

# Bacterial Induction of Early Response Genes and Activation of Proapoptotic Factors in Pleural Mesothelial Cells

Kamal A. Mohammed · Najmunnisa Nasreen ·  
Veena B. Antony

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**Abstract** In bacterial empyema the pleural mesothelium is constantly exposed to microorganisms. *Staphylococcus aureus* (*S. aureus*) is one of the most frequent pathogens associated with empyema. In an earlier study we demonstrated that *S. aureus* induced barrier dysfunction in pleural mesothelial cell monolayers. In the present study we report that *S. aureus* activates the early response genes *c-fos* and *c-jun* and activator protein-1 (AP-1), and induces proapoptosis genes *Bad* and *Bak* in primary mouse pleural mesothelial cells (PMCs). Our data indicate that in PMCs *S. aureus* induces apoptosis in a time- and multiplicity of infection (MOI)-dependent manner. *Staphylococcus aureus* induced *Bcl<sub>2</sub>*, *Bcl-X<sub>L</sub>*, *c-fos*, *c-jun*, and AP-1 expression in PMCs during the initial phase of infection. In *S. aureus*-infected PMCs, *Bad* and *Bak* gene expression was increased and correlated with DNA fragmentation and cytochrome-c release. *Bcl<sub>2</sub>* and *Bcl-X<sub>L</sub>* gene expression was significantly lower in *S. aureus*-infected PMCs than in uninfected PMCs 12 h postinfection. We conclude that at the initial stage of infection *S. aureus* modulates expression of early response genes *c-fos* and *c-jun*, and in the late phase of infection *S. aureus* induces expression of proapoptotic genes *Bak* and *Bad* in PMCs. Silencing AP-1

significantly inhibited *S. aureus*-induced *Bak* and *Bad* expression in PMCs. The upregulation of early response genes during the early phase of infection may contribute to the activation of proapoptotic genes *Bak* and *Bad* and release of cytochrome-c, caspase-3 thereby resulting in apoptosis in PMCs.

**Keywords** Apoptosis · TUNEL · EMSA · *c-fos* · *c-jun* · *Bak* · *Bad* · Cytochrome-c

## Introduction

*Staphylococcus aureus* is a leading cause of the development of parapneumonic effusions in pleural space [1]. 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. *Staphylococcus aureus* accounts for a large number of cases of pediatric empyema [2] and is a significant cause (19.7%) of bacterial pneumonia in patients with acquired immunodeficiency syndrome [3]. It is estimated that 0.8% of childhood pneumonia progresses to empyema, affecting 3.3% per 100,000 children [4, 5]. Early diagnosis of infected pleural effusion and rapid drainage is necessary to reduce the morbidity and mortality in patients with empyema [6].

In empyema the pleura is exposed to invading pathogens. In earlier studies we demonstrated that *S. aureus* induces an acute inflammatory response in the pleural space [7] and compromises pleural mesothelial cell (PMC) barrier function [8]. Pleural mesothelial integrity is necessary for maintenance of barrier function [7]. It is unclear if *S. aureus* has any deleterious effect on PMCs. Interaction of *S. aureus* with host cells often results in the

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Kamal A. Mohammed, Najmunnisa Nasreen contributed equally to this work.

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K. A. Mohammed · N. Nasreen · V. B. Antony  
Division of Pulmonary, Critical Care, and Sleep Medicine,  
Department of Medicine, College of Medicine, University of  
Florida, JHMHC, P.O. Box 100225, Gainesville,  
Florida 32610-0225, USA

K. A. Mohammed (✉) · N. Nasreen  
Malcom Randall VA Medical Center, 1601 Archer Rd,  
Gainesville, Florida 32608-1135, USA  
e-mail: mkamal@medicine.ufl.edu

internalization of bacteria [9, 10], and intracellular *S. aureus* induces apoptosis in epithelial and endothelial cells [11–13]. However, the pathway through which *S. aureus* induces apoptosis is not clear.

Apoptosis is an active cellular process of gene-directed self-destruction [14]. The process of apoptosis is regulated mainly by two gene family members, namely, the *Bcl2*-related genes and caspases. Caspases are a family of cysteine proteases that are activated during apoptosis [15]. The *Bcl2* family regulates apoptosis by controlling the activity of caspases via cytochrome-c from mitochondria [16]. The members of this family are separated functionally into two groups, apoptotic agonists (*Bad*, *Bix*, *Bak*) and apoptotic antagonists (*Bcl2*, *Bcl-X<sub>L</sub>*, *Bcl-w*). Proapoptotic and antiapoptotic family members mediate their proapoptotic or antiapoptotic signals through their relative abundance. They dimerize through their *Bcl2* homology domains, neutralizing each other's function [17]. *Bad* regulates the execution of apoptosis and *Bak* promotes cell death and counteracts the protection from apoptosis provided by *Bcl2* [18].

Recent studies revealed that cells undergoing apoptosis express the early response genes *c-fos* and *c-jun*, which code for transcriptional factors [19, 20]. The *fos/jun* early response genes encode proteins that are major components of the AP-1 transcription factor. These proteins are members of the bZIP family of transcription factors, capable of forming homo- and heterodimeric AP-1 complexes with each other as well as with other nuclear factors [21]. AP-1 is a key regulator of major biological processes, including cell proliferation, differentiation, and apoptosis [22]. Although *S. aureus* is known to induce apoptosis in a variety of cell types, the underlying mechanisms by which *S. aureus* induces apoptosis in PMCs have not been investigated. In the present study we evaluated the mechanisms of *S. aureus*-induced apoptosis in primary cultures of mice PMCs. Our data indicate that *S. aureus* induces the early response genes *c-fos* and *c-jun*; moreover, the expression of antiapoptotic genes *Bcl2* and *Bcl-X<sub>L</sub>* spiked at 12 h at the initial stage of infection. *S. aureus* induced the expression of proapoptotic genes *Bak* and *Bad* of the *Bcl2* family and cytochrome-c release in the later phase of infection.

## Material and Methods

### Reagents

Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). F12K media, AP-1 stealth RNAi, and the primers for *Bcl-X<sub>L</sub>*, *Bcl2*, *Bak*, and *Bad* were synthesized by Invitrogen (Carlsbad, CA). An *in situ* Apoptag labeling kit was obtained from Oncor

(Gaithersburg, MD). Biodyne® nylon membranes were purchased from Pierce Biotechnology (Rockford, IL). *c-fos*- and *c-jun*-specific polyclonal antibodies were obtained from Oncogene (Boston, MA). The cytochrome-c-specific monoclonal antibody and caspase-3-specific cellular activity kits were from Calbiochem (San Diego, CA) and the chemiluminescence ECL kit was from Amersham Pharmacia Biotech (Piscataway, NJ).

### Isolation and Characterization of Primary Cultures of Mice PMCs

Female mice with C57BL/6 background (5–6 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). PMCs from normal healthy (control) animals were obtained by collagenase digestion of visceral and parietal pleura as reported earlier [23]. Briefly, the pleural surfaces were exposed to collagenase (10 mg/ml) and PMCs were harvested, resuspended in F12K culture medium, and grown to confluence in tissue culture flasks. The PMCs were characterized by the presence of classic cobblestone morphology [24], the absence of factor VIII antigen, and the presence of cytokeratin [25].

### Assessment of Viable Intracellular *S. aureus*

PMCs were grown to confluence in 24-well tissue culture plates. *Staphylococcus aureus* with various multiplicities of infection (MOI) (1:1, 1:10, and 1:100) was added to each well and incubated for 3–72 h. At the end of the incubation period, uningested bacteria were removed by washing three times with HBSS. The bacteria that were not ingested but attached to PMCs were removed by lysing with Lyso-staphin (10 µg/ml for 20 min in HBSS). PMC culture was lysed by treating it with hypotonic buffer; the number of viable intracellular *S. aureus* (colony-forming units, CFU) was then estimated. The cell culture was vortex agitated briefly to disperse *S. aureus* clumps. Appropriate dilutions were performed on each sample, and duplicate aliquots of each dilution were plated on tryptic soy agar (TSA; Difco Labs, Detroit, MI) spread plates. Spread plates were incubated at 37°C for 24 h and the colonies were counted. Each experiment was performed three times in duplicates.

### Preparation of *S. aureus* Culture and PMC Stimulation

A mouse virulent strain of *S. aureus* (ATCC) was cultured as reported earlier [8]. The bacteria were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), and resuspended in F12K serum-free media (SFM).

PMCs ( $5 \times 10^5$ /ml) were incubated in the presence of *S. aureus* with various MOI (1:1, 1:10, and 1:100, PMC : *S. aureus*) in SFM for 6, 12, 24, and 48 h at 37°C in 5% CO<sub>2</sub>. The induction of apoptosis was determined with all three MOI. Further experiments were performed using 1:10 MOI.

#### Quantitative Detection of Apoptosis by TUNEL Assay

Mice PMCs were cultured in chambered glass slides and were incubated with either SFM or *S. aureus* for a desired period of time. The PMC culture was fixed with 4% paraformaldehyde. PMC apoptosis was estimated by the TUNEL method (*In situ* Apoptag Labeling kit, Oncor, Gaithersburg, MD) as reported earlier [27]. The percentage of apoptotic cells (percent positively stained nuclei to total nuclei) was determined by counting a total of 100 cells per slide and at least three slides per group.

#### Determination of Apoptotic DNA (Ladder) by Electrophoresis

DNA extraction and electrophoresis were performed as described earlier [28]. In brief,  $5 \times 10^6$  PMCs were incubated with either SFM or *S. aureus* (1:10 MOI, PMC : *S. aureus*) for 6, 12, 24, and 48 h. The isolated DNA was subjected to electrophoresis in 1.6% agarose gel at a concentration of 12 µg per lane and visualized after ethidium bromide staining.

#### Western Blot Analysis for Estimation of *c-fos*, *c-jun*, and Cytochrome-c

PMCs after activation with *S. aureus* were washed three times by centrifugation. The cell pellet was lysed and nuclear lysates were used to detect the expression of early response genes. Nuclear lysates ( $10^6$  cells/sample) were subjected to SDS-PAGE on gels containing 12% (wt/vol) acrylamide under reducing conditions. Separated proteins were transferred to Biodyne® nylon membranes (Pierce). *c-fos* and *c-jun* expression was detected by *c-fos/c-jun*-specific polyclonal antibodies obtained from Oncogene (Boston, MA). The cytochrome-c expression was detected by using a monoclonal antibody specific to cytochrome-c (Calbiochem, San Diego, CA). Specific protein detection was performed by chemiluminescence using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). The relative densities of *c-fos*, *c-jun*, and cytochrome-c were estimated using Quantity One software on GS-710 calibrated imaging densitometer (BioRad, Hercules, CA).

#### Estimation of AP-1 by Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared as described previously [29]. LightShift (Chemiluminescent EMSA kit; Pierce, Rockford, IL) was used according to the manufacturer's instructions. In brief, for each sample, 50 µM biotin-labeled (biotin 3' end DNA labeling kit; Pierce) double-stranded oligonucleotide sequence for AP-1 (Promega, Madison, WI) was incubated with 6 µg of nuclear extract. To assess the binding specificity, an excess of unlabeled AP-1/DNA complexes was added to appropriate tubes at the start of the binding reaction. Protein-DNA complexes were quantified as above.

#### Stealth RNAi and Transfection of PMCs

Stealth RNAi targeting the transcription factor AP-1 sense GGUGUGGUGGAAGUGUACCGCGUAA, anti-sense UUACGCGGUACACUCCACCACACC, and scrambled (control) RNAi were designed using Block-iT RNAi Designer (Invitrogen, Carlsbad, CA) and synthesized. PMCs were plated in a 60-mm culture dish and incubated at 37°C in 5% CO<sub>2</sub> for 24 h. The transfection of PMCs with RNAi (100 nm) was performed using lipofectamine-2000 (Invitrogen). After 4 h of transfection the culture was incubated at 37°C in 5% CO<sub>2</sub> in media containing 10% serum. Twenty-four hours after transfection the PMCs were infected with *S. aureus* but some cultures were left uninfected. The total RNA was extracted for quantitative PCR.

#### Total RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA from PMCs was isolated by using a highly pure RNA isolation kit (Roche Applied Science, Indianapolis, IN). RNA samples were diluted with RNase-free water to 100 ng/µl, and 10 µl of each sample with 1 µl of 3.5 µM anchored oligo(dT)23 (Sigma, St. Louis, MO) were denatured at 70°C for 10 min followed by 2 min of incubation on ice. The cDNA strand was generated using AMV reverse transcriptase and 20 units of RNase inhibitor. The reaction mix was incubated for 50 min at 42°C, followed by an enzyme inactivation step of 15 min at 70°C. For PCR, 50 µl of reaction mix contained cDNA, 25 µl of SYBR Green JumpStart Taq ReadyMix, 0.5 µl of internal reference dye, and 14.5 µl of corresponding oligonucleotide primers (80 nM final concentration). The *Bcl<sub>2</sub>*, *Bcl-X<sub>L</sub>*, *Bak*, and *Bad* primer pairs were purchased from R & D Systems (Minneapolis, MN). Amplification and detection were performed using the SYBR Green method and the

Applied Biosystems (Foster City, CA) 7500 Real-Time PCR System with the following profile: 1 cycle at 94°C for 2 min, 40 cycles at 94°C for 15 s, 60°C for 1 min, 72°C for 1 min. The fluorescence resulted from the incorporation of SYBR Green dye into the double-stranded DNA that was produced during the PCR reaction, and emission data were quantified using the threshold cycle (CT) value.

### Caspase-3 Cellular Activity Assay

Caspase-3 activity was measured in PMC extracts as recommended by the manufacturer (Calbiochem, San Diego, CA). In brief, mouse PMCs ( $20 \times 10^6/\text{ml}$ ) were grown to confluence and infected with *S. aureus* (1:10, PMC : *S. aureus*) or left uninfected. Cell lysates were prepared in ice-cold lysis buffer, left on ice for 10 min, and centrifuged. The protein concentrations of the cell lysates were determined using the BCA reagent method. Supernatants were collected and frozen at  $-70^\circ\text{C}$  until use. Before starting the assay, the plate conversion factor was determined. The enzyme activity was expressed as pmol substrate/min. Standard substrate *p*-nitroaniline (*p*NA) (50  $\mu\text{M}$ ) was used. The samples were run in triplicate and 10  $\mu\text{l}$  of caspase-3 inhibitor (Ac-DEVD-CHO) was added to prepare the inhibitor-treated samples. To 10  $\mu\text{l}$  of test samples 40  $\mu\text{l}$  of assay buffer and 50  $\mu\text{l}$  of caspase-3 substrate I (Ac-DEVE-*p*NA) were added and incubated at 37°C for 30 min. The absorbance was read at 405-nm excitation and 460-nm emission using a plate reader. The data were collected from 5 to 120 min and analyzed using following equation:

$$\text{caspase - 3 specific activity} = \frac{\text{absorbance}/\text{min}}{\times \text{conversion factor} \times 100 \mu\text{l}}$$

The caspase-3 activity was expressed as pmol/min/ $\mu\text{g}$  protein.

### Statistical Analysis

Data were analyzed using SigmaStat statistical software (Apple Computer, Cupertino, CA). Results are expressed as mean  $\pm$  SE. The data were analyzed by analysis of variance (ANOVA) with the use of the Student-Newman-Keuls test for multiple comparisons and was considered significant if  $p < 0.05$ .

## Results

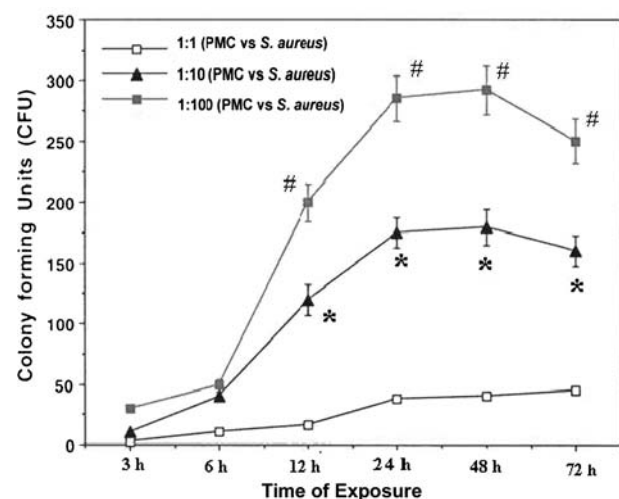
### Intracellular *S. aureus* Viability in PMCs over Time

The live intracellular bacteria were determined by counting CFUs. The infected cells were lysed and plated on agar

plates. We enumerated the number of intracellular bacteria at 3, 6, 12, 24, 48, and 72 h after infection with *S. aureus* using 1:1, 1:10, and 1:100 MOI. The intracellular CFUs increased with increasing MOI. Each MOI demonstrated a distinct and significantly increased CFU after 6 h. After 12 h of infection a significant increase in CFUs was noted with both 1:10 and 1:100 MOI. The intracellular viability of *S. aureus* increased over time until 24 h, after which it plateaued (Fig. 1).

### *S. aureus* Infection with Increasing MOI Demonstrated Increased Apoptosis in PMCs

To evaluate the percentage of apoptosis of PMCs, cells were infected with *S. aureus* or left uninfected for 6, 12, 24, and 48 h. The *in situ* detection of apoptotic DNA was estimated by TUNEL, which is based on the specific staining of the 3' hydroxyl ends of the fragmented DNA within apoptotic cells. We estimated the percentage of apoptotic cells over time after infection with *S. aureus* using 1:1, 1:10, and 1:100 MOI (PMC : *S. aureus*). *Staphylococcus aureus* induced apoptosis in PMCs in a time-dependent manner, and the percentage of apoptotic cells increased with increasing MOI (Fig. 2A). In *S. aureus*-infected PMCs (1:10 MOI), the percentage of apoptosis was  $15.08 \pm 1.58\%$  after 12 h, which is statistically significant ( $p < 0.001$ ) compared to that of uninfected PMCs. After 24 h of *S. aureus* infection a further increase in apoptosis ( $32.49 \pm 2.05\%$ ) was noted; however, no



**Fig. 1** Intracellular viable *S. aureus* (CFU) in pleural mesothelial cells over time. PMC cultures were incubated with 1, 10, or 100 MOI of *S. aureus* per mesothelial cell for 3, 6, 12, 24, 48, and 72 h. The data represent mean  $\pm$  SE CFU of three separate experiments run as duplicates. The data also compared in each MOI over time and the  $p$  values are indicated as \* $p < 0.001$  compared with 1:1 MOI and # $p < 0.001$  compared with 1:10 MOI

apoptosis was noted in uninfected PMCs. The percentage of apoptotic cells at 48 h in *S. aureus*-infected and uninfected PMCs were  $41.49 \pm 3.9\%$  and  $7.28 \pm 0.91\%$ , respectively. Figure 2B is a representative example of *in situ* TUNEL labeling of apoptotic cells in *S. aureus*-infected and uninfected PMCs at 24 h. TUNEL positive staining was observed in nuclei and nuclear fragments with the morphologic characteristics of apoptosis in *S. aureus*-infected PMCs compared to uninfected PMCs. The identification of stained apoptotic bodies was confirmed by nuclear condensation and cytoplasmic compaction.

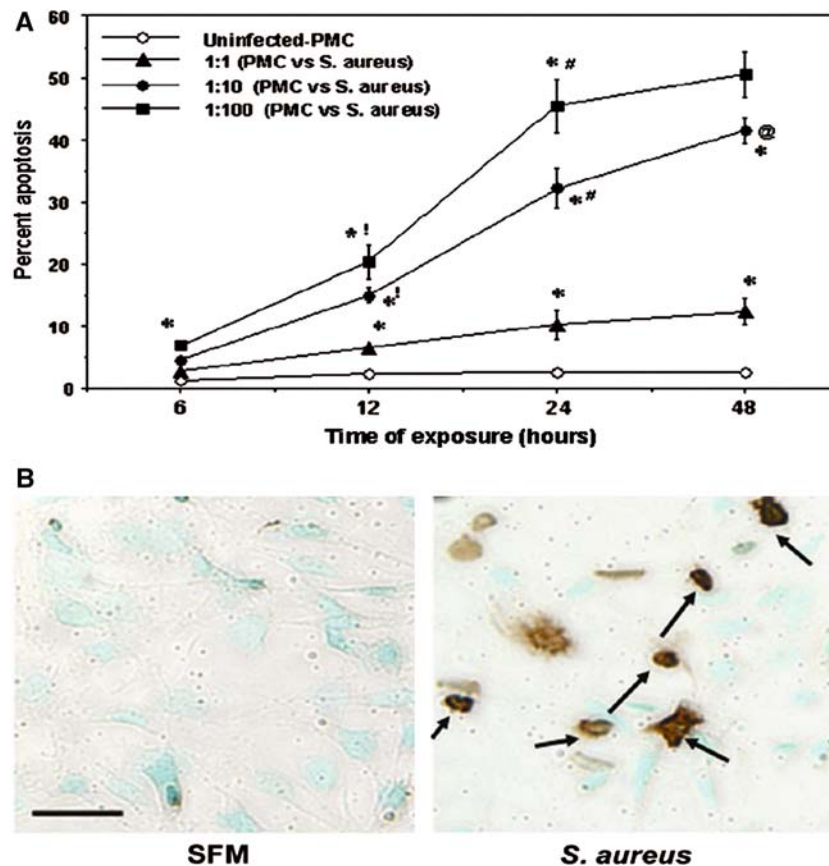
#### *S. aureus* Induces DNA Fragmentation in PMCs

DNA fragmentation is a hallmark of apoptosis. DNA fragmentation was evaluated by ladder analysis in agarose gels. Total DNA extracted from PMCs infected with

*S. aureus* and uninfected PMCs was visualized (Fig. 3). Six hours after *S. aureus* infection, the laddering pattern was observed and it increased over time. No DNA laddering was noted in uninfected PMCs.

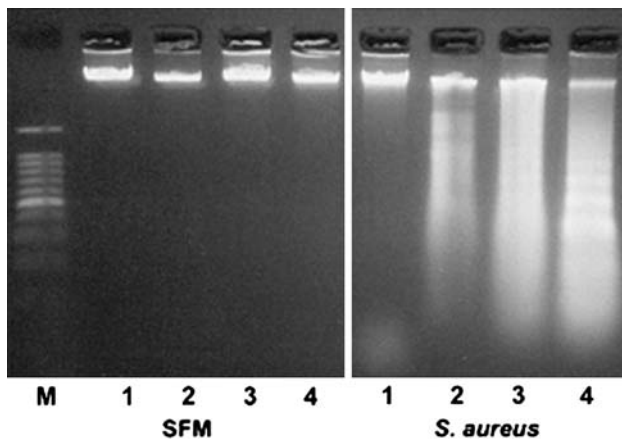
#### *S. aureus* Induces the Expression of *c-fos* and *c-jun*

The expression of early response genes *c-fos* and *c-jun* increased in *S. aureus*-treated PMCs from 3 to 12 h. We noted a steady increase in both *c-fos* and *c-jun* expression in *S. aureus*-infected PMCs up to 12 h. The maximum expression of *c-fos* and *c-jun* was noted at 12 h. However, after 24 h a significant decrease in the expression of *c-fos* and *c-jun* was noted in *S. aureus*-infected PMCs (Fig. 4). There was no significant *c-fos* expression noted in uninfected PMCs; however, there was some faint expression of *c-jun* but it was 12-fold lower than *S. aureus*-induced



**Fig. 2** *S. aureus*-induced pleural mesothelial cell apoptosis as estimated by TUNEL. **A** Increasing *S. aureus* MOI induced PMC apoptosis over time. PMC cultures were incubated with 1, 10, or 100 CFU of *S. aureus* per mesothelial cell and stained by TUNEL as described in the Methods section. \* $p < 0.001$  compared to uninfected and *S. aureus*-infected PMCs with listed MOI. The data also compared in each MOI over time and the  $p$  values are indicated as † $p < 0.001$  compared with 6 h, # $p < 0.001$  compared with 12 h, and

@ $p < 0.001$  compared with 24 h. Data are presented as mean  $\pm$  SE of three separate experiments estimated at three different times. **B** Representative image of TUNEL positive staining to demonstrate PMC apoptosis. PMCs incubated in the presence of SFM or *S. aureus* (1:10, PMC : *S. aureus*) for 24 h. PMC apoptosis was detected *in situ* by TUNEL-immunoperoxidase reaction. The data shown are single representatives of three independent experiments. Magnification:  $\times 400$ . Scale bar = 50  $\mu\text{m}$

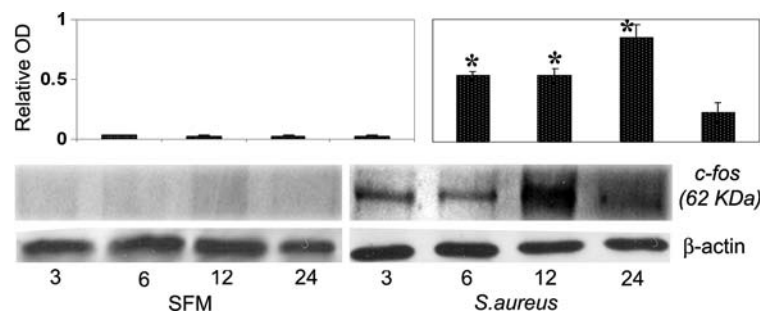


**Fig. 3** *Staphylococcus aureus*-induced DNA fragmentation in PMCs. The data presented are DNA gels stained with ethidium bromide demonstrating the typical apoptosis laddering pattern. PMCs cultured in the presence of SFM or *S. aureus* (1:10, PMC : *S. aureus*). Lane M = 100-bp DNA ladder. Lanes 1–4 are 6, 12, 24, and 48 h of exposure, respectively. The data shown are representative of three independent experiments

response (Fig. 5). The housekeeping gene  $\beta$ -actin was used to demonstrate equal loading of protein.

#### *S. aureus* Induces AP-1 DNA Binding Protein Expression

The effect of *S. aureus* on AP-1 DNA binding activity in PMCs was estimated by gel shift assay. PMCs were incubated for 12 h in the presence or absence of *S. aureus* and then the cells were harvested for nuclear protein. In cells treated with *S. aureus* a significant increase in AP-1 DNA binding complex was noted. The DNA binding complex was completely inhibited by coincubation of nuclear extracts, with an excess of unlabeled AP-1 oligonucleotide. The pretreatment of nuclear lysate with anti-*c-fos* and anti-*c-jun* antibodies decreased the expression of AP-1 DNA binding complex (Fig. 6).



**Fig. 4** *Staphylococcus aureus*-induced *c-fos* expression in PMCs. Western Blot analysis of resting and *S. aureus*-infected PMC nuclear lysates. *Upper panel*: relative optical densities, *middle panel* = autoradiograph showing *c-fos* expression, *lower panel* = autoradiograph

#### *S. aureus* Induces Proapoptosis Genes in PMCs

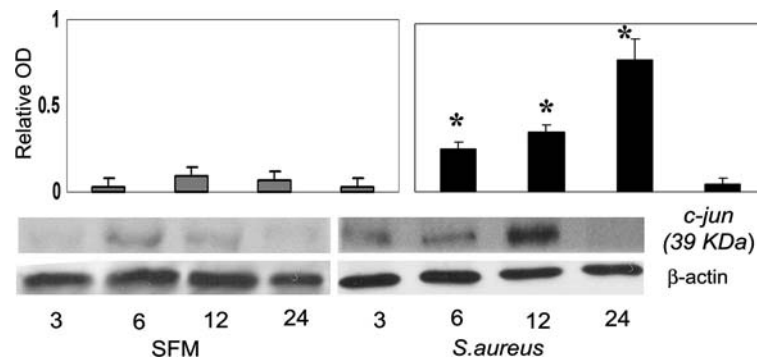
*S. aureus* infection induced significantly higher expression of proapoptotic genes compared to the antiapoptotic genes *Bcl2* and *Bcl-X<sub>L</sub>* in PMCs. In *S. aureus*-infected PMCs *Bcl2* and *Bcl-X<sub>L</sub>* gene expression spiked at 12 h; however, this response was short-lived and declined to basal levels thereafter (Fig. 7A, B). In contrast, there was no significant increase in *Bak* and *Bad* gene expression 12 h of *S. aureus* infection. However, there was a significant increase in *Bak* and *Bad* gene expression in *S. aureus*-infected PMCs after 24 and 48 h (Fig. 7C, D). In uninfected PMCs *Bcl-X<sub>L</sub>* gene expression was threefold higher, whereas *Bcl2* expression was fivefold higher than in *S. aureus*-infected cultures at 24 and 48 h. In uninfected PMCs both *Bcl-X<sub>L</sub>* and *Bcl2* expression was maintained and remained higher than that in *S. aureus*-infected PMCs after 24 h.

To understand the role of AP-1 in the regulation of proapoptotic genes *Bak* and *Bad*, PMCs were transfected with AP-1 stealth RNAi (100  $\mu$ M) or scrambled RNAi (RNAi-Sc) and infected with *S. aureus* for 24 h. Silencing the AP-1 gene with Ap1-RNAi significantly inhibited the *S. aureus*-induced expression of the proapoptotic *Bak* and *Bad* genes in PMCs compared to scrambled RNAi-treated PMCs (Fig. 8). This suggests that AP-1 plays a key role in the regulation of proapoptotic genes during *S. aureus*-induced PMC apoptosis.

#### *S. aureus* Infection Induces Cytochrome-c Release in PMCs

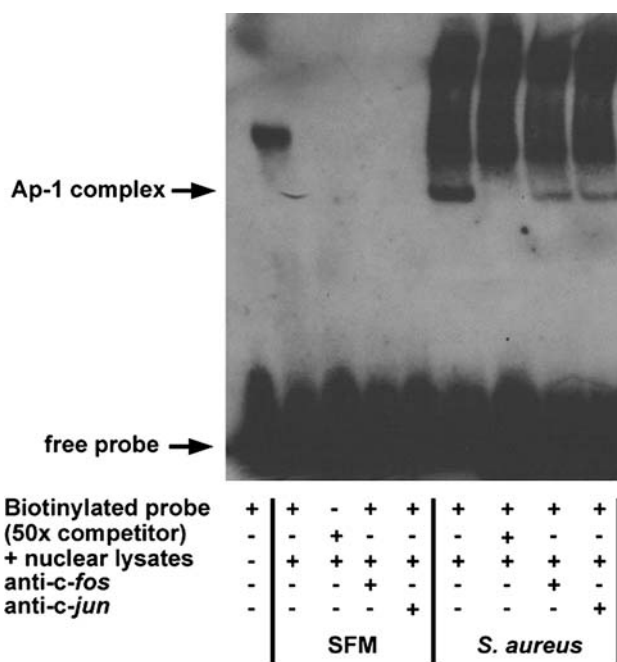
*Staphylococcus aureus* infection enhanced cytochrome-c release in PMCs. In uninfected PMCs there was no evidence of cytochrome-c release throughout the study period. In *S. aureus*-infected PMCs a significant increase in the cytochrome-c release was noted as early as 6 h and maximum cytochrome-c release was noted after 12 h of

showing  $\beta$ -actin expression demonstrating equal sample loading. Data shown are representative of three independent experiments. Relative OD = relative optical density as estimated by densitometry. \* $p < 0.001$  compared with uninfected PMCs



**Fig. 5** *Staphylococcus aureus*-induced *c-jun* expression in PMCs. Western Blot analysis of resting and *S. aureus*-infected PMC nuclear lysates. *Upper panel* = relative optical densities, *middle panel* = autoradiograph showing *c-jun* expression, *lower panel* = autoradiograph

showing  $\beta$ -actin expression demonstrating equal sample loading. Data shown are representative of three independent experiments. Relative OD = relative optical density as estimated by densitometry. \* $p < 0.001$  compared with uninfected PMCs



**Fig. 6** AP-1 DNA binding activity in *S. aureus*-infected and uninfected PMCs. Nuclear protein extracts were prepared from uninfected control (SFM) or *S. aureus*-infected PMCs for 12 h. Protein-DNA complex was resolved by nondenaturing gel electrophoresis and visualized by autoluminograph. Formation of a specific AP-1 DNA-protein complex was completely inhibited by coincubation with a 50-fold molar excess unlabeled AP-1 oligonucleotide. The pretreatment of nuclear lysate with antibodies specific to *c-fos* and *c-jun* decreased the expression of AP-1 DNA complex

*S. aureus* infection. However, this response plateaued 24 h after *S. aureus* infection (Fig. 9).

*S. aureus* Infection Induces Caspase-3 Activity in PMCs

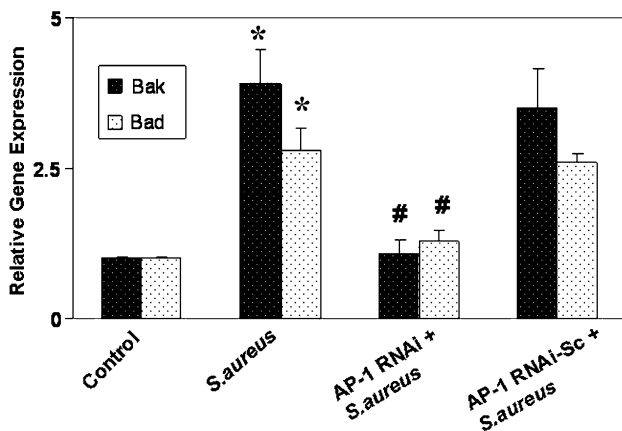
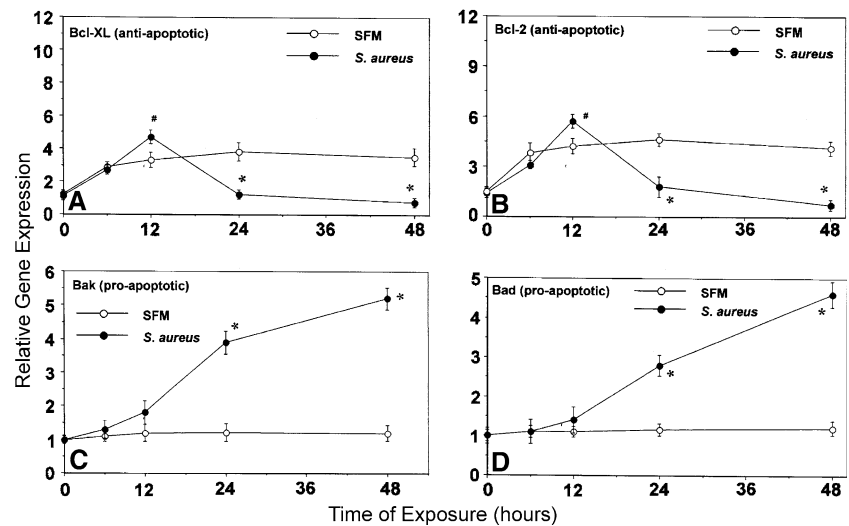
Caspase-3 activity was estimated in PMCs 12 h after *S. aureus* infection along with parallel uninfected PMC

cultures. In *S. aureus*-infected PMCs a significant increase in the caspase-3 activity was noted. In uninfected PMCs there was no evidence of caspase-3 activity. We also monitored the kinetics of caspase-3 activity in *S. aureus*-infected and uninfected PMCs. We noted a significant increase in the caspase-3 activity as early as 10 min in *S. aureus*-infected PMCs and maximum activity was noted after 1 h; the activity decreased thereafter. However, this response was significantly inhibited in caspase-3 inhibitor-treated samples, indicating a caspase-3-specific response. These results suggest that *S. aureus* induces apoptosis in PMCs in a caspase-3-dependent manner (Fig. 10).

**Discussion**

*Staphylococcus aureus* is a leading cause of the development of complicated parapneumonic effusions, especially in children. Early diagnosis and rapid drainage of infected pleural effusions is necessary to reduce the morbidity and mortality in empyema. The pleural mesothelial cells play a critical role in pleural integrity, and loss of pleural mesothelial integrity secondary to infection leads to pleural barrier dysfunction. In empyema, pleural mesothelial cells are constantly exposed to invading microbial pathogens. In earlier studies from this laboratory, we reported that *S. aureus* induced acute inflammatory responses in the pleural space via induction of murine KC and MIP-2 [7]. We also demonstrated that *S. aureus* infection results in pleural mesothelial barrier dysfunction [8]. We now provide evidence that *S. aureus* infection induces apoptosis in PMCs via induction of *c-fos* and *c-jun* early response genes that regulate downstream modulators of the apoptosis pathway. *S. aureus* induced apoptosis in PMCs in a time- and MOI-dependent manner as evaluated by TUNEL assay and apoptotic DNA ladder analysis. The number of intracellular bacteria in PMCs increased dramatically after 3 h

**Fig. 7** Relative proapoptotic and antiapoptotic gene expression in resting and *S. aureus*-infected PMCs as estimated by real-time quantitative PCR. PMCs were infected with *S. aureus* or left untreated for 0, 6, 12, 24, and 48 h. **A** *Bcl-X<sub>L</sub>* and **B** *Bcl<sub>2</sub>* (antiapoptotic genes). **C** *Bak* and **D** *Bad* (proapoptotic genes). The data are mean  $\pm$  SE of three independent experiments. \* $p < 0.001$  for *S. aureus*-infected PMCs vs. SFM (serum-free medium)



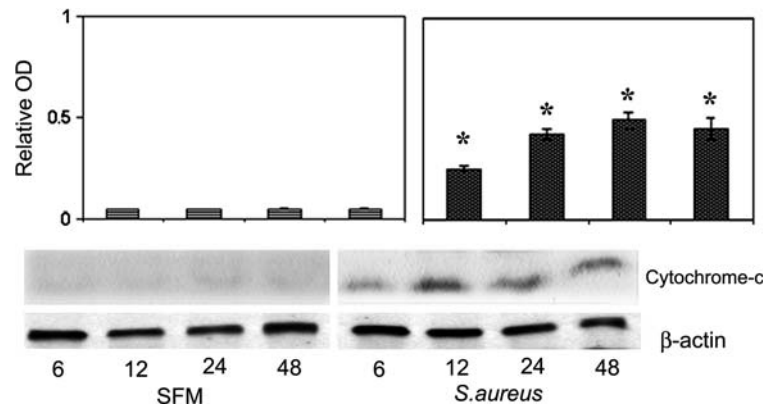
**Fig. 8** Silencing AP-1 expression inhibits *S. aureus*-induced proapoptotic gene expression in PMCs. Pleural mesothelial cells were infected for 24 h with *S. aureus* in the presence and absence of AP-1 RNAi, and *Bak* and *Bad* gene expression was estimated by quantitative PCR as discussed in the Methods section. The data are mean  $\pm$  SE of three independent experiments. \* $p < 0.001$  compared with uninfected PMCs and <sup>#</sup> $p < 0.001$  compared with *S. aureus*-induced response

of infection. This increased intracellular survival of *S. aureus* was directly correlated with PMC apoptosis as evidenced by TUNEL staining. *S. aureus* infection induced expression in PMCs of the proapoptotic genes *Bad* and *Bak*. Concomitantly, the expression of the antiapoptotic genes *Bcl<sub>2</sub>* and *Bcl-X<sub>L</sub>* was inhibited in these cells. We also noted an increase in cytochrome-c release in *S. aureus*-infected PMCs, which directly correlated with apoptosis.

During bacterial infection internalized pathogens multiply in the cytoplasm of the cell and are transmitted from cell to cell and thus achieve the infection-growth cycle [30]. In epithelial and endothelial cells, intracellular survival of *S. aureus* was noted to induce apoptosis [11, 13]. It is evident that apoptosis of *S. aureus*-infected PMCs is due

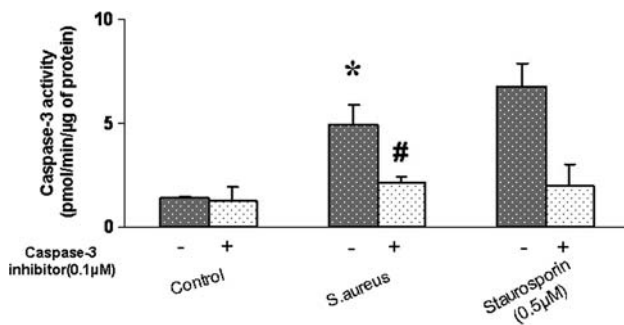
to intracellular bacterial growth over time. It is possible that free and multiplying bacteria in the cytoplasm may be responsible for inducing proapoptosis signals in PMCs. Increased expression of the early response genes *c-fos* and *c-jun* has been related to the development of apoptosis [31, 32]. In addition, signaling pathways for apoptosis have been linked to activation of specific signaling cascades and early accumulation of *c-fos*, *c-jun*, and *c-myc* [33–35]. Our data demonstrate that *S. aureus* induced expression of *c-fos* and *c-jun* early response genes in the early phase of infection in primary mouse PMCs. In uninfected PMCs no significant increase in *c-fos* and *c-jun* expression was noted. In a similar study with IL-2-dependent mouse melanoma cells, interleukin deprivation induced apoptosis via *c-fos* and *c-jun* induction, and inhibition of *c-fos* and *c-jun* expression by anti-sense oligonucleotides in these cells protected them from apoptosis induced by interleukin deprivation [33]. Preston et al. [36] demonstrated that *c-fos* gene expression causes apoptosis. High levels of *c-fos* were observed in mouse tissue in which apoptosis was part of normal development [35]. In addition, *S. aureus* infection was noted to induce *c-jun* expression in normal mouse osteoblasts [37]. A study on lymphoid cell lines also demonstrated that increased expression of *c-jun* induced apoptosis in response to various stimuli [33]. The mitogen-activated protein kinases (MAPK) and extracellular signal regulated protein kinases (ERK1 and ERK2) are activated during the invasion of bacteria, including *S. aureus* [37, 38]. MAPK-activated JNK phosphorylates the *c-jun*, which in turn enhances transcription of *FasL* and *TNF- $\alpha$*  genes which results in apoptosis [31]. In addition, JNK activation was also implicated as essential for induction of apoptosis because it is known to induce *c-jun* transcription [39]. During *S. aureus* infection the induction of early response genes *c-fos* and *c-jun* may regulate downstream events leading to apoptosis in PMCs.





**Fig. 9** Cytochrome-c response in *S. aureus*-infected and uninfected PMCs. Pleural mesothelial cell lysates were subjected to SDS-PAGE and Western blot analysis as described in the Methods section. *Upper panel* = relative optical densities, *middle panel* = autoradiograph showing cytochrome-c expression, *lower panel* = autoradiograph

showing  $\beta$ -actin expression demonstrating equal sample loading. Data presented are representative of three independent observations. Relative OD = relative optical density as estimated by densitometry. \* $p < 0.001$  compared with uninfected PMCs



**Fig. 10** *Staphylococcus aureus* induces caspase-3 expression in pleural mesothelial cells. Pleural mesothelial cells were infected with *S. aureus* for 12 h and caspase-3 activity was estimated. The data represent mean  $\pm$  SE of three independent experiments. \* $p < 0.001$  compared with uninfected PMCs and # $p < 0.001$  compared with *S. aureus*-induced response in the absence of caspase-3 inhibitor

AP-1 is considered the nuclear decision-maker that determines survival or cell death in response to extracellular stimuli. Cell death induced by *Fas* ligand (*FasL*) and its cell surface receptor *Fas* is a classical example of apoptosis induced by external signal. The *FasL* and *TNF- $\alpha$*  genes contain AP-1 binding sites. The combination of *c-fos* and *c-jun* to form the AP-1 complex is essential for the apoptotic process. We noted increased expression of *c-fos* and *c-jun* in *S. aureus*-infected cells after 6 and 12 h of infection. When we measured the AP-1 protein DNA binding with *c-fos/c-jun* in PMCs, we noticed an increase in AP-1 DNA binding complex in the *S. aureus*-infected PMCs. In addition, pretreatment of nuclear lysate with anti-*c-fos* and anti-*c-jun* antibodies significantly decreased the expression of AP-1 DNA binding complex. In *S. aureus*-infected PMCs the expression of AP-1 DNA binding complex directly correlated with the expression of *c-fos* and *c-jun*. These components of AP-1 are involved in

apoptosis; AP-1 is known to modulate apoptosis and the upregulation of *c-fos* and *c-jun* correlated with increased apoptosis [40]. *S. aureus*-induced apoptosis in PMCs was multiplicity-of-infection dependent. In PMCs the intracellular growth of *S. aureus* was directly correlated with the expression of *c-fos* and *c-jun* early response genes. Taken together, these data indicate that uncontrolled growth of bacteria in PMCs induces *c-fos/c-jun* genes via activation transcription activator factor AP-1. In addition, silencing AP-1 gene expression with RNAi-inhibited *S. aureus* induced *Bak* and *Bad* gene expression, suggesting that AP-1 activation initiates the apoptotic pathway during *S. aureus* infection in PMCs. Thus, during *S. aureus* infection the expression of AP-1 complex may reflect a functional role in activating putative downstream genes involved in later apoptosis events in PMC.

The apoptosis process is controlled mainly by *Bcl<sub>2</sub>* family proteins [18]. *Bcl<sub>2</sub>* and *Bcl-X<sub>L</sub>* function as death antagonists and *Bad* and *Bax* function as apoptotic agonists within the apoptosis pathway [18, 41]. We noted significantly higher *Bcl<sub>2</sub>* and *Bcl-X<sub>L</sub>* gene expression in resting PMCs than in *S. aureus*-infected PMCs. Interestingly, after 12 h of *S. aureus* infection a spike in *Bcl<sub>2</sub>* and *Bcl-X<sub>L</sub>* gene expression was noticed. *S. aureus*-induced expression of *Bcl<sub>2</sub>* and *Bcl-X<sub>L</sub>* during the initial phase of infection may be related to a survival mechanism to counter the infection. In contrast, *Bad* and *Bak* mRNA expression was significantly higher in *S. aureus*-infected PMCs than in resting PMCs. *Bcl<sub>2</sub>* is a mitochondrial protein and when expressed it inhibits the cell to undergo apoptosis [42], whereas *Bak* expression accelerates proapoptotic activity in cells [43]. Our data suggest that *S. aureus* infection modulates expression of pro- and antiapoptotic genes, especially by selective induction of the proapoptotic genes that mediate apoptosis in PMCs.

wMitochondria play a major role in cell death by releasing the proapoptotic proteins into cytosol. The cytochrome-c is released from mitochondria complexes with apoptosis protease-activating factor-1 (Apaf-1) and activates caspase-9 that eventually results in caspase-3 activation [44]. In cells undergoing apoptosis, caspase-3 activation is required for DNA fragmentation [45]. Caspases are primary effector molecules of apoptosis [15]. A recent study reported that in peritoneal mesothelial cells *S. aureus* induced apoptosis in the caspase-3-independent pathway [46]. However, we noted significant increases in caspase-3 activity in *S. aureus*-infected PMCs. This indicates that *S. aureus* infection induces distinct apoptotic pathways in pleural mesothelial and peritoneal mesothelial cells. In addition, *Bak* and *Bax* directly mediate the release of cytochrome-c into the cytoplasm to initiate apoptosis [47, 48]. In the late phase of *S. aureus* infection we noted an increase in *Bak* and *Bad* expression. Our results indicate that during *S. aureus* infection *Bak* and *Bad* expression is implicated in the PMC apoptosis and it is in agreement with previous studies [49–51].

In conclusion, the present data demonstrate that invading bacteria (*S. aureus*) induce early response genes *c-fos* and *c-jun* and proapoptosis genes *Bad* and *Bak* that lead to PMC apoptosis. The higher risk of morbidity in patients with empyema may, in part, be due to pathogen-induced pleural mesothelial apoptosis and loss of mesothelial monolayer integrity. However, further studies are needed to understand the role of the *c-fos* and *c-jun* early response genes in the regulation of proapoptosis and antiapoptosis genes during *S. aureus* infection in PMCs.

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