

# *Staphylococcus aureus* Phenol-Soluble Modulins Impair Interleukin Expression in Bovine Mammary Epithelial Cells

Martine Deplanche,<sup>a,b</sup> Ludmila Alekseeva,<sup>a,b,c</sup> Ksenia Semenovskaya,<sup>a,b</sup> Chih-Lung Fu,<sup>d</sup> Frederic Dessauge,<sup>e</sup> Laurence Finot,<sup>e</sup> Wolfram Petzl,<sup>f</sup> Holm Zerbe,<sup>f</sup> Yves Le Loir,<sup>a,b</sup> Pascal Rainard,<sup>g,h</sup> David G. E. Smith,<sup>i,\*</sup> Pierre Germon,<sup>g,h</sup> Michael Otto,<sup>d</sup> Nadia Berkova<sup>a,b</sup>

INRA, UMR1253 STLO, Rennes, France<sup>a</sup>; Agrocampus Ouest, UMR1253 STLO, Rennes, France<sup>b</sup>; Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russian Federation<sup>c</sup>; Laboratory of Bacteriology, National Institutes of Health, Bethesda, Maryland, USA<sup>d</sup>; INRA, UMR1348 PEGASE, Saint Gilles, France<sup>e</sup>; Clinic for Ruminants at the Centre for Clinical Veterinary Medicine, Ludwig-Maximilians University Munich, Munich, Germany<sup>f</sup>; INRA, UMR1282, Infectiologie et Santé Publique, Nouzilly, France<sup>g</sup>; Université François Rabelais de Tours, UMR1282, Infectiologie et Santé Publique, Tours, France<sup>h</sup>; Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom<sup>i</sup>

**The role of the recently described interleukin-32 (IL-32) in *Staphylococcus aureus*-induced mastitis, an inflammation of the mammary gland, is unclear. We determined expression of IL-32, IL-6, and IL-8 in *S. aureus*- and *Escherichia coli*-infected bovine mammary gland epithelial cells. Using live bacteria, we found that in *S. aureus*-infected cells, induction of IL-6 and IL-8 expression was less pronounced than in *E. coli*-infected cells. Notably, IL-32 expression was decreased in *S. aureus*-infected cells, while it was increased in *E. coli*-infected cells. We identified the staphylococcal phenol-soluble modulin (PSM) peptides as key contributors to these effects, as IL-32, IL-6, and IL-8 expression by epithelial cells exposed to *psm* mutant strains was significantly increased compared to that in cells exposed to the isogenic *S. aureus* wild-type strain, indicating that PSMs inhibit the production of these interleukins. The use of genetically complemented strains confirmed this observation. Inasmuch as the decreased expression of IL-32, which is involved in dendritic cell maturation, impairs immune responses, our results support a PSM-dependent mechanism that allows for the development of chronic *S. aureus*-related mastitis.**

Mastitis of ruminants is an inflammation of the mammary gland commonly caused by bacterial infection; *Staphylococcus aureus* and *Escherichia coli* are among the most prevalent pathogens (1). *E. coli* intramammary infections often result in acute mastitis with severe clinical manifestations, while symptoms induced by *S. aureus* infection are usually less severe and the infection can become persistent (2). It is well documented that there are weaker proinflammatory responses to *S. aureus* than to *E. coli* in ruminants, inasmuch as differential immune responses were observed *in vitro* in infected bovine epithelial cells and *in vivo* in infected cows (3, 4).

Mammary gland epithelial cells (MEC) are the first cells that are in contact with pathogens. Pathogens can be internalized into epithelial cells and hijack host cell functions to facilitate their own propagation or circumvent host defenses (5, 6).

The cytokine network, which is fine-tuned by many regulatory steps, plays a pivotal role in the outcome of infection. The identification of members of the cytokine network may allow the prioritization of potential targets for the treatment of infection. The roles of several recently discovered cytokines during infection are not completely understood. Such cytokines include, for example, interleukin-32 (IL-32), which has been reported to play a pivotal role in the pathogenesis of infectious diseases. IL-32 is produced by T lymphocytes, natural killer (NK) cells, monocytes, and epithelial cells (7). The human IL-32 gene is organized into eight exons with six splice variants of the gene; these variants have been described as IL-32 $\alpha$ , IL-32 $\beta$ , IL-32 $\gamma$ , IL-32 $\delta$ , IL-32 $\epsilon$ , and IL-32 $\zeta$  (8). IL-32  $\gamma$  induces the maturation of dendritic cells (9). Furthermore, IL-32 induces IL-1 $\beta$ , IL-6, tumor necrosis factor alpha (TNF- $\alpha$ ), and IL-8 (7), which are relevant to the pathoimmunology of infections caused by *S. aureus* and *E. coli*. Thus, the comparison of IL-6, IL-8, and IL-32 gene expression by *S. aureus*-

infected MEC with the expression of those cytokines by *E. coli*-infected MEC is pertinent in regard to infectious mastitis.

*S. aureus*-associated infections are promoted by the coordinated action of various virulence factors, among which are cell wall-associated and secreted bacterial proteins such as toxins (10, 11). Recently, it was discovered that virulence of *S. aureus*, in particular of community-associated methicillin-resistant *S. aureus* strains, depends on phenol-soluble modulins (PSMs), a family of secreted amphipathic,  $\alpha$ -helical peptides with a variety of biological functions (12). *S. aureus* PSMs have potent cytolytic activity against many cell types, including neutrophils, monocytes, erythrocytes, keratinocytes, and osteoblasts (13–15). PSMs also have proinflammatory activities: they stimulate leukocytes and initiate proinflammatory responses, including neutrophil chemoattraction and activation (12, 16). Furthermore, we have

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Address correspondence to Nadia Berkova, [nadia.berkova@rennes.inra.fr](mailto:nadia.berkova@rennes.inra.fr).

\* Present address: David G. E. Smith, School of Life Sciences, Heriot-Watt University, Edinburgh, United Kingdom.

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shown that a PSM $\alpha$ -induced G<sub>2</sub>/M transition delay correlated with a decrease in the expression of several defensin genes, suggesting a role in diminution of antibacterial functions of epithelial cells (17). Moreover, PSMs are responsible for modulation of cytokine secretion. It was demonstrated that PSMs induce the release of IL-8 from neutrophils (13) and IL-18 from human keratinocytes, likely through the lytic release (15). In contrast, simultaneous treatment of dendritic cells with *S. aureus* cell lysate and PSM $\alpha$  inhibited the secretion of TNF, IL-6, and IL-12, whereas the secretion of the anti-inflammatory cytokine IL-10 was increased (18). Taking into account numerous reports and our recent findings showing that PSMs are involved in the alteration of the host defense response (17–19), we tested in this study the hypothesis that PSMs impair cytokine expression during bovine infection using an induction of specific interleukins in bovine epithelial cells as a readout.

In the present study, we found that a weaker cytokine response was associated with the exposure of bovine MEC to live *S. aureus* bacteria than to *E. coli*. We show a pivotal role for PSMs in the inhibition of the expression of major components of the cytokine network, IL-32, IL-6, and IL-8, that is induced by *S. aureus*, an additional feature that may contribute to PSM-mediated immune evasion and persistence of staphylococcal infections.

## MATERIALS AND METHODS

**Reagents.** PSM $\alpha$ 3 peptide was synthesized by solid-phase peptide synthesis and provided by CekoLabs (Tübingen, Germany). Lactate dehydrogenase (LDH) was quantified using the Pierce LDH cytotoxicity assay (ThermoFisher, Rockford, IL).

**Eukaryotic cell maintenance.** A bovine mammary epithelial cell (MEC) line, BME-UV (20), was maintained in the following medium: 50% Dulbecco's modified Eagle medium (DMEM), 30%, RPMI 1640, 20% NCTC-135 supplemented with 10% fetal calf serum (FCS) (Gibco, Saint Aubin, France), 0.1%  $\alpha$ -lactose monohydrate, 0.1% lactalbumin enzymatic hydrolysate, 1.2 mM reduced L-glutathione, 5  $\mu$ g/ml of bovine insulin, 5  $\mu$ g/ml of bovine holo-transferrin, 5  $\mu$ g/ml of progesterone, 10<sup>-7</sup> mol/liter of hydrocortisone, 10  $\mu$ g/ml of L-ascorbic acid, and 50 IU/ml of penicillin-streptomycin (Sigma-Aldrich) at 37°C with 5% CO<sub>2</sub>. PS, a newly isolated MEC line from the secretory parenchyma (21), was maintained in advanced medium: Advanced-DMEM/F12 (Gibco) containing 20 mM HEPES, 2 mM L-glutamine (Gibco), 1  $\mu$ g/ml of hydrocortisone (Sigma-Aldrich), 10 ng/ml of insulin-like growth factor (IGF), 1.5 ng/ml of fibroblast growth factor (FGF), and 5 ng/ml of epidermal growth factor (EGF). Trypsin-EDTA (Gibco) was used to release adherent BME-UV and PS cells for subculturing.

**Bacterial strains and culture conditions.** Two *S. aureus* strains isolated from cows with mastitis were used in the *in vitro* studies, RF122 and Newbould 305 (NB305). These strains reproducibly induce severe or mild mastitis under experimental conditions (22).

The methicillin-resistant *S. aureus* USA300 (LAC wt) and its isogenic mutants LAC  $\Delta$ psm $\alpha$ , which lacks the psm $\alpha$  operon encoding PSM $\alpha$ 1 to -4, and LAC  $\Delta$ psm $\alpha$  $\beta$ hld, which lacks the psm $\alpha$  and psm $\beta$  operons and in which translation of the hld gene is abolished by mutation of the start codon, were obtained from the Laboratory of Bacteriology, NIH, USA (12, 23). The construction of a wild-type (WT) LAC pTX $\Delta$ 16 strain harboring the control plasmid pTX $\Delta$ 16, PSM $\alpha$  deletion mutant LAC  $\Delta$ psm $\alpha$  pTX $\Delta$ 16, and complemented strain LAC  $\Delta$ psm $\alpha$  pTX $\Delta$ 16 $\alpha$ 1-4, as well as PSM-deficient deletion mutant LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 and complemented strains expressing either the four PSM $\alpha$  peptides (LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 $\alpha$ 1-4), PSM $\beta$  (LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 $\beta$ 1-2), or hld (LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 hld), were obtained as described previously (24).

All *S. aureus* cultures were performed as follows. Aliquots from overnight cultures in brain heart infusion (BHI) broth were diluted (1:50) in

DMEM. The mutants (LAC  $\Delta$ psm $\alpha$  and LAC  $\Delta$ psm $\alpha$  $\beta$ hld) in which the psm $\alpha$  operon was exchanged for a spectinomycin resistance cassette were grown in BHI containing 250  $\mu$ g/ml of spectinomycin before inoculation into DMEM. The tetracycline-resistant strains harboring plasmid TDX16 were grown in BHI containing 12.5  $\mu$ g/ml of tetracycline before inoculation into DMEM. The growth curves of deletion and complemented *S. aureus* mutants were similar to that of the wild-type strain. Strains were incubated at 37°C under anaerobic conditions until cultures reached an optical density of 0.6 at 600 nm, corresponding to approximately 10<sup>8</sup> CFU/ml. The staphylococci were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in the interaction medium (DMEM). Bacterial concentrations were estimated spectrophotometrically and confirmed by plate counts using a micro-method as previously described (25).

*E. coli* K-12 MG1655 (ATCC) was grown for 8 h in Luria broth medium, after which it was maintained under conditions as described for *S. aureus* strains. After dilution (1:50) in DMEM, bacterial cultures were grown to an optical density of 0.6 at 600 nm, corresponding to approximately 1.2  $\times$  10<sup>8</sup> CFU/ml.

*E. coli* (1303) and *S. aureus* (1027) strains, which were used for the induction of experimental clinical (*E. coli*) and subclinical (*S. aureus*) mastitis as described previously (21), were isolated from cases of clinical and subclinical bovine mastitis, respectively. The numbers of bacterial strains refer to the identification numbers of the animals (26–28).

**Cell culture infection.** Live *E. coli* and *S. aureus* bacteria were used for the experiments. Taking into account the difference in the doubling time between *E. coli* and *S. aureus*, we tested different multiplicities of infection (MOIs; numbers of bacteria per cell at the onset of infection) in order to determine the highest bacterial concentration that did not cause cytotoxicity toward host cells. Mock cells (cells treated the same way except without the bacteria) were used as controls.

Preliminary experiments using host cells infected with live *E. coli* for 2 h revealed that there was no alteration in cytokine expression at an MOI of 1:1 compared to the noninfected control cells. Usage of an MOI higher than 30:1 resulted in the development of cytotoxicity toward host cells. Consequently, 3 different MOIs (5:1, 15:1, and 30:1) were used for the experiments with *E. coli*-infected cells.

Preliminary assays with live *S. aureus*-infected cells showed that at an MOI of 20:1, there was no change in interleukin expression compared to that of the mock cells. The cytotoxic effect toward host cells was observed at an MOI higher than 160:1. Different MOIs (40:1, 80:1, and 160:1) were used in experiments.

DMEM without antibiotics was used as an infection medium. Eukaryotic cell concentrations were determined using one of the four replicate samples. The remaining samples were used for the analysis in triplicate. Unbound bacteria were removed 90 min postinfection by washing wells with PBS, followed by incubation in medium containing 20  $\mu$ g/ml of lysostaphin and 100  $\mu$ g/ml of gentamicin for 2 h, which eliminates the extracellular bacteria, followed by a washing in PBS and then by incubation in medium containing 25  $\mu$ g/ml of gentamicin for the periods indicated below. Mock cells were used as controls.

**Adhesion and internalization assays.** Adhesion assays were performed as described previously (29). Briefly, confluent monolayers of PS cells in 96-well plates (2.5  $\times$  10<sup>5</sup> cells/well) were incubated for 90 min with an *S. aureus* suspension at an MOI of 80:1. After the lysis of cells using 0.01% Triton X-100, the number of CFU of adherent bacteria was determined using a micromethod (25). In order to account for possible variations in staphylococcal suspensions, the portion of the inoculum adhered to or internalized into PS cells was indicated as a percentage of the initial inoculum (i.e., of the number of input CFU).

For internalization assays, cells were exposed to *S. aureus* as described for the adhesion assay. After 90 min of incubation with bacteria, cells were incubated in DMEM containing 100  $\mu$ g/ml of gentamicin and 20  $\mu$ g/ml of lysostaphin for 2 h in order to remove extracellular bacteria and to measure internalized bacteria only. The percentage of internalized bacteria

TABLE 1 Primer sequences for qRT-PCR<sup>a</sup>

Primer	Sequence	Product size (bp)	NCBI accession no.
IL-32f	5'-TCAAGAGAACAGTCCCGAAACC-3'	71	Consensus sequence
IL-32r	5'-AGCGTACTTCTTGCTGTGCTTC-3'		
IL-8f	5'-TGGGCCACACTGTGAAAAT-3'	92	NM_173925.2
bIL-8r	5'-TCATGGATCTTGCTTCTCAGC-3'		
IL-6f	5'-TGCTGGTCTTCTGGAGTATC-3'	153	EU276071
IL-6r	5'-GTGGCTGGAGTGGTTATTAG-3'		
RPL19f	5'-TACTGCCAATGCTCGAATGC-3'	114	NM_001040516.1
RPL19r	5'-TGATACATGTGGCGGTCAATC-3'		
PPIAf	5'-ATGGCAAGACCAGCAAGAAG-3'	201	XM_002690515.2
PPIAr	5'-CTTGAGGGGGATAAGGAAA-3'		

<sup>a</sup> Primer sequences were designed using primer 3. The RPL19 and PPIA genes were used as housekeeping genes. All melting temperatures were 60°C. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager based on the threshold cycle method.

from the initial inoculum was determined as described for the adhesion assay.

There were no statistically significant differences between percentages of either adhered or internalized LAC wt strain or LAC  $\Delta$ psm $\alpha$  and LAC  $\Delta$ psm $\alpha$ hhd mutants (see the supplemental material).

**Evaluation of the viability of epithelial cells.** The viability of eukaryotic cells was assessed at the end of the incubation with *E. coli* or *S. aureus*. The viability was estimated by cell counts using a hemocytometer combined with the trypan blue exclusion assay (30). The results were calculated as the percentage of live cells out of the total number of cells. There were no significant differences between percentages of infected and control noninfected cells at any of the concentrations used (data not shown).

**Gene expression analysis by qRT-PCR.** Infection of bovine MEC was performed as indicated above. Interleukin expression was evaluated by quantitative real-time PCR (qRT-PCR), as described previously (17). Briefly, total RNA was isolated with an RNA II kit (Macherey-Nagel), and cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences). Reaction mixtures devoid of reverse transcriptase and reaction mixtures containing H<sub>2</sub>O instead of cDNA were used as negative controls. Consensus sequence from an alignment of bovine mRNA IL-32 sequences available at <http://www.ncbi.nlm.nih.gov> (NCBI reference sequence accession numbers XM\_002697870.3, XM\_001790359.3, XM\_002697939.2, and XM\_005224638.1) were used for primer design (Table 1). The peptidyl-prolyl *cis-trans* isomerase A (PPIA) and RPL19 (ribosomal protein 19) housekeeping genes were used as reference genes for normalization. Amplification was performed on a CFX96 real-time system (Bio-Rad, Marne la Coquette, France).

Relative quantification after normalization refers to the PCR signal of the target transcript in a treatment group divided by the values obtained from mock cells, arbitrarily set to 1. When the expression was decreased compared to that in mock cells, data were presented as negative values.

**HPLC-MS.** To analyze PSM production in culture filtrates of the bovine mastitis isolates, high-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis of culture filtrates was performed as described previously (23).

**Udder samples from *E. coli*- or *S. aureus*-infected cows.** Samples from infected animals were obtained from previous experiments conducted as described previously (21). Experiments were performed at the Centre for Clinical Veterinary Medicine, Munich, Germany, with the approval of the ethics committee of the regional government (no. 55.2-1-54-2531-108-05). Briefly, 10 healthy German Holstein Friesian heifers in midlactation were inoculated in one quarter with 500 CFU of *E. coli* strain 1303 ( $n = 5$ ) or with 10,000 CFU of *S. aureus* strain 1027 ( $n = 5$ ). Five heifers that received no treatment served as untreated controls. All *E. coli*-inoculated animals developed clinical mastitis in the affected quarter.

The diagnosis of subclinical *S. aureus*-induced mastitis was assessed for all 5 animals based on an increase of the milk somatic cell count of  $>10^6$ /ml and repeated recovery of *S. aureus* in milk of the inoculated quarters. Animals were slaughtered 24 h postinfection (p.i.) (*E. coli*) or 72 h p.i. (*S. aureus*). RNA was isolated from 100 mg of udder tissue using TRIzol (Invitrogen) (21). Samples were reverse transcribed to cDNA using random hexamers and SuperScript RT III (Invitrogen) and were analyzed by qRT-PCR. After normalization using RPL19 and PPIA, expression of each gene was calculated relative to the values obtained from unstimulated samples.

**Analysis of cytotoxic effects of PSM $\alpha$ 3.** Bovine PS cells ( $15 \times 10^3$ /well) were grown to 80% confluence in 96-well tissue culture plates overnight. The cells then were exposed to PSM $\alpha$ 3 for 8 h, 24 h, and 48 h in DMEM without FCS, as described above. PSM concentrations ranged from 0.01 to 2  $\mu$ g/ml. The cytotoxic activity of PSM was estimated by release of cytoplasmic lactate dehydrogenase (LDH) (LDH cytotoxicity assay kit; Pierce, USA), as described previously (17).

For controls, untreated cells were included for measurements of spontaneous LDH release and maximum LDH release was induced by the addition of lysis reagent. LDH activity in DMEM at A<sub>492</sub> (Xenius, Safas, Monaco) alone was used as the background control. Cytotoxicity levels were calculated as follows: LDH release of treated cells (percent) = (LDH of PSM-treated cells – LDH of control untreated cells)/(maximum LDH release – LDH of control untreated cells)  $\times$  100.

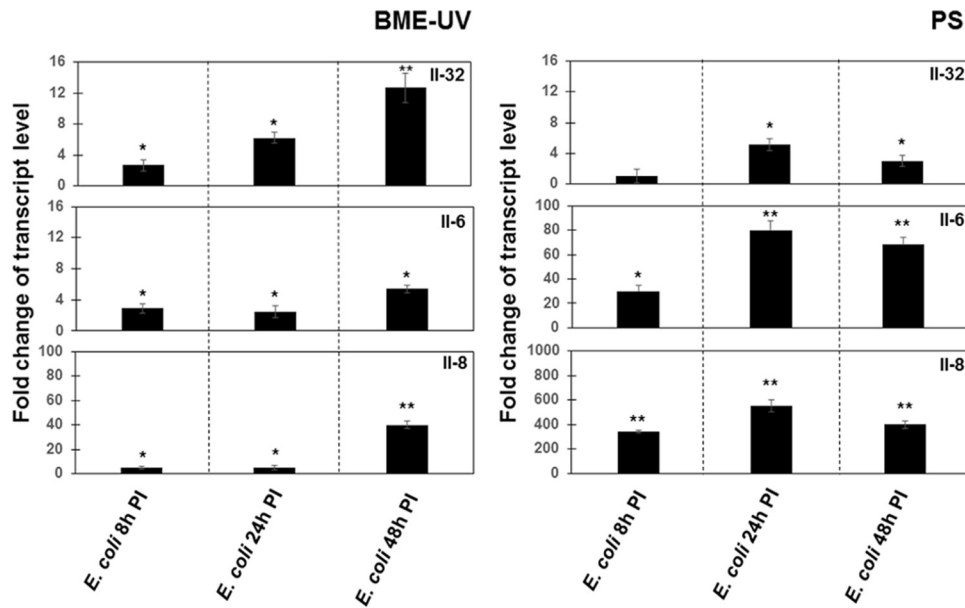
An exposure to synthetic PSM $\alpha$ 3 for 8 h and 24 h in a range of concentrations from 0.01 to 2  $\mu$ g/ml and for 48 h in a range of concentrations from 0.01 to 0.5  $\mu$ g/ml did not reveal a cytotoxic effect, while 10% of cytotoxic activity was observed after 48 h of incubation with 2  $\mu$ g/ml of PSM $\alpha$ 3 (data not shown).

**Exposure of PS cells to synthetic PSM $\alpha$ 3.** Bovine PS cells were exposed to synthetic PSM $\alpha$ 3 ranging from 0.01 to 2  $\mu$ g/ml for 8 h, 24 h, or 48 h. Interleukin expressions in PS cells were evaluated by qRT-PCR, as described above.

**Statistical analysis.** At least three different assays were performed per experiment. The differences among the groups in *in vitro* experiments were assessed by analysis of variance (ANOVA). *P* values of  $<0.05$  were considered to be significant. Tukey's honestly significant difference (HSD) test was applied for comparison of means between groups. The values are expressed as means  $\pm$  standard deviations (SD). To analyze the tissue samples, the permutation test was performed using the StatXact software. *P* values of  $<0.05$  were considered significant.

## RESULTS

**Upregulation of IL-32, IL-6, and IL-8 expression in *E. coli*-infected cells.** To analyze whether there is a difference in IL-32 ex-



**FIG 1** Upregulation of IL-32, IL-6, and IL-8 expression in *E. coli*-infected cells. BME-UV or PS cells ( $2 \times 10^5$ ) were grown in six-well plates for 24 h. The cells were then exposed to the *E. coli* K-12 strain (MOI, 30:1) for 8 h, 24 h, and 48 h. Isolation of total RNA, synthesis of cDNA, and qRT-PCR were performed as described in Materials and Methods. After normalization using PPIA and RPL19 genes, interleukin expression at 8 h, 24 h, and 48 h p.i. was calculated relative to the values obtained from mock cells, arbitrarily set to 1. Data were calculated from three different experiments performed in triplicate. *P* values of <0.05 (\*) and <0.01 (\*\*) were considered significant.

pression in MEC exposed to live *S. aureus* versus live *E. coli*, we first investigated the gene expression in *E. coli*-infected cells, taking into account the capacity of *E. coli* to induce strong immune responses. Different MOIs (5:1, 15:1, and 30:1) were used for experiments with *E. coli*-infected cells. Results obtained with an MOI of 30:1, the highest *E. coli* concentration without a cytotoxic effect, are presented in Fig. 1, while Table S1 in the supplemental material shows the results obtained with other MOIs.

There were no differences in IL-32 expression between *E. coli*-infected (MOI, 30:1) and uninfected BME-UV or PS cells 2 h, 4 h, and 6 h p.i. (data not shown). The expression of IL-32 by BME-UV cells was increased 3-fold after 8 h, 6-fold after 24 h, and 12-fold after 48 h of exposure compared to that in mock cells; an increase of IL-32 expression in *E. coli*-infected PS cells was observed after 24 h and after 48 h of exposure (Fig. 1).

To gain a more comprehensive picture of the MEC response to *E. coli* infection, the expression levels of IL-6 and IL-8, key regulatory mediators of immune responses, were evaluated under the same conditions. As shown in Fig. 1, the relative levels of mRNA expression of IL-6 and IL-8 were increased in both types of cells 8 h, 24 h, and 48 h p.i.; however, the level of expression was much higher in PS cells.

Similar results were obtained using MOIs of 5:1 and 15:1, as shown in Table S1.

**Downregulation of IL-32 expression and upregulation of IL-6 and IL-8 expression in *S. aureus*-infected cells.** Since the responsiveness of PS cells was higher than that of BME-UV cells (Fig. 1), PS cells were employed for the analysis of cytokine expression after challenge with the two *S. aureus* strains RF122 and NB305. MOIs of 40:1, 80:1, and 160:1 were used in the experiments. The results obtained with an MOI of 80:1 (the highest *S. aureus* concentration without a cytotoxic effect toward host cells)

are presented in Fig. 2, while Table S2 in the supplemental material shows results obtained with the 3 different MOIs.

There was no alteration in IL-32 expression at 2 h, 4 h, or 6 h postinfection; hence, the expression of IL-32 was investigated from 8 h to 48 h postinfection. As shown in Fig. 2, there were slight decreases in IL-32 expression after 8 h or 24 h of exposure of PS cells to either *S. aureus* strain (Fig. 2). The decreases in IL-32 expression were stronger after 48 h of exposure, namely, 7-fold and 3-fold for strains RF122 and NB305, respectively. To gain a more complete picture of the cytokine response during *S. aureus* infection, the levels of expression of IL-6 and IL-8 were analyzed at 8 h, 24 h, and 48 h postinfection. A slight increase in IL-6 expression was observed in cells after 8 h of exposure to either the NB305 or RF122 strain. There were no differences between infected and noninfected cells at 24 h or 48 h postinfection (Fig. 2). The level of IL-8 expression was approximately 4-fold higher in cells infected for 8 h than in noninfected control cells. After 24 h and 48 h, the IL-8 expression was only twice as high as the expression in control cells (Fig. 2). Similar results were obtained using MOIs ranging from 40:1 to 160:1 (see Table S2 in the supplemental material).

**IL-32 expression in *E. coli*- and *S. aureus*-infected udder tissue resembles *in vitro* responses.** To verify whether IL-32 expression was increased in the udders of experimentally infected cows, *E. coli*- or *S. aureus*-infected udder samples, prepared as described previously (21), were analyzed by qRT-PCR. A significant increase of IL-32 expression was observed in *E. coli*-infected quarters. There was a slight increase in IL-32 expression in uninfected quarters from *E. coli*-infected cows; however, the difference did not reach statistical significance (Fig. 3). In contrast, there were no differences in IL-32 expression between udder samples of control cows and samples of either infected or uninfected quarters of udders of *S. aureus*-infected cows (Fig. 3).

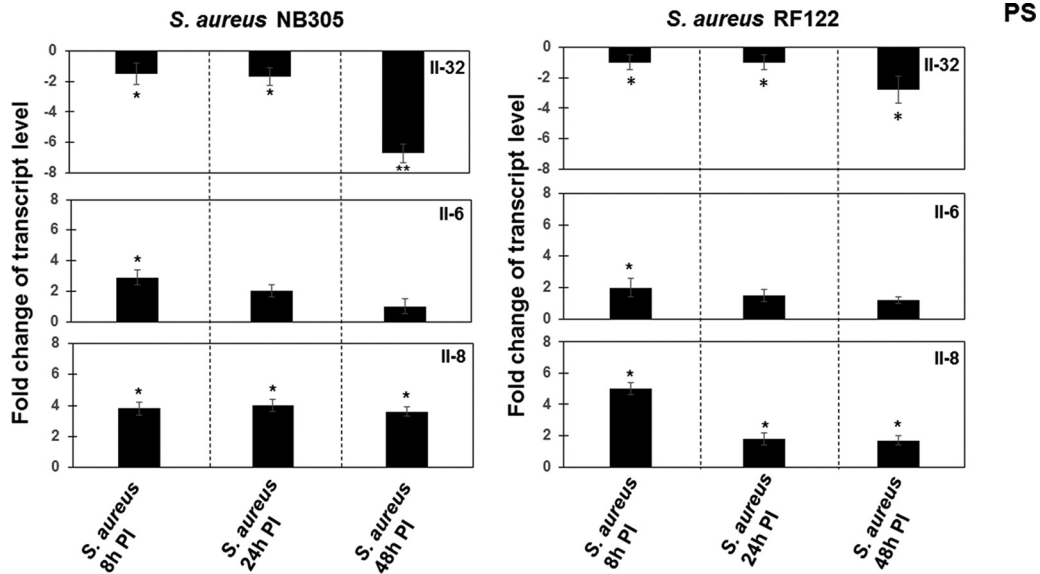


FIG 2 Analysis of IL-32, IL-6, and IL-8 expression in *S. aureus*-infected cells. PS cells ( $2 \times 10^5$ ) were grown for 24 h. The cells were then exposed to *S. aureus* NB305 or RF122 strains (MOI, 80:1) for 8 h, 24 h, and 48 h. Isolation of total RNA, synthesis of cDNA, and qRT-PCR were performed as described in Materials and Methods. After normalization using PPIA and RPL19 genes, interleukin expression at 8 h, 24 h, and 48 h p.i. was calculated relative to the values obtained from mock cells, arbitrarily set to 1. When the expression was decreased compared to that in unstimulated mock cells, data were presented as negative values. Data were calculated from three different experiments performed in triplicate. *P* values of <0.05 (\*) and <0.01 (\*\*) were considered significant.

**The bovine mastitis isolates have low-level expression of PSM $\alpha$  peptides but high expression of  $\delta$ -toxin.** To analyze PSM production in the bovine mastitis isolates used in this study, we performed HPLC-MS analysis of culture filtrates (23) in comparison to a culture filtrate of the USA300 isolate LAC, which was previously described as a high PSM producer (12). Taking into account that all tested bovine mastitis isolates and LAC USA300 have similar growth curves, all strains were grown as described in Materials and Methods until cultures reached an optical density of 0.6 at 600 nm.

As shown in Fig. 4, the bovine mastitis isolates showed production of  $\delta$ -toxin in a range similar to that of strain LAC, but PSM $\alpha$

and PSM $\beta$  levels were considerably reduced compared to those in that strain.

***S. aureus* PSM deletion mutants induce higher interleukin expression than the wild-type strain.** Interleukin expression levels exposed to various strains were compared to those of mock cells, and then the differences between those of cells exposed to the LAC wt strain and deletion mutants were evaluated. Expression levels of IL-32, IL-6, and IL-8 in cells exposed either to LAC wt or to corresponding deletion mutants with MOI 80:1 from 8 h to 48 h differ from those of mock cells. As shown in Fig. 5, the LAC wt strain decreased IL-32 expression 24 h and 48 h postinfection, while its isogenic mutants LAC  $\Delta$ psm $\alpha$  and LAC  $\Delta$ psm $\alpha$  $\beta$ hld in-

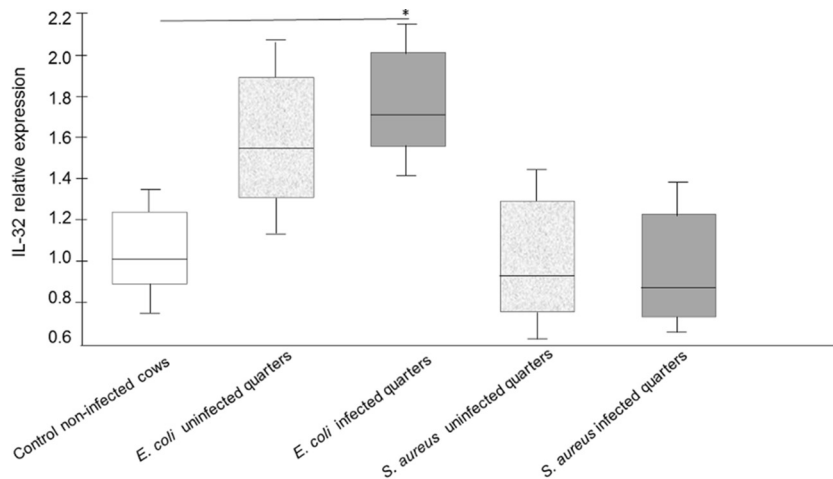


FIG 3 Increased IL-32 expression during experimentally *E. coli*-induced mastitis. Cows were infected either with *E. coli* or *S. aureus* as described in Materials and Methods. IL-32-specific qRT-PCRs were performed with RNA extracted from tissue samples of udder quarters from uninfected or bacteria-infected cows. Data presented are values from five animals; a statistical significance ( $P < 0.05$  [\*]) of *E. coli*-infected versus uninfected cows was observed using exact permutation tests after global comparison using a Kruskal-Wallis test.

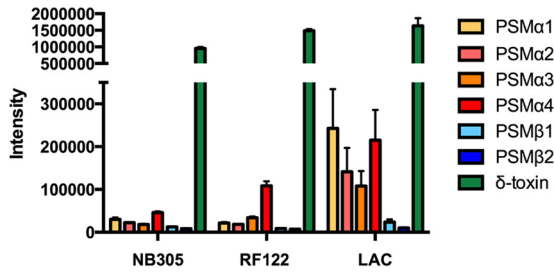


FIG 4 PSM production in bovine mastitis isolates. PSMs were analyzed in culture filtrates of cultures inoculated from precultures and grown for 8 h in tryptic soy broth. HPLC-MS detection was performed essentially as described previously (23). Intensity values are based on the integration of the extracted ion chromatogram using the two most abundant  $m/z$  peaks of every single PSM. Intensities can thus be directly compared between strains for a specific PSM, but only in a limited way for different PSMs. The assay was performed in triplicate. Error bars show standard deviations.

creased expression levels compared to those in mock cells (Fig. 5). To assess the impact of PSMs on interleukin expression, we compared interleukin expressions in LAC wt-treated cells to those in cells stimulated by the LAC  $\Delta psm\alpha$  or LAC  $\Delta psm\alpha\beta hld$ . ANOVA analysis followed by Tukey's HSD test showed that there was a significant difference between IL-32 expression levels in cells when stimulated either by the LAC  $\Delta psm\alpha$  mutant or LAC  $\Delta psm\alpha\beta hld$  compared to LAC wt at 24 h and 48 h postinfection (Fig. 5).

Additionally, there were statistically significant differences between IL-6 and IL-8 expression levels in cells exposed to either LAC wt or deletion mutants: IL-6 expression was twice as great in cells exposed to LAC  $\Delta psm\alpha$  or LAC  $\Delta psm\alpha\beta hld$  mutants than in

cells exposed to LAC wt 24 h postinfection and 3-fold higher at 48 h postinfection. IL-8 was increased 2.8-fold in LAC  $\Delta psm\alpha$ -treated cells and 4-fold in LAC  $\Delta psm\alpha\beta hld$ -treated cells compared to LAC wt-treated cells. Similar results were obtained with other MOIs (see Table S3 in the supplemental material).

To confirm the findings, we compared interleukin expression levels induced by a wild-type (WT) LAC pTX $_{\Delta}$ 16 strain harboring the control plasmid pTX $_{\Delta}$ 16 to those of corresponding deletion mutants as well as genetically complemented strains, which are described in Materials and Methods. It has to be noted that interleukin expression levels in cells exposed to all strains from 8 h to 48 h were different from those of mock cells: the IL-32 expression level was downregulated, while IL-6 and IL-8 expression levels were upregulated (Fig. 6). Interleukin expression levels in cells exposed to a LAC (WT) pTX $_{\Delta}$ 16 strain was, similar to those induced by LAC wt (USA300). Exposure to  $psm\alpha$  deletion mutant LAC  $\Delta psm\alpha$  pTX $_{\Delta}$ 16 resulted in increased expression of all tested interleukins, similar to the increase of expression induced by the deletion mutant LAC  $\Delta psm\alpha$ , while exposure to the complemented strain LAC  $\Delta psm\alpha$  pTX $_{\Delta}$ 16-4 resulted in a decrease of expression levels. Comparison of expression levels in cells exposed to LAC (WT) pTX $_{\Delta}$ 16 to those in cells exposed to corresponding deletion and complemented mutants showed that interleukin expression levels were significantly higher in cells exposed to complemented strain LAC  $\Delta psm\alpha$  pTX $_{\Delta}$ 16-4 than in cells infected with LAC (WT) pTX $_{\Delta}$ 16 but lower than those from cells infected with deletion mutant LAC  $\Delta psm\alpha$  pTX $_{\Delta}$ 16.

To examine the role of other PSMs, interleukin expression levels in cells exposed to LAC (WT) pTX $_{\Delta}$ 16 were compared to expression levels in cells exposed to the PSM-deficient deletion mutant LAC  $\Delta psm\alpha\beta hld$  pTX $_{\Delta}$ 16 and to the complemented mutants

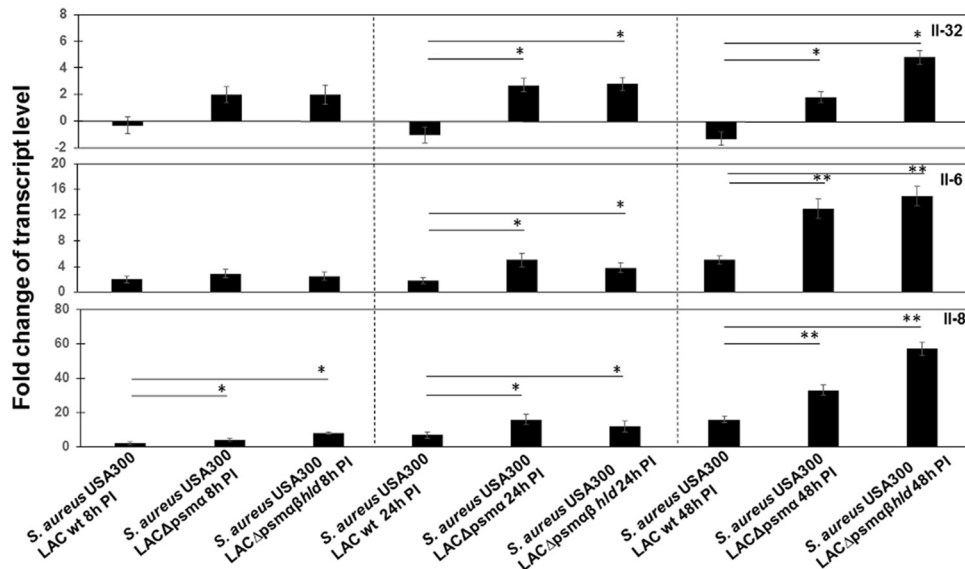
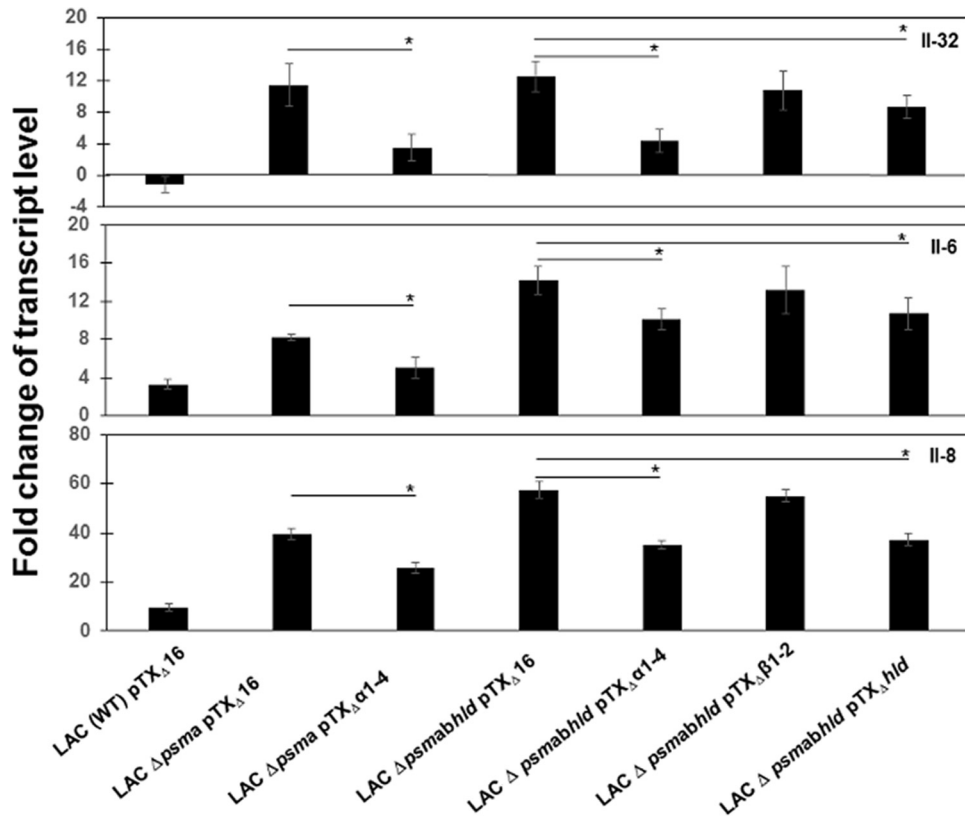


FIG 5 Analysis of the expression of IL-32, IL-6, and IL-8 in cells exposed to *S. aureus* LAC wt and its mutants. PS cells ( $2 \times 10^5$ ) were grown for 24 h. The cells were then exposed to LAC wt and to the deletion mutants LAC  $\Delta psm\alpha$  and LAC  $\Delta psm\alpha\beta hld$  at an MOI of 80:1 for 8 h, 24 h, and 48 h. An mRNA expression was measured in total RNA preparation by qRT-PCR. After normalization using PPIA and RPL19 genes, interleukin expression was calculated relative to the values obtained from mock cells, arbitrarily set to 1. According to ANOVA with *post hoc* Tukey's HSD test, there was a statistically significant difference between interleukin expression levels in cells exposed either to LAC wt or deletion mutants and mock cells, except for IL-32 expression in LAC wt-treated cells for 8 h. There were also statistically significant differences between interleukin expression levels in cells exposed to LAC wt and corresponding deletion mutants as indicated with asterisks. Data were calculated from three different experiments performed in triplicate.  $P$  values of  $<0.05$  (\*) and  $<0.01$  (\*\*) were considered significant.



**FIG 6** Analysis of the expression of IL-32, IL-6, and IL-8 in cells exposed to *S. aureus* LAC WT pTXD16, corresponding deletion mutants, and complemented strains. PS cells ( $2 \times 10^5$ ) were grown for 24 h. The cells were then exposed to LAC (WT) pTX $\Delta$ 16, to the deletion PSM $\alpha$ -deficient mutant LAC  $\Delta$ psm $\alpha$  pTX $\Delta$ 16, and to the complemented LAC  $\Delta$ psm $\alpha$  pTX $\Delta$  $\alpha$ 1-4 strain, as well as to the PSM-deficient mutant LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 and complemented strains LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$  $\alpha$ 1-4, LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$  $\beta$ 1-2, and LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ hld at an MOI of 80:1 for 24 h. mRNA expression was measured in total RNA preparations by qRT-PCR. After normalization using PPIA and RPL19 genes, interleukin expression was calculated relative to the values obtained from mock cells, arbitrarily set to 1. According to ANOVA with *post hoc* Tukey's HSD test, there was a statistically significant difference between interleukin expression levels in cells exposed to either LAC (WT) pTX $\Delta$ 16, the deletion mutants, or complemented strains and mock cells. Statistical analysis demonstrated that interleukin expression levels were significantly higher in cells infected with deletion mutants than in those from cells infected either with LAC (WT) pTX $\Delta$ 16 or complemented strains. Statistically significant differences between interleukin expression levels in cells exposed to deletion mutants and corresponding complemented strains are indicated with asterisks. Data were calculated from three different experiments performed in triplicate. *P* values of <0.05 (\*) and <0.01 (\*\*) were considered significant.

LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$  $\alpha$ 1-4, LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$  $\beta$ 1-2, and LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ hld. As shown in Fig. 6, exposure to deletion mutant LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 increased interleukin expression compared to that of the LAC (WT) pTX $\Delta$ 16 strain expressing PSMs. Exposure to the complemented strains LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$  $\alpha$ 1-4 and LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ hld resulted in lower interleukin expression than did exposure to the PSM-deficient LAC  $\Delta$ psm $\alpha$  $\beta$ hld pTX $\Delta$ 16 strain, while no significant difference was observed when the *psm* $\beta$  locus was complemented (with plasmid pTX $\Delta$ psm $\beta$ 1-2) (Fig. 6).

Additionally to the analysis of interleukin expression by the cells exposed to the mutant and genetically complemented bacterial strains, interleukin expression was evaluated in the cells treated with PSM $\alpha$ 3, one of the most cytolytically active and well-characterized PSMs (12). As shown in Fig. 7, expression levels of IL-32, IL-6, and IL-8 were significantly increased after 8 h of exposure to 2  $\mu$ g/ml of PSM $\alpha$ 3.

## DISCUSSION

Mammary epithelial cells (MEC) represent the first line of contact with microorganisms and are crucial for the early defense against

intramammary pathogens. They produce various antimicrobial substances and inflammatory mediators that enhance effector functions of innate immunity and stimulate adaptive immunity, playing a pivotal role in the resolution of infection (27). A great number of studies dedicated to the analysis of the defensive response of MEC used killed bacteria or bacterial supernatants; however, those models have a significant limitation, since there is a great difference in the immune responses initiated by live bacteria, killed bacteria, bacterial supernatants, and purified bacterial components (26, 31, 32). Using live *E. coli* and *S. aureus* bacteria, we set up *in vitro* infection models that assessed the effects of these Gram-negative and Gram-positive bacteria on expression of IL-32, IL-6, and IL-8.

In *in vitro* MEC challenges, the increased level of *E. coli*-induced IL-6 and IL-8 expression compared to the reduction induced by *S. aureus* observed in our experiments was in agreement with data obtained by others (26).

The role of the newly discovered proinflammatory IL-32 during inflammation and in several types of infections is well documented (33, 34). However, to our knowledge there is no informa-

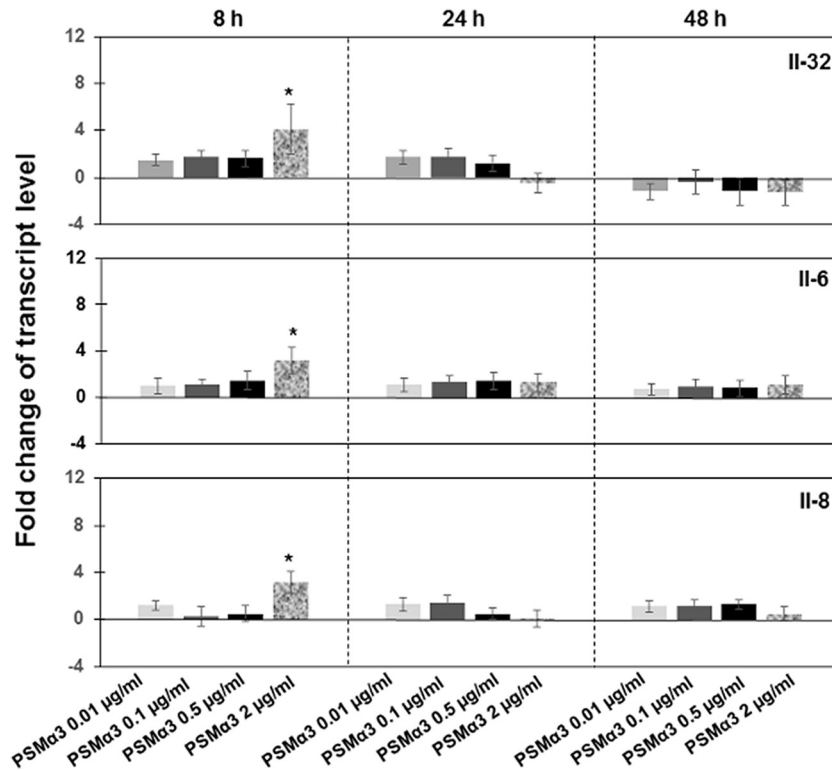


FIG 7 Analysis of the expression of IL-32, IL-6, and IL-8 in cells treated with synthetic PSM peptides. PS cells ( $2 \times 10^5$ ) were grown for 24 h and then exposed to synthetic PSM $\alpha$ 3 ranging from 0.01 to 2  $\mu$ g/ml for 8 h, 24 h, or 48 h. mRNA expression was measured in total RNA preparation by qRT-PCR. After normalization using PPIA and RPL19 genes, interleukin expression was calculated relative to the values obtained from mock cells, arbitrarily set to 1. According to ANOVA with *post hoc* Tukey's HSD test, there were statistically significant differences between interleukin expression levels in cells treated with 2  $\mu$ g/ml of PSM $\alpha$ 3 for 8 h compared to those in mock cells. Data were calculated from three different experiments performed in triplicate. *P* values of <0.05 (\*) were considered significant.

tion about the involvement of IL-32 during exposure to live *S. aureus*, and there are no data regarding infections associated with *S. aureus* or *E. coli* in the context of mastitis. We observed decreased IL-32 expression in *S. aureus*-infected cells compared to that in *E. coli*-infected cells. This is important new information contributing to our understanding of the molecular mechanisms that underlie the divergent host responses during *S. aureus* versus *E. coli* infection.

The increase of IL-32 expression in *E. coli*-infected cells or in the udder tissue of *E. coli*-infected cows was consistent with other studies of IL-32 in response to Gram-negative pathogens and endotoxin (35–37). In contrast, expression of IL-32 by *S. aureus*-infected cells was decreased. Taking into account that IL-32 $\alpha$  plays an intracellular mediatory role in IL-6 production in promonocytic cells (34) and considering the capacity of human IL-32 $\gamma$  to induce the maturation of dendritic cells (38), we speculate that decreased IL-32 expression during *S. aureus* infection could be associated with an attenuated immune response and may promote the progression of chronic infection.

The extraordinary virulence of *S. aureus* depends on its ability to effectively compromise host defense mechanisms by a variety of strategies: *S. aureus* may inhibit complement activation, block and destroy phagocytic cells, and modify host B-cell and T-cell responses (39). Several factors contribute to the pathogenicity of *S. aureus*, including the well-established virulence determinants accessory gene regulator and alpha-hemolysin (Hla) (40, 41). The

immunomodulatory actions of staphylococcal enterotoxins are also reported (42, 43). Additionally, PSMs have recently emerged as a novel toxin family that contributes to increased virulence and the spread of *S. aureus* infection (12, 44). PSMs are divided into two groups, depending on their size: the short (20- to 25-amino-acid)  $\alpha$ -type peptides (PSM $\alpha$ 1 to PSM $\alpha$ 4 and  $\delta$ -toxin) and the long (44-amino-acid)  $\beta$ -type peptides (PSM $\beta$ 1 and PSM $\beta$ 2) (13). Prompted by those results, we examined in this study the role of PSMs in *S. aureus*-induced interleukin expression. Comparison of clinically related LAC wt strain to PSM deletion mutants allows the correlation of the findings with those obtained by using of RF122 and NB305 strains isolated from cows with mastitis. Infection of cells with PSM deletion mutants resulted in an increase of IL-6, IL-8, and IL-32 expression, in contrast to the findings with LAC wt (Fig. 5). Utilization of wild-type, deletion, and complemented strains harboring plasmids allowed us to comprehend the impact of different PSMs. Exposure of cells to a strain expressing the four PSM $\alpha$  peptides significantly decreased interleukin expression compared to that obtained with a PSM $\alpha$  deletion mutant (Fig. 6), suggesting that PSM $\alpha$  expression by internalized *S. aureus* inhibits interleukin expression. Since the LAC  $\Delta$ psm $\alpha$  $\beta$ hld mutant induced a higher level of interleukin expression than the LAC  $\Delta$ psm $\alpha$  mutant, we examined whether PSM $\beta$  peptides or, more likely, the highly expressed  $\delta$ -toxin may be involved in the inhibition of interleukin expression in addition to the PSM $\alpha$  peptides. Results from strains specifically expressing only the  $\delta$ -toxin or the



PSM $\beta$  peptides showed that also the  $\delta$ -toxin has a significant effect, while no decrease of interleukin expression was observed with the PSM $\beta$ -expressing strain, a result that may be explained either by the different structure of the considerably longer PSM $\beta$  peptides or by their overall lower expression levels. The observed inhibitory effect likely depends on the presence of PSMs and not different numbers of bacteria, which were adhered to or internalized into cells, since we observed similar adherence and internalization rates between LAC wt and its mutants.

The observed inhibition of IL-6, IL-8, and IL-32 expression in bovine epithelial cells by staphylococcal PSMs differs from the observations showing increased release of IL-8 by human neutrophils upon addition of PSMs (12, 45). This difference could be explained by different conditions of experiments, including the exposure time and PSM concentrations, or by the different transduction pathways induced by PSMs in different types of cells. Furthermore, cell lysis may also explain the release of IL-8, as the PSM concentrations used in those experiments were in the cytolytic range (12). Similarly, the stimulation of PSM-induced IL-1 $\beta$  and IL-18 release from human keratinocytes, highly specialized epithelial cells, was also likely associated with cell lysis (15). Treatment of bovine PS cells with PSM $\alpha$ 3 at 2  $\mu$ g/ml for 8 h resulted in increased interleukin expression in the absence of a cytotoxic effect (Fig. 7). These findings suggest a difference between transduction pathways induced by a synthetic PSM and a PSM produced by internalized *S. aureus* bacteria.

An analysis of epithelial intestinal HT-29 cells showed an increase in IL-8 secretion after exposure to TNF- $\alpha$  in contrast to the absence of IL-8 secretion in PSM-stimulated cells (46). Interleukin inhibition by internalized bacteria, observed in our experiments, is in accordance with data showing the inhibition of IL-6, IL-12, and TNF- $\alpha$  expression in dendritic cells by PSM $\alpha$  and data demonstrating the inhibition of TNF- $\alpha$  secretion by the USA300 wild-type strain harboring PSMs in contrast to the mutant strain deficient in all PSMs (18), and it shows that this effect extends to the “front line” defenses of the epithelium. The epithelium is in permanent contact with various microorganisms, resulting in the host’s defense mechanisms, including expressing and secreting inflammatory cytokines that recruit inflammatory cells. Thus, it was shown that TNF- $\alpha$  and IL-1 $\beta$  were secreted by the primary cultured rat epithelia infected with *S. aureus* (47). Human corneal epithelial cells react to *S. aureus* infection by enhancing the secretion of IL-6 (48).

In conclusion, we found that in mammary epithelial cells *S. aureus* PSMs decrease the expression of the cytokine IL-32, which is responsible for the maturation of dendritic cells and plays an essential role in linking innate and adaptive immunity. This mechanism may contribute to the attenuated immune response generally observed during staphylococcal infection. These findings may be important for the development of new anti-infective and anti-inflammatory strategies. Finally, we cannot exclude the existence of various isoforms of bovine IL-32 (with differential similarity to human IL-32) with different expression levels of the expression; a more detailed assessment of those differences forms an avenue for future investigation. As a final point, it has to be noted that the findings obtained with bovine cells and tissue likely may be extended to human medicine: it would be appropriate to determine the level of IL-32 in patients with mastitis.

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## REFERENCES

1. Thompson-Crispi K, Atalla H, Miglior F, Mallard BA. 2014. Bovine mastitis: frontiers in immunogenetics. *Front Immunol* 5:493. <http://dx.doi.org/10.3389/fimmu.2014.00493>.
2. Schukken YH, Gunther J, Fitzpatrick J, Fontaine MC, Goetze L, Holst O, Leigh J, Petzl W, Schuberth HJ, Sipka A, Smith DG, Quesnell R, Watts J, Yancey R, Zerbe H, Gurjar A, Zadoks RN, Seyfert HM. 2011. Host-response patterns of intramammary infections in dairy cows. *Vet Immunol Immunopathol* 144:270–289. <http://dx.doi.org/10.1016/j.vetimm.2011.08.022>.
3. Fu Y, Zhou E, Liu Z, Li F, Liang D, Liu B, Song X, Zhao F, Fen X, Li D, Cao Y, Zhang X, Zhang N, Yang Z. 2013. *Staphylococcus aureus* and *Escherichia coli* elicit different innate immune responses from bovine mammary epithelial cells. *Vet Immunol Immunopathol* 155:245–252. <http://dx.doi.org/10.1016/j.vetimm.2013.08.003>.
4. Jensen K, Gunther J, Talbot R, Petzl W, Zerbe H, Schuberth HJ, Seyfert HM, Glass EJ. 2013. *Escherichia coli*- and *Staphylococcus aureus*-induced mastitis differentially modulate transcriptional responses in neighbouring uninfected bovine mammary gland quarters. *BMC Genomics* 14:36. <http://dx.doi.org/10.1186/1471-2164-14-36>.
5. Alekseeva L, Huet D, Femenia F, Mouyna I, Abdelouahab M, Cagna A, Guerrier D, Tichanne-Seltzer V, Baeza-Squiban A, Chermette R, Latge JP, Berkova N. 2009. Inducible expression of beta defensins by human respiratory epithelial cells exposed to *Aspergillus fumigatus* organisms. *BMC Microbiol* 9:33. <http://dx.doi.org/10.1186/1471-2180-9-33>.
6. Almeida RA, Dogan B, Klaessing S, Schukken YH, Oliver SP. 2011. Intracellular fate of strains of *Escherichia coli* isolated from dairy cows with acute or chronic mastitis. *Vet Res Commun* 35:89–101. <http://dx.doi.org/10.1007/s11259-010-9455-5>.
7. Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. 2005. Interleukin-32: a cytokine and inducer of TNF $\alpha$ . *Immunity* 22:131–142. <http://dx.doi.org/10.1016/j.immuni.2004.12.003>.
8. Ferlazzo G, Pack M, Thomas D, Paludan C, Schmid D, Strowig T, Bougras G, Muller WA, Moretta L, Munz C. 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci U S A* 101:16606–16611. <http://dx.doi.org/10.1073/pnas.0407522101>.
9. Jung MY, Son MH, Kim SH, Cho D, Kim TS. 2011. IL-32 $\gamma$  induces the maturation of dendritic cells with Th1- and Th17-polarizing ability through enhanced IL-12 and IL-6 production. *J Immunol* 186:6848–6859. <http://dx.doi.org/10.4049/jimmunol.1003996>.
10. Painter KL, Krishna A, Wigneshweraraj S, Edwards AM. 2014. What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol* 22:676–685. <http://dx.doi.org/10.1016/j.tim.2014.09.002>.
11. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* 28:603–661. <http://dx.doi.org/10.1128/CMR.00134-14>.
12. Wang R, Broughton KR, Kretschmer D, Bach TH, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, Deleo FR, Otto

- M. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 13:1510–1514. <http://dx.doi.org/10.1038/nm1656>.
13. Peschel A, Otto M. 2013. Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol* 11:667–673. <http://dx.doi.org/10.1038/nrmicro3110>.
  14. Rasigade JP, Trouillet-Assant S, Ferry T, Diep BA, Sapin A, Lhoste Y, Ranfaing J, Badiou C, Benito Y, Bes M, Couzon F, Tigaud S, Lina G, Etienne J, Vandenesch F, Laurent F. 2013. PSMs of hypervirulent *Staphylococcus aureus* act as intracellular toxins that kill infected osteoblasts. *PLoS One* 8:e63176. <http://dx.doi.org/10.1371/journal.pone.0063176>.
  15. Syed AK, Reed TJ, Clark KL, Boles BR, Kahlenberg JM. 2015. *Staphylococcus aureus* phenol-soluble modulins stimulate the release of proinflammatory cytokines from keratinocytes and are required for induction of skin inflammation. *Infect Immun* 83:3428–3437. <http://dx.doi.org/10.1128/IAI.00401-15>.
  16. Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, Bohn E, Schoneberg T, Rabiet MJ, Boulay F, Klebanoff SJ, van Kessel KA, van Strijp JA, Otto M, Peschel A. 2010. Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. *Cell Host Microbe* 7:463–473. <http://dx.doi.org/10.1016/j.chom.2010.05.012>.
  17. Deplanche M, Filho RA, Alekseeva L, Ladier E, Jardin J, Henry G, Azevedo V, Miyoshi A, Beraud L, Laurent F, Lina G, Vandenesch F, Steghens JP, Le LY, Otto M, Gotz F, Berkova N. 2015. Phenol-soluble modulin alpha induces G2/M phase transition delay in eukaryotic HeLa cells. *FASEB J* 29:1950–1959. <http://dx.doi.org/10.1096/fj.14-260513>.
  18. Schreiner J, Kretschmer D, Klenk J, Otto M, Buhning HJ, Stevanovic S, Wang JM, Beer-Hammer S, Peschel A, Autenrieth SE. 2013. *Staphylococcus aureus* phenol-soluble modulin peptides modulate dendritic cell functions and increase in vitro priming of regulatory T cells. *J Immunol* 190:3417–3426. <http://dx.doi.org/10.4049/jimmunol.1202563>.
  19. Forsman H, Christenson K, Bylund J, Dahlgren C. 2012. Receptor-dependent and -independent immunomodulatory effects of phenol-soluble modulin peptides from *Staphylococcus aureus* on human neutrophils are abrogated through peptide inactivation by reactive oxygen species. *Infect Immun* 80:1987–1995. <http://dx.doi.org/10.1128/IAI.05906-11>.
  20. Zavizion B, van DM, Schaeffer W, Politis I. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. *In Vitro Cell Dev Biol Anim* 32:138–148. <http://dx.doi.org/10.1007/BF02723679>.
  21. Roussel P, Cunha P, Porcherie A, Petzl W, Gilbert FB, Riollet C, Zerbe H, Rainard P, Germon P. 2015. Investigating the contribution of IL-17A and IL-17F to the host response during *Escherichia coli* mastitis. *Vet Res* 46:56. <http://dx.doi.org/10.1186/s13567-015-0201-4>.
  22. Vautour E, Cockfield J, Le MC, Le LY, Chevalier M, Robinson DA, Thiery R, Lindsay J. 2009. Difference in virulence between *Staphylococcus aureus* isolates causing gangrenous mastitis versus subclinical mastitis in a dairy sheep flock. *Vet Res* 40:56. <http://dx.doi.org/10.1051/vetres/2009039>.
  23. Joo HS, Otto M. 2014. The isolation and analysis of phenol-soluble modulins of *Staphylococcus epidermidis*. *Methods Mol Biol* 1106:93–100. [http://dx.doi.org/10.1007/978-1-62703-736-5\\_7](http://dx.doi.org/10.1007/978-1-62703-736-5_7).
  24. Chatterjee SS, Joo HS, Duong AC, Dieringer TD, Tan VY, Song Y, Fischer ER, Cheung GY, Li M, Otto M. 2013. Essential *Staphylococcus aureus* toxin export system. *Nat Med* 19:364–367. <http://dx.doi.org/10.1038/nm.3047>.
  25. Baron F, Cochet MF, Ablain W, Grosset N, Madec MN, Gonnet F, Jan S, Gautier M. 2006. Rapid and cost-effective method for micro-organism enumeration based on miniaturization of the conventional plate-counting technique. *Lait* 86:251–257. <http://dx.doi.org/10.1051/lait:2006005>.
  26. Günther J, Esch K, Poschadel N, Petzl W, Zerbe H, Mitterhuemer S, Blum H, Seyfert HM. 2011. Comparative Kinetics of *Escherichia coli*- and *Staphylococcus aureus*-specific activation of key immune pathways in mammary epithelial cells demonstrates that *S. aureus* elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1A or tumor necrosis factor alpha. *Infect Immun* 79:695–707. <http://dx.doi.org/10.1128/IAI.01071-10>.
  27. Mitterhuemer S, Petzl W, Krebs S, Mehne D, Klanner A, Wolf E, Zerbe H, Blum H. 2010. *Escherichia coli* infection induces distinct local and systemic transcriptome responses in the mammary gland. *BMC Genomics* 11:138. <http://dx.doi.org/10.1186/1471-2164-11-138>.
  28. Petzl W, Zerbe H, Gunther J, Yang W, Seyfert HM, Nurnberg G, Schubert HJ. 2008. *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Vet Res* 39:18. <http://dx.doi.org/10.1051/vetres:2007057>.
  29. Almeida RA, Patel D, Friton GM, Oliver SP. 2007. Intracellular killing of mastitis pathogens by penethamate hydriodide following internalization into mammary epithelial cells. *J Vet Pharmacol Ther* 30:151–156. <http://dx.doi.org/10.1111/j.1365-2885.2007.00830.x>.
  30. Berkova N, Gilbert C, Goupil S, Yan J, Korobko V, Naccache PH. 1999. TNF-induced haptoglobin release from human neutrophils: pivotal role of the TNF p55 receptor. *J Immunol* 162:6226–6232.
  31. Gilbert FB, Cunha P, Jensen K, Glass EJ, Foucras G, Robert-Granie C, Rupp R, Rainard P. 2013. Differential response of bovine mammary epithelial cells to *Staphylococcus aureus* or *Escherichia coli* agonists of the innate immune system. *Vet Res* 44:40. <http://dx.doi.org/10.1186/1297-9716-44-40>.
  32. Moreilhon C, Gras D, Hologne C, Bajolet O, Cottrez F, Magnone V, Merten M, Groux H, Puchelle E, Barbry P. 2005. Live *Staphylococcus aureus* and bacterial soluble factors induce different transcriptional responses in human airway cells. *Physiol Genomics* 20:244–255. <http://dx.doi.org/10.1152/physiolgenomics.00135.2004>.
  33. Hou J, OGvan Groothuisminck ZM, Claassen MA, Kreefft K, Zaaraoui-Boutahar F, van IJcken WF, Osterhaus AD, Janssen HL, Andeweg AC, de Knegt RJ, Boonstra A. 2014. Gene expression profiling to predict and assess the consequences of therapy-induced virus eradication in chronic hepatitis C virus infection. *J Virol* 88:12254–12264. <http://dx.doi.org/10.1128/JVI.00775-14>.
  34. Kang JW, Park YS, Lee DH, Kim JH, Kim MS, Bak Y, Hong J, Yoon DY. 2012. Intracellular interaction of interleukin (IL)-32alpha with protein kinase Cepsilon (PKCepsilon) and STAT3 protein augments IL-6 production in THP-1 promonocytic cells. *J Biol Chem* 287:35556–35564. <http://dx.doi.org/10.1074/jbc.M112.400911>.
  35. Nakayama M, Niiki Y, Kawasaki T, Takeda Y, Ikegami H, Toyama Y, Miyamoto T. 2013. IL-32-PAR2 axis is an innate immunity sensor providing alternative signaling for LPS-TRIF axis. *Sci Rep* 3:2960. <http://dx.doi.org/10.1038/srep02960>.
  36. Ouhara K, Kawai T, Silva MJ, Fujita T, Hayashida K, Karimbux NY, Kajiya M, Shiba H, Kawaguchi H, Kurihara H. 2012. Expression levels of novel cytokine IL-32 in periodontitis and its role in the suppression of IL-8 production by human gingival fibroblasts stimulated with *Porphyromonas gingivalis*. *J Oral Microbiol* 4:10.3402/jom.v4i0.14832. <http://dx.doi.org/10.3402/jom.v4i0.14832>.
  37. Sakitani K, Hirata Y, Hayakawa Y, Serizawa T, Nakata W, Takahashi R, Kinoshita H, Sakamoto K, Nakagawa H, Akanuma M, Yoshida H, Maeda S, Koike K. 2012. Role of interleukin-32 in *Helicobacter pylori*-induced gastric inflammation. *Infect Immun* 80:3795–3803. <http://dx.doi.org/10.1128/IAI.00637-12>.
  38. Schenk M, Krutzik SR, Sieling PA, Lee DJ, Teles RM, Ochoa MT, Komisopoulou E, Sarno EN, Rea TH, Graeber TG, Kim S, Cheng G, Modlin RL. 2012. NOD2 triggers an interleukin-32-dependent human dendritic cell program in leprosy. *Nat Med* 18:555–563. <http://dx.doi.org/10.1038/nm.2650>.
  39. Thammavongsa V, Kim HK, Missiakas D, Schneewind O. 2015. Staphylococcal manipulation of host immune responses. *Nat Rev Microbiol* 13:529–543. <http://dx.doi.org/10.1038/nrmicro3521>.
  40. Berube BJ, Bubeck WJ. 2013. *Staphylococcus aureus* alpha-toxin: nearly a century of intrigue. *Toxins (Basel)* 5:1140–1166. <http://dx.doi.org/10.3390/toxins5061140>.
  41. Bubeck WJ, Bae T, Otto M, Deleo FR, Schneewind O. 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat Med* 13:1405–1406. <http://dx.doi.org/10.1038/nm1207-1405>.
  42. Krakauer T, Stiles BG. 2013. The staphylococcal enterotoxin (SE) family: SEB and siblings. *Virulence* 4:759–773. <http://dx.doi.org/10.4161/viru.23905>.
  43. Pinchuk IV, Beswick EJ, Reyes VE. 2010. Staphylococcal enterotoxins. *Toxins (Basel)* 2:2177–2197. <http://dx.doi.org/10.3390/toxins2082177>.
  44. Cheung GY, Joo HS, Chatterjee SS, Otto M. 2014. Phenol-soluble modulins—critical determinants of staphylococcal virulence. *FEMS Microbiol Rev* 38:698–719. <http://dx.doi.org/10.1111/1574-6976.12057>.
  45. Nakamura Y, Oscherwitz J, Cease KB, Chan SM, Munoz-Planillo R, Hasegawa M, Villaruz AE, Cheung GY, McGavin MJ, Travers JB, Otto M, Inohara N, Nunez G. 2013. *Staphylococcus delta*-toxin induces aller-

- gic skin disease by activating mast cells. *Nature* 503:397–401. <http://dx.doi.org/10.1038/nature12655>.
46. Melmed G, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, Zhou Y, Hu B, Arditi M, Abreu MT. 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol* 170:1406–1415. <http://dx.doi.org/10.4049/jimmunol.170.3.1406>.
47. Zhao YT, Guo JH, Wu ZL, Xiong Y, Zhou WL. 2008. Innate immune responses of epididymal epithelial cells to *Staphylococcus aureus* infection. *Immunol Lett* 119:84–90. <http://dx.doi.org/10.1016/j.imlet.2008.05.002>.
48. Arranz-Valsero I, Schulze U, Contreras-Ruiz L, Garcia-Posadas L, Lopez-García A, Paulsen F, Diebold Y. 2013. Involvement of corneal epithelial cells in the Th17 response in an in vitro bacterial inflammation model. *Mol Vis* 19:85–99.