Phenol-Soluble Modulin Peptides Contribute to Influenza A Virus-Associated *Staphylococcus aureus* Pneumonia

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ABSTRACT Influenza A virus (IAV) infection is often followed by secondary bacterial lung infection, which is a major reason for severe, often fatal pneumonia. Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains such as USA300 cause particularly severe and difficult-to-treat cases of IAV-associated pneumonia. CA-MRSA strains are known to produce extraordinarily large amounts of phenol-soluble modulin (PSM) peptides, which are important cytotoxins and proinflammatory molecules that contribute to several types of *S. aureus* infection. However, their potential role in pneumonia has remained elusive. We determined the impact of PSMs on human lung epithelial cells and found that PSMs are cytotoxic and induce the secretion of the proinflammatory cytokine interleukin-8 (IL-8) in these cells. Both effects were boosted by previous infection with the 2009 swine flu pandemic IAV H1N1 strain, suggesting that PSMs may contribute to lung inflammation and damage in IAV-associated *S. aureus* pneumonia. Notably, the PSM-producing USA300 strain caused a higher mortality rate than did an isogenic PSM-deficient mutant in a mouse IAV-*S. aureus* pneumonia coinfection model, indicating that PSMs are major virulence factors in IAV-associated *S. aureus* pneumonia and may represent important targets for future anti-infective therapies.

KEYWORDS influenza, pneumonia, *Staphylococcus aureus*, toxins

Bacterial pneumonia is a severe disease that can be caused by bacterial superinfection of virus-damaged mammalian lungs. Viruses commonly involved in the predisposition to bacterial pneumonia include influenza A virus (IAV), influenza B virus, respiratory syncytial virus, and human parainfluenza virus (1). Normally, the motility of cilia localized in the tracheal epithelium actively removes mucus, thereby eradicating entrapped foreign particles that would otherwise end up in the lungs. However, when a viral lung infection is already established, the function of the cilia is impaired, paving the way for further invaders (2, 3). Pandemic influenza virus infections such as those of the 2009 H1N1 swine flu pandemic correlated with bacterial superinfections with devastating consequences, such as high morbidity and mortality rates (4–6).

Two forms of pneumonia have been distinguished: community-associated pneumonia (CAP) and hospital-associated pneumonia (HAP). In most cases of CAP, *Streptococcus pneumoniae* has been determined to be the cause of pneumonia (7). *Staphylococcus aureus*, another aggressive Gram-positive pathogen, causes a variety of diseases in humans, including HAP, especially in ventilated patients (8). CAP is less often caused by *S. aureus*, except for pneumonia following influenza virus infection, which constitutes a severe secondary infection often resulting in fatal outcomes (9, 10). Moreover, *S. aureus*

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causes influenza-associated CAP more frequently in children than in adults (11). Gram-negative bacteria, such as *Escherichia coli* (12), *Haemophilus influenzae* (13), *Klebsiella pneumoniae* (14), or *Pseudomonas aeruginosa* (15), are less common causative agents of CAP. CAP and HAP caused by *S. aureus* have become more frequent with the emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) (16, 17), probably because most antibiotics that are active against CA-MRSA are less effective than β -lactams (18). Moreover, the manifold arsenal of aggressive toxins produced by CA-MRSA appears to be a contributing factor (19–22).

Phenol-soluble modulins (PSM) peptides belong to the most potent staphylococcal toxins (23). The inactivation of PSM genes leads to strongly reduced *S. aureus* virulence (24), and there is a tendency for the level of PSM production to correlate with the severity of typical infections caused by a specific *Staphylococcus* species (25). PSM peptides have been described to be highly cytotoxic for a variety of mammalian cells, including leukocytes (24), at micromolar concentrations. Moreover, PSMs have immunomodulatory properties because they are chemoattractants for leukocytes and can induce the release of proinflammatory cytokines such as interleukin-8 (IL-8) from neutrophils at nanomolar concentrations (24). This activity depends on the capacity of PSMs to stimulate mammalian formyl-peptide receptor 2 (FPR2), a G-protein-coupled chemotaxis receptor expressed in leukocytes and other human cells (26, 27).

The role of staphylococcal PSM peptides in inflammation has been established for abscess formation and systemic infection. However, their potential direct influence on staphylococcal pneumonia has remained unclear (28). Moreover, it has remained unclear if lung epithelial cells are as equally as susceptible to the cytotoxic activity of PSMs as leukocytes.

In our study, we challenged the human lung epithelial cell line A549 with PSM peptides alone or in combination with IAV strain H1N1pdm09. We report that PSMs cause both cytotoxicity and IL-8 release in a dose-dependent manner. When applied to A549 cells, upon IAV infection, the frequencies of both phenotypes increased. The PSM receptor FPR2 is most likely not involved in the above-mentioned processes because nontoxic FPR2 agonists had no such activity. In an *in vivo* infection model, mortality was enhanced when mice suffering from IAV infection were superinfected with a nonlethal dose of a strongly PSM-producing *S. aureus* strain. This effect was significantly reduced when mice were superinfected with an isogenic mutant strain lacking all PSM peptides, indicating that PSM peptides damage and induce inflammation in lung epithelia and contribute to severe outcomes of IAV-associated pneumonia.

RESULTS

PSMs exhibit dose-dependent cytotoxicity toward A549 epithelial cells, which is increased by preceding IAV infection. PSM peptides are known to be cytotoxic for leukocytes at concentrations in the micromolar range. Since they do not seem to require a specific receptor, it appeared likely that they might also damage other human cells encountered during *S. aureus* infections, as shown for epithelial cells. To address this question, we tested the ability of PSMs to lyse the human lung epithelial cell line A549 by measuring the release of cytoplasmic lactate dehydrogenase (LDH) upon incubation with PSMs. Three synthetic PSM peptides, PSM α 2, PSM α 3, and delta-toxin, were applied at concentrations of 3, 10, and 30 μ M. A dose-dependent increase in the LDH level was detected, revealing the cytotoxic activity of PSMs against A549 cells (Fig. 1A).

Both PSMs and IAV have the capacity to damage epithelial cells, which may contribute to severe courses of superinfection. The combined effect of IAV infection and PSM peptides on A549 cells was elucidated by first challenging cells with the particularly virulent IAV strain A/Regensburg/D6/2009 H1N1pdm09 (RB1) at a multiplicity of infection (MOI) of 0.5, followed by the addition of the PSM peptide PSM α 3 or delta-toxin. Cell viability was analyzed by measuring adsorption resulting from the succinate-tetrazolium reductase system of live cells cleaving water-soluble tetrazolium (WST-1) into formazan. At the MOI used, the cytotoxicity of IAV alone did not exceed the

FIG 1 Micromolar concentrations of PSM peptides are toxic for A549 cells. (A) The cytotoxicity of synthetic PSM peptides on A549 cells was monitored by measuring the release of LDH from dead cells. Values are presented as percentages of the value for the Triton X-100 positive control. Background LDH activity in the absence of PSMs was subtracted. (B) Cytotoxicity after infection with IAV (MOI of 0.5) and subsequent incubation with PSM peptides was determined by measuring the mitochondrial ability of live cells to cleave WST-1 into formazan. Values are displayed as percentages of the value for the Triton X-100 positive control. Data represent the means $\pm$ standard errors of the means of results from at least three independent experiments. *P* values were determined by Student's two-tailed, unpaired *t* test. *, *P* < 0.05; **, *P* < 0.005.

background cell decay of A549 cells observed in buffer-treated samples after a 24-h incubation period. However, the combination of the virus and higher micromolar concentrations of either PSMα3 or delta-toxin resulted in the increased killing of A549 cells compared to that with PSMs alone (Fig. 1B).

PSM peptides induce IL-8 secretion in lung epithelial cells. Tissue damage associated with pneumonia is largely due to exuberant inflammation. To test whether PSM peptides have a proinflammatory effect on epithelial cells, we challenged A549 cells with increasing concentrations of different PSM peptides and measured IL-8 release. PSMα3, PSMα2, and delta-toxin induced dose-dependent IL-8 release measured 24 h after stimulation. Of note, this activity was observed at lower concentrations (1 μM) than those for cytotoxic effects (3 μM or higher) (Fig. 2A). PSM peptides induce the release of IL-8 in neutrophils and monocytes at nanomolar concentrations via FPR2 (26), which can also be expressed by airway epithelial cells (29). However, incubation with the nontoxic synthetic FPR2 agonist MMK1 (30) did not lead to IL-8 production even at very high concentrations (Fig. 2A), indicating that the proinflammatory activity of PSMs toward A549 cells does not depend on FPR2 and rather may be associated with the capacity of PSMs to affect the cytoplasmic membrane of human cells.

Stimulation of cells with culture filtrates of the PSM-producing wild-type (WT) USA300 strain and the nonproducing isogenic PSM-deficient mutant (ΔΔΔ mutant) revealed that the WT induced strong, dose-dependent IL-8 release, while the ΔΔΔ mutant elicited only a basal level of IL-8 at the highest concentration used (0.75% culture filtrate), which may be mediated by other toxins or microorganism-associated molecular patterns such as Toll-like receptor ligands (Fig. 2B). Thus, PSM production has a profound impact on the capacity of *S. aureus* to stimulate lung epithelial cells, and this activity probably results from the capacity of PSMs to perturb and damage host cell membranes.

Superinfection of lung epithelial cells with IAV and *S. aureus* potentiates IL-8 production. We mimicked the combined impact of IAV and *S. aureus* on proinflammatory responses in lung epithelial cells by first infecting A549 cells with IAV prior to stimulation with PSMs. Stimulation with PSMs alone led to no (PSMα3) or much weaker (delta-toxin) IL-8 induction, as described above (Fig. 2A versus C), probably because the cells had to be preincubated with IAV for 1 day before PSMs were added. Some IL-8 release was also observed for IAV alone. However, the combined challenge of A549 cells with IAV (MOI of 0.5) and PSMα3 resulted in a significant increase in the amount of

FIG 2 PSMs stimulate IL-8 release in A549 cells. Shown is the secretion of IL-8 by A549 cells after stimulation with increasing concentrations of PSM peptides or the synthetic FPR2 agonist MMK1 (A) or culture filtrates of *S. aureus* USA300 strains (B), after infection with IAV (MOI of 0.5) and subsequent PSM peptide stimulation (C), or after infection with IAV (MOI of 0.5) and subsequent stimulation with culture filtrates of *S. aureus* USA300 strains (D). Background IL-8 release in the absence of a stimulus was subtracted in panel A. The sterile bacterial culture medium TSB was used as a control in panels B and D. Data represent means $\pm$ standard errors of the means of results from at least three independent experiments. *P* values were determined by one-way ANOVA with Bonferroni's posttest. *, *P* < 0.05; **, *P* < 0.001; ***, *P* < 0.0005; ****, *P* < 0.0001.

secreted IL-8 compared to that with PSM or IAV alone. A 1 μ M concentration was sufficient to significantly elevate IL-8 production in the presence of IAV but not when PSMs were used alone. At low concentrations, PSM α 3 elicited a higher level of IL-8 release than did delta-toxin (Fig. 2C).

WT USA300 culture filtrates induced significantly more IL-8 in combination with IAV than did the WT culture filtrate alone. In contrast, no increased IL-8 secretion was

measured for the combination of IAV with the culture filtrate of the $\Delta\Delta\Delta$ mutant (Fig. 2D). In summary, both PSM peptides and IAV elicit the secretion of IL-8, and the combination of the two stimuli increases this proinflammatory response.

PSM peptides exacerbate pneumonia in a murine IAV-*S. aureus* lung infection model. To elucidate the influence of PSM peptides on IAV- and *S. aureus*-induced pneumonia, the lungs of mice were first infected via the nasal route with a dose of IAV that led to mortality at between days 9 and 12. The lungs of IAV-infected animals showed typical signs of pneumonia-associated tissue destruction and inflammation, with large numbers of immigrated macrophages. Moreover, infected lungs contained significantly increased levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF- α) and IL-6 and of the chemokine monocyte chemoattractant protein 1 (MCP-1) compared to those in the lungs of control animals (Fig. 3A and B). Two days after infection, subgroups of mice were additionally challenged in the same way with a dose of the USA300 WT or $\Delta\Delta\Delta$ mutant strain, which hardly caused any mortality in the absence of previous IAV infection until day 14. Of note, the health of mice challenged with both IAV and the WT USA300 strain worsened dramatically, resulting in the obligation to euthanize them already at between days 5 and 7. In contrast, mice challenged with IAV plus the $\Delta\Delta\Delta$ strain lived significantly longer than did those infected with IAV and the WT strain but were sicker than those infected with IAV alone. (Fig. 3C).

These *in vivo* results show that PSM peptides aggravate staphylococcal pneumonia after previous IAV infection and contribute to mortality. However, *S. aureus* produces additional virulence factors contributing to IAV-associated pneumonia, as the USA300 PSM mutant was also pathogenic in mouse IAV-*S. aureus* superinfection albeit with lower virulence than the corresponding WT strain.

DISCUSSION

In this study, we report that PSMs play an important role in the development of *S. aureus* pneumonia because of a preceding IAV infection. The most aggressive and most abundant PSMs, PSM α 2, PSM α 3, and delta-toxin, efficiently killed human epithelial cells at high micromolar concentrations in a way similar to that found previously for leukocytes (24). In combination with a preceding IAV infection, which also led to epithelial cell death, the toxic activities of PSMs were potentiated. Furthermore, PSM peptides led to dose-dependent IL-8 secretion by airway epithelial cells. This effect was boosted by previous infection with IAV, indicating that both agents, staphylococcal PSMs and IAV, can combine their proinflammatory activities in superinfections. We verified our findings in an *in vivo* infection model. A sublethal dose of the WT USA300 strain severely aggravated the condition of mice, which had been infected with IAV previously. The corresponding mutant strain lacking genes for PSM production was significantly less pathogenic. Our findings show that PSM peptides affect the severity of *S. aureus* pneumonia and are crucial virulence factors in the lung.

The potent receptor-dependent protein toxins Pantone-Valentine leukocyte (PVL) and alpha-toxin have been implicated in *S. aureus* pneumonia (19, 31–33), with alpha-toxin being capable of lysing a variety of host cells (28) and PVL being specific for human or rabbit neutrophils (22, 34, 35). However, conflicting data have raised doubts about the importance of PVL in human *S. aureus* lung infections (36). The USA300 mutant lacking all PSMs induced a basal level of IL-8 release and killed mice albeit not to the same extent as the WT strain, indicating that further toxins apart from PSMs such as alpha-toxin contribute to *S. aureus* virulence in mouse pneumonia.

It has yet to be determined whether PSMs aggravate lung infection by their proinflammatory or cytotoxic activities or by both activities. However, impairing the integrity of lungs by previous infection with aggressive IAV appears to predispose an individual to the development of *S. aureus* pneumonia, which is rare in patients without preceding IAV infection. In line with this notion, a recent study using a nonlethal IAV variant failed to demonstrate an impact of IAV infection on subsequent *S. aureus* lung infection in a nonhuman primate model (37).

FIG 3 PSMs contribute to the severity of IAV-associated *S. aureus* pneumonia in a mouse infection model. The lungs of C57BL/6 mice were infected with IAV in PBS via the nasal route. Two days later, 6.4×10^8 to 7.0×10^8 CFU/ml of either the *S. aureus* USA300 WT or $\Delta\Delta\Delta$ mutant strain in PBS were applied in the same way. (A and B) Lungs of infected C57BL/6 mice were removed at the end of the experiment, photographically documented (A, left), and analyzed by histopathology and tissue macrophage-specific immunohistological staining using a mouse CD107b-specific antibody (A, right) or by the detection of cytokine levels (B). Significant differences versus uninfected controls in panel B were determined by Student's two-tailed, unpaired *t* test (**, $P < 0.001$; ***, $P < 0.0005$; ****, $P < 0.0001$ [means are indicated by horizontal lines]). (C) The survival of infected mice was monitored for up to 2 weeks, and statistical analysis was performed by using the log rank test (**, $P < 0.005$ when IAV-WT strain- or IAV- $\Delta\Delta\Delta$ mutant strain-infected mice were compared with mice that were infected with IAV alone; *, $P < 0.05$ when IAV-WT strain-infected mice were compared with IAV- $\Delta\Delta\Delta$ mutant strain-infected mice).

Pneumonia-associated lung injury is believed to be caused by so-called cytokine storms, mediated by the massive accumulation of cytokines and the tissue-damaging activity of neutrophils or other immune cells (38). Upon the lysis of neutrophils by the cytolytic activity of PSMs or other toxins, reactive oxygen species and other molecules are released, which in turn may damage surrounding lung tissue. Exuberant inflammation is a common pattern in *S. aureus* infection (39). Our study demonstrates that PSMs are cytotoxic to not only leukocytes but also epithelial cells, which is in agreement with data from a recent study (31). This finding underscores the cytotoxic versatility of PSMs and supports the notion that PSMs kill host cells in a receptor-independent manner (26). Human G-protein-coupled FPR2 is highly sensitive to PSMs (26). However, the activation of FPR2 by the nontoxic synthetic ligand MMK1 did not induce IL-8 production, suggesting that FPR2 is not involved in PSM-mediated IL-8 induction in A549 cells.

In conclusion, we propose that PSMs are of importance in *S. aureus*-induced pneumonia following seasonal flu, in addition to other recently investigated virulence factors. Future studies on the role of PSMs and their interplay with lung cells will be required for a full understanding of PSM-mediated lung damage. The development of PSM-blocking agents such as vaccines or inhibitors of the essential PSM exporter Pmt (40) may be a promising therapeutic approach for staphylococcal pneumonia and other diseases.

MATERIALS AND METHODS

Human cells, bacteria, and peptides. Adenocarcinomic human alveolar basal epithelial cells (A549; ATCC CCL-185), lung epithelial cancer cells, were grown in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (Life Technologies, Carlsbad, CA, USA) at 37°C with 5% CO₂. Confluent A549 cells were challenged with PSM peptides or bacterial culture filtrates diluted as indicated in DMEM without FCS and incubated for 24 h at 37°C with 5% CO₂. PSM peptides were dissolved in dimethyl sulfoxide (DMSO), whose final concentrations did not affect the A549 cytotoxicity or IL-8 assays. Cells were used up to passage 10.

S. aureus WT strain USA300 LAC and the isogenic PSM-deficient mutant ($\Delta\Delta\Delta$ mutant) were kindly provided by Michael Otto, NIH, Bethesda, MD (24). These strains were grown at 37°C in tryptic soy broth (TSB) (AppliChem, Darmstadt, Germany) for 17 h under agitation. Culture filtrates were prepared as described previously (41). For infection experiments, bacterial cultures grown overnight in TSB were inoculated at an optical density (OD) of 0.1. The cultures were incubated at 37°C for 3 h. Bacteria were pelleted by centrifugation for 10 min at 5,000 × *g*, washed twice with phosphate-buffered saline (PBS) (Life Technologies, USA), and diluted to the desired CFU per milliliter in PBS. Synthetic PSM peptides were kindly provided by Stefan Stefanović, Institute of Cell Biology, Department of Immunology, University of Tübingen. The synthetic FPR2 agonist MMK1 was obtained from EMC Microcollections, Tübingen, Germany.

Influenza virus. Human influenza virus A/Regensburg/D6/2009 H1N1pdm09 (RB1) (IAV) was used in all experiments. A549 cells were infected at the indicated multiplicities of infection in PBS supplemented 0.6% bovine serum albumin (BSA) (MP Biomedicals, Solon, OH, USA) for 30 min at 37°C with 5% CO₂. The cells were further incubated in DMEM supplemented with trypsin (1.2 µl ml⁻¹) for 24 h at 37°C with 5% CO₂ to enable the attachment of virus particles to all cells.

IL-8 detection and cytotoxicity assays. IL-8 was detected in the culture supernatants of A549 cells by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. In brief, 1 × 10⁵ A549 cells per well in 1 ml were seeded into a 24-well plate. On day 2, cells were stimulated with PSMs or MMK1 at the indicated concentrations for 24 h. On day 3, cells were centrifuged for 10 min at 250 × *g*, and cell-free culture filtrates were used for measuring either IL-8 release or LDH release. For stimulation with IAV, cells were seeded on day 1 (10⁵ cells per well). After removal of the medium on day 2, IAV or fresh medium was added. On day 3, complete medium and unbound IAV particles were removed, and either medium or PSMs were added. On day 4, cells were centrifuged for 10 min at 250 × *g*, and cell-free culture filtrates were used for measuring either IL-8 release or LDH release. LDH release in the cell culture supernatant was measured according to the manufacturer's protocol (Roche, Basel, Switzerland). The latter was obtained by centrifugation at 250 × *g* for 10 min. The WST-1 assay was conducted according to the manufacturer's recommendations (Roche, Basel, Switzerland). For all assays, 10% Triton X-100 (Carl Roth, Karlsruhe, Germany) in DMEM served as the positive control.

Mice. Eight-week-old wild-type C57BL/6 mice were obtained from Janvier (St. Berthevin, France). The lungs of three groups of five mice each were infected via the nasal route with IAV at a concentration of 1.5 × 10⁵ PFU/mouse, diluted in PBS. Two days after infection, two groups were superinfected via the nasal route with 6.4 × 10⁸ to 7 × 10⁸ CFU/ml of the USA300 LAC WT or $\Delta\Delta\Delta$ mutant strain. Two additional groups were infected with bacteria only. The mice were monitored twice daily, and body weight was measured daily. The mice were euthanized when their weight loss reached 20% of their initial body weight. The remaining mice were sacrificed at day 14. Mice were sedated before intranasal

inoculation of the virus or bacteria by using identical amounts of a solution containing 2% xylazine (Rompun; Bayer, Leverkusen, Germany) and 10% ketamine (Sanofi, Paris, France) mixed 1:10 with PBS. One hundred fifty microliters of this solution was injected intraperitoneally. Animal experiments were performed in strict accordance with the European Health Law of the Federation of Laboratory Animal Science Associations. The protocol was approved by the Regierungspräsidium Tübingen (approval IM 01/17).

Analysis of lung pathology. Mouse lungs were removed and either analyzed by immunohistology as described below or shredded by using the FastPrep FP 120 system (Savant) to analyze the cytokines TNF- α , IL-6, and MCP-1 with a custom-made Luminex assay, including magnetic beads (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The samples were analyzed on a Bio-Plex Magpix multiplex reader (Bio-Rad Laboratories, Munich, Germany) with Bio-Plex Manager MP software (Bio-Rad) and an ELISA (R&D Systems, Minneapolis, MN, USA). In addition, some lungs were fixed overnight in 4% buffered paraformaldehyde and embedded in paraffin. Five-micrometer-thick tissue sections were used for hematoxylin and eosin (HE) stainings and *in situ* staining of mononuclear tissue macrophages with the rat anti-mouse CD107b-specific antibody (Mac3, clone M3/84; BD Pharmingen, USA) followed by the rat-on-mouse alkaline phosphatase polymer system (Biocare Medical, Pacheco, CA, USA) and HistoGreen (Linaris, Dossenheim, Germany) as a substrate. The slides were counterstained with hematoxylin.

Statistical analysis. All statistical analyses were performed by using Prism 5.04 software (GraphPad, La Jolla, CA, USA). Differences were analyzed for significance by using Student's *t* test, one-way analysis of variance (ANOVA) with Bonferroni's posttest, or a log rank test, as indicated.

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