CC, Hsu MJ, Liao YT, Lin CC, Sheu JR, Lin CH: Peptidoglycan-induced IL-6 production in RAW 264.7 macrophages is mediated by cyclooxygenase-2. PGE_2/PGE_4 receptors, protein kinase A, IκB kinase, and NF-κB. J Immunol 2006, 177:681–693
39. Park JY, Pillinger MH, Abramson SB: Prostaglandin E_2 synthesis and secretion: the role of PGE_2 synthases. Clin Immunol 2006, 119:229–240
40. Keller R, Fischer W, Keist R, Bassetti S: Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect Immun 1992, 60:3664–3672
41. Ellingsen E, Morath S, Flo T, Schromm A, Hartung T, Thiemermann C, Espevik T, Golenbock D, Foster D, Solberg R, Aasen A, Wang J: Induction of cytokine production in human T cells and monocytes by highly purified lipoteichoic acid: involvement of Toll-like receptors and CD14. Med Sci Monit 2002, 8:BR149–BR156
42. Jordan MS, Singer AL, Koretzky GA: Adaptors as central mediators of signal transduction in immune cells. Nat Immunol 2003, 4:110–116
43. Williams DL, Ozment-Skelton T, Li C: Modulation of the phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. Shock 2006, 25:432–439
44. Ji RR, Gereau RW, Malcangio M, Strichartz GR: MAP kinase and pain. Brain Res Rev 2009, 60:135–148
45. Eckert RL, Efimova T, Balasubramanian S, Crish JF, Bone F, Dashti S: p38 Mitogen-activated protein kinases on the body surface - a function for p38δ. J Invest Dermatol 2003, 120:823–828
46. Chen Y, Chen P, Hanaoka M, Droma Y, Kubo K: Enhanced levels of prostaglandin E_2 and matrix metalloproteinase-2 correlate with the severity of airflow limitation in stable COPD. Respirology 2008, 13:1014–1021