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# Peptidoglycan-mediated IL-8 expression in human alveolar type II epithelial cells requires lipid raft formation and MAPK activation

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**Abstract**

*Staphylococcus aureus*, a major sepsis-causing Gram-positive bacterium, invades pulmonary epithelial cells and causes lung diseases. In the lung, alveolar type II epithelial cells play an important role in innate immunity by secreting chemokines and antimicrobial peptides upon bacterial infection whereas type I cells mainly function in gas-exchange. In this study, we investigated the ability of *S. aureus* peptidoglycan (PGN) to induce expression of a chemokine, IL-8, in a human alveolar type II epithelial cell line, A549. PGN induces IL-8 mRNA and protein expression in a dose- and time-dependent manner. Supplementation of soluble CD14 further enhanced the PGN-induced IL-8 expression. Interestingly, PGN-induced IL-8 expression was inhibited by nystatin, a specific inhibitor for lipid rafts, but not by chlorpromazine, a specific inhibitor for clathrin-coated pits. Furthermore, PGN-induced IL-8 expression was attenuated by inhibitors for MAP kinases such as ERK, p38 kinase, and JNK/SAPK, whereas no inhibitory effect was observed by inhibitors for reactive oxygen species or protein kinase C. Electrophoretic mobility shift assay demonstrates that PGN increased the DNA binding of the transcription factors, AP-1 and NF-κB while minimally, NF-IL6, all of which are involved in the transcription of IL-8. Taken together, these results suggest that PGN induces IL-8 expression in a CD14-enhanced manner in human alveolar type II epithelial cells, through the formation of lipid rafts and the activation of MAP kinases, which ultimately leads to activation of AP-1, NF-κB, and NF-IL6.


*Keywords:* Peptidoglycan; *Staphylococcus aureus*; IL-8; Epithelial cells; A549; Signaling; MAP kinase; Transcription factor

## 1. Introduction

Despite frequent exposure to various pathogens during respiration, the lung maintains mucosal integrity in part through the innate immune system. Once invading pathogens enter the tracheobronchial tract, the host mucociliary movement moves the pathogens out of the respiratory tract (Machen, 2006). Pathogens that escape the host mucociliary movement can be inactivated by various antimicrobial sentinel molecules such as β-defensins, LL-37, and surfactant proteins (Kuroki et al., 2007; Rogan et al., 2006). Surviving pathogens cause disease by adhering to and invading the host epithelium or by colonizing the mucus layers (Gillespie and Balakrishnan,

*Abbreviations:* AP-1, activating protein 1; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; IL-8, interleukin-8; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; NF-IL6, nuclear factor for IL-6; NAC, *N*-acetyl-L-cystein; NF-κB, nuclear factor-kappa B; PAMPs, pathogen-associated molecular patterns; PGN, peptidoglycan; ROS, reactive oxygen species; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; sCD14, soluble CD14.

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2000). However, epithelial cells activate diverse immunoregulatory functions in response to pathogens. For example, when alveolar type II epithelial cells sense pathogen-associated molecular patterns (PAMPs), they secret chemokines, such as interleukin-8 (IL-8) to recruit and activate neutrophils during the early stage of infection. Upon recruitment, neutrophils phagocytose pathogens and mediate inflammatory responses (Fehrenbach, 2001).

*Staphylococcus aureus* is a major pulmonary pathogen and is associated with pneumonia and other airway diseases such as cystic fibrosis (Krivan et al., 1988). Without proper treatment, *S. aureus* infection often leads to severe inflammatory syndrome, septic shock, or multi-organ failure (Wang et al., 2003). This cascade is mediated by bacterial cell wall components such as peptidoglycan (PGN) and lipoteichoic acid (LTA) (Kumar et al., 2004; Wang et al., 2004). These cell wall components can trigger an excessive release of pro-inflammatory cytokines including TNF-α, IL-6, and IL-8 (Kumar et al., 2004; Wang et al., 2000) and nitric oxide (Hattor et al., 1997). Notably, PGN constitutes about 90% of the cell wall components of Gram-positive bacteria (Palaniyar et al., 2002), suggesting that PGN may play a critical role in the manifestation of bacterial infection. Indeed, PGN enhances production of the pro-inflammatory cytokines to a greater degree than LTA (Hessle et al., 2005). Also, *S. aureus* PGN strongly induces IL-8 release in whole blood (Hadley et al., 2005) and in monocytes (Nadesalingam et al., 2005).

Although PGN is known to induce various pro-inflammatory cytokines, the signaling pathways involved are under debate. For instance, one study suggested that PGN is recognized by Toll-like receptor 2 (TLR2) (Schwandner et al., 1999), while another suggested that highly purified PGN is not sensed by TLR2, TLR2/1, or TLR2/6 (Travassos et al., 2004). However, CD14 (Weidemann et al., 1997) and nucleotide-binding oligomerization domain (NOD) family members are needed for PGN recognition, though Gram-positive bacterial PGN preferentially activates NOD2 (Strober et al., 2006). Once PAMPs are recognized by the receptors, formation of lipid rafts or clathrin-coated pits is necessary to transmit signals to the intracellular compartment (Dykstra et al., 2003; Stoddart et al., 2002). For example, LPS signaling is mediated through the formation of lipid rafts (Triantafilou et al., 2002). Furthermore, intracellular signaling pathways for IL-8 induction require mitogen-activated protein kinases (MAPKs) such as JNK/SAPK, ERK, and p38 kinase (Hoffmann et al., 2002). MAPK activation subsequently leads to the activation of transcription factors such as NF-κB, AP-1, and NF-IL6, all of which bind to the promoter of IL-8 gene, thereby regulating IL-8 transcription (D'Angio et al., 2004). Interestingly, NF-κB appears to be essential for the induction of IL-8 transcription (Mukaida et al., 1994), while AP-1 and NF-IL6 are required for the maximal gene expression (Hoffmann et al., 2002).

Although a number of studies have focused on the effect of PGN to the antigen-presenting cells such as macrophages and dendritic cells, little is known about the role of PGN in stimulating respiratory epithelial cells. We therefore explored the action of *S. aureus* PGN on IL-8 expression in a human alveolar type II epithelial cell model.

## 2. Materials and methods

### 2.1. Reagents and chemicals

*S. aureus* PGN and soluble CD14 (sCD14) were purchased from InVivoGen (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Nystatin, chlorpromazine, *N*-acetyl-L-cysteine (NAC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Calphostin C and all the specific MAPK inhibitors were purchased from Calbiochem (La Jolla, CA, USA).

### 2.2. Cell culture

A549 cells were obtained from the American Type Culture Collection (Mannas, VA, USA) and cultured in Ham's F-12 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and a commercial antibiotics mixture, Antibiotic-Antimycotics (Invitrogen) at 37 °C in a humidified incubator with 5% $CO_2$. Before stimulation, cells were cultured for 6 h in the same media but containing 0.1% FBS.

### 2.3. IL-8 ELISA

A549 cells were plated on 48-well culture plates at $1 \times 10^5$ cells/ml in 500 μl of the aforementioned media containing 0.1% FBS. Cells were stimulated with *S. aureus* PGN at various doses for the indicated time period. For the experiments using inhibitors, cells were pre-treated with the designated inhibitors for 1 h followed by a 24-h PGN treatment. Media were then collected and analyzed for IL-8 production by a commercial IL-8 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### 2.4. Measurement of cell viability

Cell viability was determined by the MTT-based cytotoxicity assay as previously described (Plumb et al., 1989). Briefly, MTT solution (0.5 mg/ml) was added to the cells in a microtiter plate and incubated at 37 °C in 5% $CO_2$ for 40 min. Then, the culture media containing the MTT solution was removed, and cells were dissolved in dimethylsulfoxide (Sigma–Aldrich Chemical Co.). Then, absorbance at 550 nm was measured using a microtiter plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

### 2.5. RT-PCR

Expression of IL-8 mRNA was evaluated by RT-PCR. Cells were seeded on 60 mm-dishes and treated with *S. aureus* PGN for the indicated time period. Following treatment, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA)