Innate immune responses in murine pleural mesothelial cells: Toll-like receptor-2 dependent induction of β-defensin-2 by staphylococcal peptidoglycan

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Hussain T, Nasreen N, Lai Y, Bellew BF, Antony VB, Mohammed KA. Innate immune responses in murine pleural mesothelial cells: Toll-like receptor-2 dependent induction of β-defensin-2 by staphylococcal peptidoglycan. Am J Physiol Lung Cell Mol Physiol 295: L461–L470, 2008. First published July 11, 2008; doi:10.1152/ajplung.00276.2007.—The innate immune response is mediated in part by pattern recognition receptors including Toll-like receptors (TLRs). The pleural mesothelial cells (PMCs) that line the pleural surface are in direct contact with pleural fluid and accordingly carry the risk of exposure to infiltrating microorganisms or their components in an event of a complicated parapneumonic effusion. Here we show that murine primary PMCs constitutively express TLR-1 through TLR-9 and, upon activation with peptidoglycan (PGN), mouse PMC produce antimicrobial peptide β-defensin-2 (mBD-2). Treatment of PMCs with staphylococcal PGN, a gram-positive bacterial cell wall component and a TLR-2 agonist, resulted in a significant increase in TLR-2 and mBD-2 expression. Silencing of TLR-2 expression by small interfering RNA led to the downregulation of PGN-induced mBD-2 expression, thereby establishing causal relationship between the activation of TLR-2 receptor and mBD-2 production. PMCs exposed to PGN showed increased p38 MAPK activity. In addition, PGN-induced mBD-2 expression was attenuated by SB203580, a p38 MAPK inhibitor, underlining the importance of p38 MAPK in mBD-2 induction. PMCs exposed to PGN showed increased p38 MAPK activity. In addition, PGN-induced mBD-2 expression was attenuated by SB203580, a p38 MAPK inhibitor, underlining the importance of p38 MAPK in mBD-2 induction. Inhibition of erk1/erk2 or phosphatidylinositol 3-kinase did not block PGN-induced mBD-2 expression in PMC. PGN-activated PMC-derived mBD-2 significantly killed Staphylococcus aureus, and mBD-2-neutralizing antibodies blunted this antimicrobial activity. Taken together, these data indicate that PMCs may contribute to host innate immune defense upon exposure to gram-positive bacteria or their products within the pleural space by upregulating TLR-2 and mBD-2 expression.

p38 mitogen-activated protein kinase; pleural effusion; empyema; pleural infection

DEFENSE AGAINST AN INFECTIOUS pathogen is initiated by the host innate immune system. The broad classes of pathogens, including viruses, bacteria, and fungi, constitutively express a set of molecules called pathogen-associated molecular patterns. These microbial molecular markers may be composed of proteins, carbohydrates, lipids, or nucleic acids and are intracellular or surface bound (29). The host innate immune system takes advantage of these constitutively expressed pathogen-associated molecular patterns to recognize the invading pathogens with the help of highly conserved pattern recognition receptors such as TLRs (22). To date nine Toll-like receptors (TLR-1 through TLR-9) have been well defined in mammals, and agonists for most of the TLRs have been described previously (20, 31). TLR signaling induces innate and adaptive immune responses (9). Upon binding with their respective TLRs, various agonists elicit downstream signaling leading to the production of cytokines, chemokines, or peptides with antimicrobial activity (44, 58). TLR-induced responses lead to NF-κB activation that is pivotal in TLR-mediated induction of innate immune responses (9, 31, 34). TLR-2 is an important member of TLR family recognizing bacteria (75) and a wide range of microbial components, including peptidoglycan (PGN; Ref. 74), LPS (48), and nucleic acids (48, 54). Both gram-positive and gram-negative bacteria upregulated TLR-2 mRNA expression in tissues (75). TLR-2-deficient mice are highly susceptible to the infection of Staphylococcus aureus, underlining the importance of TLR signaling in the host defense (65).

Defensins are small cationic peptides involved in innate immunity with antimicrobial function and considered as natural antibiotics, as they directly kill the pathogenic microbes by permeabilizing the microbial membranes (24). Defensins are expressed by the leukocytes and epithelial surfaces in the body in response to various microbial pathogens (37). Defensins are classified as α- and β-defensins (BDs) based on the position of three intramolecular disulfide bonds. Four well-characterized BDs are reported in humans expressed predominantly in the epithelia. The human BD-1 (hBD-1) is constitutively expressed (76), while the expressions of hBD-2, -3, and -4 are inducible (15, 18, 19). Antimicrobial activities of hBDs have been well demonstrated both in vivo and in vitro against diverse pathogens (15, 16, 18, 19, 26). In addition to a role in innate immunity, hBDs promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial invasion (72). BDs also chemoattract human neutrophils (49) and are implicated in cell differentiation and tissue remodeling (1, 68). BDs are also reported in mice and have been shown to possess bactericidal activity against gram-positive and gram-negative bacteria (6, 7, 27, 43, 44, 71). Murine-BD-2, -BD-3, and -BD-6 were shown to be inducible by endotoxin stimuli (6, 7, 42, 52, 71).

The pleural cavity is a space between visceral and parietal mesothelial membranes that surround the lung and chest. It
normally contains a small quantity of pleural fluid that facilitates movement of the lung during inspiration and expiration. Pleural effusion formation is defined as excess fluid accumulation in the pleural cavity and is an abnormal condition resulting from infection, malignancy, or heart failure (3). Empyema is a serious complication of pulmonary parenchymal infection associated with the presence of bacteria in the pleural space and rarely resolves without appropriate medical therapy. It is well recognized that *S. aureus*, a gram-positive bacteria, is a leading cause for the development of parapneumonic effusions (4) and as a result infiltrates into the pleural space. PMCs covering the pleural surface come in direct contact with infiltrated pathogens or their products. Since PMCs are in close proximity with the invading pathogens in the pleural space, they may express the receptors and produce mediators of innate immunity to keep the infection at bay. We (39–41) have previously shown that PMCs indirectly contribute to innate immunity by augmenting neutrophil functions and secreting inflammatory mediators in response to *S. aureus* infection in empyema. However, whether PMCs directly participate in the host innate immune defenses is not known. Since *S. aureus* is frequently associated with empyema effusions, we sought to determine whether this pathological condition results in the production of antibacterial peptides such as BDs by PMCs in response to staphylococcal PGN, a gram-positive bacterial cell wall component and natural ligand to TLR-2.

The TLR ligand receptor interaction results in the activation of MAPK (31). MAPKs play a key role in the transduction of extracellular signals to cellular response. The MAPK cascade participates in regulation of gene expression by connecting the extracellular signal to intracellular transcriptional elements and regulatory proteins (64). The p44/42 and p38 MAPK phosphorylation activates a group of MAPK-activated protein kinases that control a variety of cellular functions. In a recent study (5), increased defensins were noticed in the pleural fluids of patients with empyema. In the present study, we demonstrate that murine primary PMCs upon exposure to bacterial PGN upregulate the expression of the antibacterial peptide mBD-2, thereby directly contributing to the host innate immune defense. In addition, we show that PMCs constitutively express TLR-1 through TLR-9 and after treatment with PGN upregulate TLR-2 expression. Further, we demonstrate that mBD-2 expression in PMCs is dependent on TLR-2 and p38 MAPK signaling.

**MATERIALS AND METHODS**

Reagents and cell culture. The insoluble staphylococcal PGN, SYBR Green Jump Start ready mix and AMV-RT enzyme, and actinomycin D were purchased from Sigma Aldrich (St. Louis, MO). SB203580 (P38 MAPK inhibitor), PD98059 (p44/42 MAPK inhibitor), and LY294002 [a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor] were purchased from Calbiochem (La Jolla, CA). The PGN (1 ng/ml) suspension was prepared in PBS and used to activate the PMC cultures. The p38 MAPK activity assay kit was obtained from Cell Signaling (Beverly, MA). TLR-2-specific and control small interfering RNAs (siRNAs), transfection reagent, goat polyclonal TLR-2, and goat polyclonal mBD-2 antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Micro BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). The RNA isolation kit was purchased from Qiagen (Valencia, CA). Mouse primary PMC cultures were established as detailed earlier (40) and maintained at 37°C with 5% CO2 in F-12K complete medium supplemented with antibiotics and 10% FBS. PMC cultures with ~70% confluence were incubated in serum-free F-12K medium without antibiotics for 16 h before the indicated treatments.

Reverse-transcription and real-time PCR. One microgram of total RNA isolated from PMCs after indicated treatments was reverse transcribed to cDNA using oligo dT primer and enhanced AMV-RT enzyme in a 20-μl reaction volume. The cDNA template equivalent to 100 ng of total RNA in triplicate was subjected to real-time PCR using SYBR Green JumpStart ready mix in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Primers (Table 1) to amplify mouse TLR-1 through TLR-9 and mBD-2, mBD-3, and mBD-6 were synthesized from Sigma. Mouse β-actin was amplified as an endogenous control. PCR conditions included an initial denaturation of the template at 94°C for 2 min followed by 45 amplification cycles each consisting of denaturation at 94°C for 15 s followed by annealing and extension at 60°C for 1 min. Data were analyzed by the ΔΔCt method and expressed as relative gene expression compared with the lowest expressed gene among the test samples.

**siRNA transfection.** Transfection of the TLR-2-specific and control siRNAs was carried out as reported earlier (45), following the manufacturer instructions (Santa Cruz Biotechnology). Briefly, 25 pmol of siRNA duplex targeting TLR-2 or control siRNA were mixed with the 100 μl of transfection reagent and left at room temperature for 30 min.

**Table 1. Specific primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>mTLR-1</td>
<td>GTTGTCACAGTGTTTCAGCC</td>
<td>CTGCTACTTAGAGAATTCTGC</td>
</tr>
<tr>
<td>mTLR-2</td>
<td>AGCTTTGAAAGGGCGGGTACAG</td>
<td>TGGAGACGCGACGCTGCTCA</td>
</tr>
<tr>
<td>mTLR-3</td>
<td>CATATGGTACATGCTAGG</td>
<td>TGGGCGATGGGCAGCC</td>
</tr>
<tr>
<td>mTLR-4</td>
<td>ATGTCGCTACAGCAATTGAGC</td>
<td>CTTATAGGCTAGATTCGAC</td>
</tr>
<tr>
<td>mTLR-5</td>
<td>GACTTCTAAGCGCGTAA</td>
<td>AGGAGATATAGCGGGA</td>
</tr>
<tr>
<td>mTLR-6</td>
<td>AGTCGGCCAGTTGGCA</td>
<td>AGCAAAACCGAGATAAGG</td>
</tr>
<tr>
<td>mTLR-7</td>
<td>CCTTCTCACTGGGTCAGAA</td>
<td>GGGTCAAGCTGCAAGATG</td>
</tr>
<tr>
<td>mTLR-8</td>
<td>GGCATACGCTTGGGTGATT</td>
<td>CATTCTGGTGGTGGTGG</td>
</tr>
<tr>
<td>mTLR-9</td>
<td>CGCCTTGAGCTGGGATACAC</td>
<td>GTATATAGAAGTGGGGTG</td>
</tr>
<tr>
<td>mBD-1</td>
<td>GACGTGCGATCTTCGTCCGAC</td>
<td>CAGATCCGTTGGTGGTGG</td>
</tr>
<tr>
<td>mBD-2</td>
<td>GGTGCGAGTTCCCTGCTGCTC</td>
<td>AGGGCTTTCTGCTGGGAA</td>
</tr>
<tr>
<td>mBD-3</td>
<td>TCTGTTGAGATTCTCGTCCG</td>
<td>TAAACCTCCGAGATCGTATG</td>
</tr>
<tr>
<td>mBD-4</td>
<td>TCTGTTGAGATTCTCGTCCG</td>
<td>TTCAGCTGAAAGCTGGAAGT</td>
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<tr>
<td>Defl-1</td>
<td>ATTTCTCCTCGTGGCAGGTG</td>
<td>GGTTACGAGAGATCCGTC</td>
</tr>
<tr>
<td>mBD-6</td>
<td>TCGAGGAGATCCATGAC</td>
<td>TTGGCAATCAAGCTGGAAGT</td>
</tr>
<tr>
<td>mβ-actin</td>
<td>GTGTCGGGCGGCTGCA</td>
<td>CTCTTCTGATCGTCAGATTC</td>
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TLR, Toll-like receptor; BD, β-defensin; m, mouse.
The contents were added to PMCs in serum-free-F-12K media at 70% confluence and incubated for 24 h before the media was changed to fresh F-12K supplemented with 10% FBS. Cells were further incubated for 24 h, and the media were changed to serum-free F-12K for 16 h before treatment with 10 μg/ml of PGN for 3, 12, or 24 h to examine TLR-2 mRNA, mBD-2 mRNA, or mBD-2 protein, respectively.

Flow cytometry. TLR-2 expression in PMCs was measured by flow cytometry as reported earlier (40). PMCs left untreated or treated with 10 μg/ml of PGN for 6, 12, or 24 h were trypsinized, washed with PBS, and blocked in 2% BSA in PBS for 20 min at 4°C temperature. To evaluate if the PMC surface expressed TLR-2 is a direct effect of induction of mRNA expression, we pretreated the PMC cultures with 2 μg of actinomycin D for 30 min and harvested the cells after 12 h of PGN activation. Cells were incubated with TLR-2 antibody or nonspecific goat IgG (Isotype) at a dilution of 1:20 in PBS for 30 min at room temperature. Cells were washed and incubated with FITC-conjugated secondary antibody diluted at 1:100 in PBS for 30 min at 4°C temperature. Cells were washed in PBS with 5% BSA, fixed in 4% paraformaldehyde, and analyzed by FACS (FACCalibur, Becton Dickinson, Mountain View, CA).

Immunofluorescence staining. The mBD-2 expression in PMCs was detected by immunofluorescence microscopy, as reported earlier with minor modifications (45). PMCs in chambered slides were transfected with TLR-2-specific or control siRNA as detailed in siRNA transfection and were left untreated or treated with 10 μg/ml of PGN for 24 h. Cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton-X-100 for 5 min, and blocked for 30 min in 2% BSA in PBS at room temperature. Cells were incubated at room temperature in mBD-2 antibody for 1 h and subsequently in the FITC-labeled secondary antibody and DAPI for 30 min at room temperature. Cells were visualized for mBD-2 and nuclear stains under a fluorescence microscope (Carl Zeiss) using AxioVision software.

Slot-blot analysis. Equal volumes (500 μl) of culture supernatants of PMCs left untreated or treated with 10 μg/ml of PGN with and without SB203580 for 24 h were slot blotted in each well onto a polyvinylidene fluoride membrane and blocked in 5% nonfat dry milk in PBS for 2 h at room temperature. Affinity purified goat anti-mBD-2 antibody (diluted 1:500 in PBS containing 5% nonfat dry milk) was added into each well, and the wells were incubated at 4°C overnight. Parallel samples were maintained with nonspecific goat IgG (1:500) to evaluate nonspecific reactions. The wells were rinsed three times with PBS and subsequently incubated with horseradish peroxidase-conjugated rabbit anti-goat secondary antibody at room temperature for 90 min. The wells were rinsed again three times with PBS, and the signal was detected using an ECL substrate followed by exposure to X-ray film.

p38 MAPK assay and Western blot analysis. The p38 MAPK activity was measured following the manufacturer’s instructions (Cell Signaling). Briefly, the whole cell lysates of PMCs treated with 10 μg/ml of PGN for the indicated times were prepared. The total protein was estimated using Micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Two hundred micrograms of protein from each sample were incubated overnight at 4°C with phospho-p38 antibody at 1:1,000 dilution in TBS-Tween 20 containing 5% BSA overnight at 4°C. The membrane was washed with TBS buffer and further incubated with horseradish peroxidase-conjugated secondary antibody for 90 min at room temperature. The phosphorylated p38 MAPK was detected with LumiGLO substrate by autoradiography.

Antimicrobial assay. The antimicrobial activity of PMC-derived mBD-2 was evaluated against S. aureus by colony formation assay on tryptic soy agar plates. A mouse virulent strain of S. aureus (ATCC-14154) was obtained from American Type Culture Collection (Rockville, MD). S. aureus was cultured as reported earlier (41). A loopful of S. aureus was grown in 50 ml of nutrient broth at 37°C for 16 h. The number of colony-forming units (CFU) of cultured bacteria in the original stock was determined by plating known volumes of serial dilutions over nutrient agar plates. The bacteria were washed three times in PBS pH 7.4, and they were adjusted to 1 × 10^6 CFU/ml. Serial dilutions of PMC conditioned medium along with 10,000 CFU of S. aureus were mixed in a total volume of 100 μl and incubated in a microtiter plate for 3 h at 37°C. In some samples, mBD-2 was immunoprecipitated with 5 μg of affinity purified goat anti-mBD antibody immobilized to agarose beads before incubation using a Protein G PLUS agarose immunoprecipitation kit purchased from Santa Cruz Biotechnology. Affinity purified nonspecific goat IgG was included in some samples to estimate the nonspecific binding. The number of viable bacteria in the culture was estimated by culturing on the nutrient agar plates. The data obtained were presented as the percentage of maximal bacterial growth in the medium alone.

Statistical analysis. The significance of differences between experimental and control groups was tested by ANOVA using Sigma-Stat 3.5 (Systat, San Jose, CA) statistical software. The significance of difference between the two groups was tested by all pair wise multiple comparison procedure (Student-Newman-Keuls method), and a P value <0.05 was considered significant.

RESULTS

Mouse primary PMCs constitutively express multiple TLR mRNAs. To determine whether mouse PMCs constitutively express TLRs, total RNA was extracted from the cells after they were incubated in serum-free F-12K media for 16 h. One microgram of total RNA from each sample was reverse transcribed and analyzed by real-time PCR in triplicate using primers specific for mouse TLR-1 through TLR-9 (Table 1). Mouse β-actin was amplified as an internal control (housekeeping gene). The data are expressed as relative gene expression compared with the lowest expressed gene (TLR-6, in this case) among the test samples. PCR analysis revealed that mouse PMCs constitutively expressed TLR-1 through TLR-9 mRNAs (Fig. 1). Among the nine TLRs studied, there was no significant difference in the expression of TLR-3 and TLR-4, but both were significantly higher than compared with other TLRs expressed in PMCs. The TLR-6 expression levels were comparable with those of TLR-9 and were least expressed in resting PMCs. Similarly, TLR-1 mRNA was expressed to a
similar extent to that of TLR-5 and TLR-7 expression was not different from TLR-8. These data indicate that resting PMCs express TLRS and hence may participate in host innate immune responses in the pleural space. Since *S. aureus* is frequently associated with empyema effusions and staphylococcal PGN is a natural ligand to TLR-2, we focused our studies on understanding the mechanisms associated with TLR-2 signaling in murine BD-2 production.

*PGN upregulates TLR-2 expression in PMCs.* The cell wall of gram-positive bacteria is largely made of PGN. Gram-positive bacterial cell membranes also contain teichoic acids that may provide structural support to the cell wall. The PGN used in this study was not tested for teichoic acid content; however, the purity of PGN was established by spectroscopy (Fluka, Switzerland). The PGN cannot be solubilized, but it is possible to make a suspension in buffer. We prepared a 1 mg/ml stock suspension in

![Graph A](image)

**Fig. 2.** PGN upregulates TLR-2 expression in PMCs. **A:** total RNA from PMCs treated with PGN (10 μg/ml) was extracted at the indicated times and subjected to real-time PCR analysis using TLR-2 specific primers (Table 1). Data are relative mRNA expression compared with 0-h sample and are means ± SD of 3 independent experiments each estimated in triplicate at different times. *P < 0.05, **P < 0.001, significant upregulation; #P < 0.001, significant decrease compared with unstimulated culture. B:** TLR-2 receptor expression in PGN-treated and untreated (control) PMCs over time determined by flow cytometry. **C:** TLR-2 receptor expression in PMC pretreated with and without actinomycin D (Act-D; 2 μg/ml) pretreatment. Data shown are a single representative of 3 similar but independent experiments, and each experiment was performed at different times.
PBS and used it to expose the PMC cultures. Bacterial PGN is a natural TLR-2 ligand, and various agonists are shown to upregulate its expression in leukocytes. Therefore, we aimed to determine whether PGN upregulates TLR-2 expression in PMCs. Real-time PCR analysis of mRNA from PMCs treated with 10 μg/ml of PGN for the indicated times showed that PGN significantly upregulated the expression of TLR-2 mRNA (Fig. 2A). Compared with levels at 0 h, TLR-2 mRNA increased within 1 h of PGN treatment and rose further at 3 h. A 2.3- and 4.5-fold induction of TLR-2 mRNA expression was noted at 1 and 3 h, respectively, compared with 0 h. Decreased mRNA levels were noted with the further treatment of cells for 6 h and were comparable with those of 0 h. The increased expression of TLR-2 mRNA after the treatment with PGN correlated with an increase in protein levels. Flow cytometry analysis revealed a significant induction of TLR-2 protein 12 h after PGN treatment, and the levels remained elevated for at least up to 24 h (Fig. 2B). In resting PMCs, we noticed an increasing trend in TLR-2 expression and it was significantly different between 6 and 24 h. This increase in TLR-2 at 24 h may be associated with PMC maturation. However, in PGN-activated PMC, we noticed a significant decrease in TLR-2 expression at 24 h. We speculate that when PMCs are activated some of the TLR-2 receptors are being internalized and hence there is less surface availability. We evaluated whether actinomycin D inhibits the PGN-induced TLR-2 expression in PMCs. We noticed that the inclusion of actinomycin D significantly decreased PGN-induced TLR-2 expression in PMC, indicating that PGN-induced TLR expression in PMC was dependent on TLR-2 mRNA synthesis (Fig. 2C).

**PGN upregulates mBD-2 expression in PMCs.** The effect of PGN on mBD-2 expression in PMCs was evaluated at the mRNA and protein levels. To examine the time-dependent response against PGN, PMCs were initially treated with 10 μg/ml of PGN for the indicated times. Total RNA was extracted and analyzed by real-time PCR using primers specific for mBD-2 (Table 1). PCR data revealed that PGN upregulated mBD-2 mRNA in a time-dependent fashion (Fig. 3A). Compared with the levels at 0 h, mRNA was increased within 3 h and remained elevated for at least up to 24 h. A maximum 7.5-fold increase in the expression of mBD-2 was measured at 12 h compared with the levels at 0 h. Changes in mBD-2 mRNA expression were also evaluated against varying concentrations of PGN. Compared with untreated control, PMCs demonstrated a dose-dependent increase in mBD-2 mRNA expression reaching maximum levels with 10 μg/ml of PGN (Fig. 3B). A 3.5-fold increase in mBD-2 mRNA levels was observed with a 2.5 μg/ml concentration of PGN compared with control. Similarly, a 5.4- and 7.3-fold induction was noted with 5 and 10 μg/ml concentrations of PGN, respectively. mBD-2 mRNA levels remained elevated in response to 20 μg/ml and were comparable with the levels at 10 μg/ml. No change in mBD-3 and mBD-6 mRNA levels was observed after treatment of PMCs with PGN for the indicated concentrations and time (Fig. 3, A and B). Changes in mBD-2 protein expression in response to PGN were also evaluated by immunofluorescence staining and found to be consistent with the mRNA data. Fluorescence analysis showed an intense staining for mBD-2 protein in PMCs treated with PGN compared with its expression in unstimulated cells (Fig. 4C). We also examined the effect of PGN on mBD-2 protein secretion in the PMC culture by dot blot analysis. The mBD-2 secretion was found to be elevated by more than threefold in the culture supernatant of cells treated with PGN compared with the levels found in the supernatant of untreated cells (Fig. 5B).

**mBD-2 expression in PMCs is TLR-2 dependent.** Since we found that PGN treatment of PMCs led to the upregulation of TLR-2 and mBD-2 expression, we sought to examine if there exists a relationship among these molecules. We silenced the TLR-2 receptor expression using specific siRNA and studied its effect on mBD-2 expression. Initially, we confirmed the silencing efficiency of TLR-2 siRNA in the reduction of TLR-2 expression. PGN activation resulted in a significant upregulation of TLR-2 expression. TLR-2 expression was completely attenuated in resting as well as in PGN-activated cells transfected with TLR-2-specific siRNA; however, it was not affected in cells transfected with control siRNA (Fig. 4B).
Similarly, the effect of TLR-2 silencing on mBD-2 expression was also assessed by real-time PCR and immunofluorescence analysis. PCR analysis revealed that PGN activation enhanced mBD-2 mRNA and silencing TLR-2 expression in these cells significantly attenuated PGN-induced mBD-2 mRNA, while the mBD-2 mRNA levels remained unaltered in control siRNA-transfected cells (Fig. 4A). Since mBD-2 is inducible gene, we noticed no increase in the mBD-2 gene expression in resting PMCs, and consequently TLR-2 siRNA treatment had no effect in these cultures. Likewise, immunofluorescence analysis demonstrated a dense positive fluorescence in PGN-activated PMC and a negative fluorescence in resting PMCs. In PGN-activated cells that were transfected with TLR-2 siRNA, mBD-2-specific immunofluorescence staining was reduced while it was unaffected in cells transfected with control siRNA (Fig. 4C).

**p38 MAPK inhibition decreases expression of mBD-2 mRNA and protein.** Cell surface receptor mediated signaling is known to activate MAPK in various cells. We examined the role of MAPK in mBD-2 induction in PMCs by inhibiting MAPK. To evaluate if the PGN-induced BD-2 expression in PMC is mediated either p44/42 or p38 or PI3-kinases, we introduced varying concentrations of pharmacological inhibitors against these kinases along with PGN activation in PMC. The concentrations of pharmacological inhibitors used in the PMC cultures in this study were found to be nontoxic as tested by cell toxicity assay (Sigma). Real-time PCR analysis of total RNA collected from PMCs showed no significant inhibition in PGN-induced BD-2 expression in the presence of PD98059, a p44/42 MAP kinase inhibitor (Fig. 5B). Similarly, no significant inhibition was noticed in the presence of LY294002, a PI3-kinase inhibitor (Fig. 5C). However, SB203580, a p38 MAPK inhibitor, significantly (P < 0.001) blocked the PGN-induced mBD-2 mRNA expression in PMCs (Fig. 5A). Similarly, slot-blot analysis of culture supernatants of PMCs treated with PGN revealed that PGN-induced mBD-2 protein levels were significantly (P < 0.001) attenuated by SB203580 (Fig. 5D). Inclusion of nonspecific goat IgG did not show a positive reaction, indicating the absence of nonspecific reaction.

**PGN phosphorylates p38 MAPK in PMCs.** As the pharmacological inhibition of p38 MAPK activation resulted in the downregulation of mBD-2 expression, we intended to examine the status of p38 MAPK activation in PGN-treated PMCs. Activation of p38 MAPK was assessed by its ability to phosphorylate its downstream signaling target ATF-2 protein in the kinase reaction. The amount of ATF-2 phosphorylation is directly proportional to that of p38 MAPK activity. Western blot analysis for phospho-ATF-2 demonstrated that PGN activation with PGN indeed resulted in ATF-2 phosphorylation (Fig. 6). Thus the phosphorylation of ATF-2 is the resultant of the activation of p38 MAPK. In PGN-activated PMCs, increased phospho-ATF-2 was found as early as 10 min and it increased linearly up to 60 min compared with the 0-min control. PGN treatment further increased ATF-2 phosphorylation to four and sixfold after 30 and 60 min of exposure,

Fig. 4. PGN-induced mBD-2 expression in PMCs is TLR-2 dependent. A: PMCs were transfected with TLR-2 small interfering RNAs (siRNAs) or control siRNA and treated with PGN (10 μg/ml) for 12 h or left untreated (control). Relative expression of mBD-2 compared with control is shown. Data are means ± SD from 3 independent experiments determined in triplicate, and each experiment was performed at different times. **P < 0.001, significant increase compared with resting PMC; #P < 0.001, significant decrease compared with PGN-activated cultures. B: PMCs were transfected with TLR-2 siRNA or control siRNA and treated with PGN (10 μg/ml) for 3 h or left untreated (control). Relative expression of mRNA for TLR-2 compared with control is shown. Data are means ± SD of 3 independent experiments determined in triplicate, and each experiment was performed at different times. **P < 0.001, significant increase with resting PMCs; #P < 0.001, significant decrease compared with PGN-activated cultures. C: murine PMCs were transfected with TLR-2 siRNA or control siRNA and treated with PGN (10 μg/ml) for 24 h or left untreated (control). Cells were fixed and analyzed by immunofluorescence staining for mBD-2 protein using FITC (green) labeled secondary antibody and DAPI (blue) for nuclear staining. Data shown are single representative of 3 similar observations each estimated at different times. SFM, serum-free medium.
components, such as PGN, LPS, and nucleic acids, have all been shown to engage TLRs (47, 48, 57, 74, 75). Upon coming in contact with the pathogenic agents, TLRs initiate downstream signaling ultimately to transactivate genes involved in resolving the impending microbial invasion (10, 58).

TLRs recognize microbes or microbial-derived products. TLR-expressing leukocytes participate in the host innate immune responses. In human keratinocytes, multiple TLRs were also implicated in the signal transduction in response to S. aureus, Mycobacterium tuberculosis, Candida albicans, LPS, and PGN (36, 53). Bronchial epithelial cells are positive for TLR-1 through TLR-10 and are responsive to influenza-A virus, flagellin, zymosan, PGN, LPS, and dsRNA in the induction of various cytokines and chemokines (17, 61). Taken together, these studies indicate that cells that are at risk of exposure to pathogenic agents express TLRs and trigger an innate immune response. This underlines the importance of TLR expression in the host defense. Consistent with the aforementioned data, the constitutive expression of multiple TLRs in the present study indicates that PMCs are well equipped to recognize and to mount an innate immune response against a diverse range of infectious agents in the pleural space. Addition ally, a surge in TLR-2 expression in PMCs by PGN, a TLR-2 agonist, suggests a mechanism for a rapid and heightened immune response against S. aureus. In macrophages, similar increases in TLR-2 expression were found in response to PGN, LPS, and nucleic acids (33, 35, 48) and after exposure to both gram-positive and gram-negative bacteria (75). Expression of multiple TLRs by PMCs reveals that PMCs are implicated in innate immune responses against pathogens in the pleural space.

Disensins are expressed in multiple tissues, most notably by the leukocytes and by the cells of epithelial surfaces in the body. Defensins are critical to the host innate immunity. The wide range of pathogens, including bacteria, viruses, and fungi, as well as microbial components, such as PGN, LPS, and nucleic acids, have all been shown to engage TLRs (47, 48, 57, 74, 75). Upon coming in contact with the pathogenic agents, TLRs initiate downstream signaling ultimately to transactivate genes involved in resolving the impending microbial invasion (10, 58).
hBDs are a subgroup of the defensin family of proteins produced mostly by the epithelial lining of the respiratory, gastrointestinal, and genitourinary tract. Predominant expression of hBDs at surfaces often prone to pathogen exposure suggests that these peptides play an important role in the host defense. Accordingly, the antimicrobial properties of hBDs have been demonstrated in vivo and in vitro against a wide range of pathogens (15, 16, 18, 19, 26, 59). The hBDs are inducible by pathogenic microbes as well as by their cellular components, including nucleic acids, LPS, and PGN (15, 18, 19, 54, 69). Similar to humans, BDs were also reported in the mouse. The mBD-1 was constitutively expressed in multiple epithelia and was bactericidal in nature (6, 27, 43). Murine BD-2 expression was found in the reproductive tract and ocular surface tissue (11, 28, 62) and was induced in enteroendocrine cells by LPS and flagellin and in airways by LPS (42, 52). Murine BD-4 is expressed in the tongue, esophagus, and trachea (30). mBD-3 and mBD-6 are inducible and bactericidal (7, 8, 60, 71). A defensin-related protein called Defr-1, which lacks one of the conserved six cysteine residues present in the other mBDs (23, 54, 69, 70). However, PMC activation with PGN did not induce mBD-1, mBD-3, mBD-4, mBD-6, or Defr-1. It is possible that these defensins are regulated by different TLR signaling mechanisms in PMC. Accordingly, upregulation of TLR-2 and mBD-2 expressions after PGN treatment and attenuation of mBD-2 expression upon silencing of TLR-2 demonstrates that TLR-2 mediates mBD-2 induction in PMCs. Further, the rise in TLR-2 mRNA and protein levels at 3 and 12 h, respectively, compared with mRNA and protein levels of mBD-2 at 12 and 24 h, respectively, after PGN treatment indicates that TLR-2 activation preceded mBD-2 production.

Regulation of mBD-2 expression in PMCs has not been studied. Upon binding with their respective TLRs, various agonists elicit downstream signaling culminating with NF-κB activation and production of cytokines (44, 58). MAPK cascades play a key role in transduction of extracellular signals to cellular responses. This kinase cascade participates in the regulation of gene expression by connecting the extracellular signals to the intracellular transcriptional elements and regulatory proteins (64). Based on agonists, TLR receptor activation results in downstream activation of p38, p44/42, and PI3 MAPK in various cells (51, 66). The inclusion of the p44/42 inhibitor PD98059 and the PI3-kinase inhibitor LY294002 did not block PGN-induced mBD-2 expression in PMCs. However, a p30 MAPK inhibitor significantly blocked PGN-induced mBD-2 expression in PMCs. The p38 MAPK is downstream of the TLR-2 signal phosphorylate and activates the group of MAPK protein kinases MK1 and MK2, the mitogen- and stress-activated kinases MSK1 and MSK2, and the MAPK-interacting kinases MNK1 and MNK2 and ultimately results in cellular activation (21, 50, 55). p38 MAPK has been shown to signal the induction of hBDs in different cell types (33, 38). In the present study, downmodulation of PGN-induced mBD-2 expression by SB203580 clearly demonstrates the critical role of p38 MAPK signaling in the induction of mBD-2 in murine PMC. Additionally, increased kinase activity of p38 MAPK in PMCs as measured by its ability to phosphorylate ATF-2 after PGN treatment substantiated this obser-

Fig. 6. PGN induces phosphorylation of p38 MAPK in PMCs. The activity of p38 MAPK was measured by immune complex protein kinase assay using activating transcription factor-2 (ATF-2) substrate. Phosphorylated ATF-2 was detected after Western blot analysis by autoradiography. Top: relative densities (means ± SD) representing each of phospho-ATF-2 bands from 3 similar but independent evaluations. *P < 0.05, **P < 0.001, significant increase compared with 0-h response; #P < 0.001, significant decrease compared with 60-min response. Bottom: single representative of 3 similar Western blots.
viation. Compared with unstimulated cells, the kinase activity of p38 MAPK in the PGN-treated cells remained elevated at least up to 4 h, suggesting that TLR-2-mediated signaling is a sustained event in PMCs. These data demonstrate that PGN-induced mBD-2 expression is dependent on p38 MAPK signaling in PMCs.

Infiltration of bacterial organisms into the pleural space during pleural effusion is a well-described complication of pneumonia (13, 14, 63). Pleural effusion may also result from viral infection of the lungs especially due to epidemic pneumo-
dynia, an infection usually caused by coxsackie viruses or echo viruses (25). The presence of Epstein-Barr virus particles in the pleural space has also been reported (67). These clinical complications indicate that PMCs that cover the pleural surface are in direct contact with the microorganisms or that their components may produce factors that counter the pathogens. We have previously shown that PMCs respond to S. aureus and produce inflammatory mediators. In addition, increased levels of defensins were reported in the pleural fluids of patients with empyema (5). This is an important observation considering that defensins are detected only in the pleural fluids of patients with empyema, while they are absent in other clinical conditions not associated with microbial infections. We noticed significantly increased bactericidal activity by the PMC-derived mBD-2 against S. aureus. These data indicate that PMCs by producing BD-2 may participate in the host innate immune response against gram-positive bacterial infection in the pleural space.

In the present study, we demonstrate that PMCs constitutively express TLR-1 through TLR-9. Staphylococcal PGN upregulated TLR-2 receptor expression and mBD-2 production in PMCs. We also demonstrate that mBD-2 production was dependent on TLR-2 activation. Further, we showed that TLR-2-mediated mBD-2 production in PMCs was dependent on p38 MAPK signaling. Finally, we show PMC via releasing BD-2 promotes bactericidal activity against S. aureus. Taken together, our results show that PMCs are capable of producing the components of innate immunity and enhancing the host innate defenses. To our knowledge, this is the first study to show that PMCs directly participate in innate immunity by virtue of the production of mBD-2 against pleural infections.

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