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Staphylococcus aureus phenol-soluble modulins impair interleukin expression in bovine mammary epithelial cells

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Running Head: Staphylococcal PSMs inhibit interleukins expression

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Abstract

The role of the recently-described interleukin-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- as compared to E.coli (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 as compared to 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.
Introduction

Mastitis of ruminants is an inflammation of the mammary gland commonly caused by bacterial infection: *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are among the most prevalent pathogens (1). *E. coli* intra-mammary 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 pro-inflammatory responses in ruminants to *S. aureus* as compared to *E. coli*, 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 is not completely understood. This includes, 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-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε and IL-32ζ (8). IL-32γ induces the maturation of dendritic cells (9). Furthermore, IL-
32 induces IL-1β, IL-6, TNF-α, 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 regards 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, α-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 pro-inflammatory activities: they stimulate leukocytes and initiate pro-inflammatory responses, including neutrophil chemoattraction and activation (12,16). Furthermore, we have shown that a PSMα-induced G2/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 interleukin-8 (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 PSMα 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 here tested 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 of 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**

Phenol-soluble modulins (PSM) PSMα3 peptide was synthesized by solid-phase peptide synthesis and provided by CecoLabs (Tübingen, Germany). LDH (lactate dehydrogenase) was quantified using the Pierce LDH Cytotoxicity Assay (Thermopierce, Rockford, IL, USA).

**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% α-lactose monohydrate, 0.1% lactalbumin enzymatic hydrolysate, 1.2 mM reduced L-glutathione, 5 µg/ml bovine insulin, 5 µg/ml bovine holo-transferrin, 5 µg/ml progesterone, $10^{-7}$ mol/l hydrocortisone, 10 µg/ml L-ascorbic acid, 50 IU/ml penicillin/streptomycin (Sigma-Aldrich) at 37°C with 5% CO$_2$. PS, a newly isolated MEC line from the secretory parenchyma (21), was maintained in advanced medium: Advanced-DMEM/F12 (Gibco, Saint Aubin, France) containing 20
mM HEPES, 2 mM L-glutamine (Gibco, Saint Aubin, France), 1 µg/ml hydrocortisone (Sigma-Aldrich), 10 ng/ml Insulin-like growth factor (IGF), 1,5 ng/ml fibroblast growth factor (FGF), 5 ng/ml epidermal growth factor (EGF). Trypsin/EDTA (Gibco, Saint Aubin, France) 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), its isogenic mutants LACΔpsma, which lacks the *psma* operon encoding PSMα1 to 4 and LACΔpsmaβhld, which lacks the *psmx* and *psmβ* operons, and 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 LAC (WT) pTXΔ16 strain harboring the control plasmid pTXΔ16, PSMα-deletion mutant LACΔpsma pTXΔ16 and complemented strain LACΔpsmaα1-4, as well as PSM-deficient deletion mutant LACΔpsmaβhld pTXΔ16 and complemented strains expressing either the four PSMα peptides (LAC Δpsmaβhld pTXΔα1-4) or PSMβ (LAC Δpsmaβhld pTXβ1-2) or *hld* (LAC Δpsmaβhld pTXΔ hld) were obtained as described (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 (LACΔpsma and LACΔpsmaβhld) in which the *psma* operon was exchanged for a spectinomycin resistance cassette were grown in BHI containing 250 µg/ml spectinomycin before inoculating into DMEM. The tetracycline resistant strains harboring plasmid TDX16 were grown in BHI containing 12.5 µg/ml
tetacycline before inoculating 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 $10^8$ CFU/ml (CFU, colony-forming unit). 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 micromethod as previously described (25).

*E. coli* K-12 MG1655 (ATCC) was grown for 8 h in Luria Broth medium, after which *E. coli* was maintained in 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 $1.2 \times 10^8$ 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 a case of clinical and subclinical bovine mastitis respectively. The number of bacterial strains refers to the identification number of animal (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 MOIs (multiplicity of infection, number of bacteria per cell at the onset of infection) in order to determine the highest bacterial concentration that did not cause cytotoxicity towards host cells. Mock cells (treated the same way except without the bacteria) at indicated time point 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 MOI 1:1 compared to the non-infected control cells. Usage of an MOI higher than 30:1 resulted in the development of cytotoxicity towards 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 MOI 20:1 there was no change in interleukin expression compared to the non-infected mock cells. The cytotoxic effect towards 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 post-infection by washing wells with PBS, followed by incubation in medium containing 20 μg/ml of lysostaphin and 100 μg/ml gentamicin for 2h, which eliminates the extracellular bacteria, followed by washing in PBS and then by incubation in medium containing 25 μg/ml of gentamicin for the periods indicated. Mock cells at indicated time point were used as controls.

**Adhesion and internalization assays**

Adhesion assays were performed as described (29). Briefly, confluent monolayers of PS cells in 96 wells plates (2.5x10^5 cells/well) were incubated for 90 min with *S. aureus* suspension at MOI 80:1. After the lysis of cells using 0.01% Triton X-100 the number of adherent bacteria CFU 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 incubation with bacteria, cells were incubated in DMEM containing 100 µg/ml of gentamicin and 20 µg/ml lysostaphin for 2 h in order to remove extracellular bacteria and to measure internalized bacteria only. The percentage of internalized bacteria from the initial inoculum was determined as described for adhesion assay.

There were no statistically significant differences between percentages of either adhered or internalized LAC wt strain or LACΔpsma and LACΔpsmαβhld mutants (Supplemental data).

**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 non-infected cells at all concentrations used during indicated periods (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 (17). Briefly, total RNA was isolated with a RNA II kit (Macherey Nagel), cDNA was synthesized using a qScript cDNA Synthesis kit (Quanta Biosciences). Reactions devoid of reverse
transcriptase and reactions containing H₂O 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 (XM_002697870.3; XM_001790359.3; XM_002697939.2; 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).

After normalization relative quantification 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 at the indicated period. When the expression was decreased compared to mock cells, data were presented as a negative value.

**High performance liquid chromatography/mass spectrometry**

To analyse 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 (23).

**Udder samples from *E. coli* or *S. aureus*-infected cows**

Samples from infected animals were obtained from previous experiments conducted as described (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, fifteen healthy German Holstein Friesian heifers in mid-lactation were inoculated in one quarter with 500 CFU *E. coli* strain 1303 (n = 5) or with 10,000 CFU *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 >10^6/ml and repeated recovery of *S. aureus* in milk of the inoculated quarters. Animals were slaughtered 24 h p.i. (*E. coli*) or 72 h p.i. (*S. aureus*). RNA was isolated from 100 mg of udder tissue using Trizol (Invitrogen) (21)(Roussel P, 2015). 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 PSMα3**

Bovine PS cells (15x10^3/well) were grown to 80% confluence in 96-well tissue culture plates overnight. The cells then were exposed to PSMα3 for 8 h, 24 h and 48 h in DMEM without FCS, as described above. PSM concentrations ranged from 0.01 to 2 µg/ml. The cytotoxic activity of PSM was estimated by release of cytoplasmic lactate dehydrogenase (LDH) (Pierce LDH Cytotoxicity Assay Kit, USA), as described (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 medium at A_{492 nm} (Xenius, Safas, Monaco) alone was used as background control. Cytotoxicity levels were calculated as follows: LDH release of treated cells (%) = \frac{(LDH \text{ PSM treated cells} - LDH \text{ Control untreated cells})}{(Maximum \text{ LDH release} - LDH \text{ Control untreated cells})} \times 100.

An exposure to synthetic PSMα3 for 8 h and 24 h in a range of concentrations from 0.01 to 2 µg/ml and for 48 h in a range of concentrations from 0.01 to 0.5 µg/ml did
not reveal a cytotoxic effect, while 10% of cytotoxic activity was observed after 48 h of incubation with 2 µg/ml of PSMα3 (data not shown).

**Exposure of PS cells to synthetic PSMα3**

Bovine PS cells were exposed to synthetic PSMα3 ranging from 0.01 to 2 µg/ml for 8 h, 24 h or 48 h. Interleukins 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 < 0.05 were considered to be significant. Tukey’s Honestly Significant Difference test was applied for comparison of means between groups. The values are expressed as mean ± standard deviation (±SD). To analyze the tissue samples the permutation test was performed using the StatXact software. P-values < 0.05 were considered to be 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 expression 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 the experiments with *E. coli*-infected cells. Results with MOI 30:1, the highest *E. coli* concentration without a
cytotoxic effect, are presented in Figure 1, while Table 1 (Supplementary data) 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 exposure compared to mock cells; an increase of IL-32 expression in *E. coli*-infected PS cells was observed after 24 h and after 48 h 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 mRNA expression of IL-6 and IL-8 was increased in both type 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 1 (Supplementary data).

**Downregulation of IL-32 expression and upregulation of IL-6 and IL-8 expression in *S. aureus*-infected cells.**

Since the responsiveness of PS 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 80:1 (the highest *S. aureus* concentration without a cytotoxic effect towards host cells) are presented in Fig. 2, while Table 2 (Supplementary data) shows results obtained with the 3 different MOIs.
There was no alteration in IL-32 expression at 2 h, 4 h or 6 h post-infection; hence the expression of IL-32 was investigated from 8 h to 48 h post-infection. As shown in Fig. 2, there were slight decreases in IL-32 expression after 8 h or 24-h exposure of PS cells to either S. aureus strain (Fig. 2). The decrease in IL-32 expression was stronger after 48-h 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 level of expression of IL-6 and IL-8 was analysed at 8 h, 24 h and 48 h post infection. 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 non-infected cells at 24 h or 48 h post-infection (Fig. 2). The level of IL-8 expression was approximately 4-fold higher in cells infected for 8 h compared to non-infected 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 (Table 2, Supplementary data).

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 udder of experimentally-infected cows, E. coli- or S. aureus-infected udder samples, prepared as described previously (21) were analysed by RT-qPCR. 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).
The bovine mastitis isolates have low-level expression of PSMα peptides, but high expression of δ-toxin.

To analyse PSM production in the bovine mastitis isolates used in this study, we performed HPLC/MS analysis of culture filtrates (23) in comparison to 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 Material 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 δ-toxin in a range similar to that of strain LAC, but PSMα and PSMβ levels were considerably reduced as compared to that strain.

S. aureus PSM deletion mutants induce higher interleukin expression compared to the wild-type strain

First, interleukin expression levels exposed to various strains were compared to those of mock cells, then the differences between those of cell exposed to the LAC wt strain and deletion mutants were evaluated. Expression levels of II-32, II-6 and II-8 in cells exposed either to LAC wt or 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 post infection while its isogenic mutants LACΔpsmα and LACΔpsmαβhld increased expression levels compared to mock cells (Fig. 5). To assess the impact of PSMs on interleukins expression, we compared interleukin expressions in LAC wt-treated cells to those in cells stimulated by the LACΔpsmα or LACΔpsmαβhld cells. ANOVA analysis followed by Tukey’s HSD test.
showed that there was a significant difference between IL-32 expressions in cells when stimulated either by the LACΔpsmA mutant or LACΔpsmAβhld as compared to the LAC wt at 24 h and 48 h post infection (Fig. 5.) Additionally there were statistically significant differences between IL-6 and IL-8 expression levels in cells exposed either to LAC wt or deletion mutants: IL-6 expression was twice as great in cells exposed to LACΔpsmA or LACΔpsmAβhld mutants compared to LAC wt 24h post-infection and 3-fold higher at 48h post-infection. IL-8 was increased 2.8 fold in LACΔpsmA-treated cells and 4-fold in LACΔpsmAβhld-treated cells compared to LAC wt-treated cells. Similar results were obtained with other MOIs (Table 3, Supplementary data).

To confirm the findings we compared interleukin expression levels induced by a wild type LAC (WT) pTXΔ16 strain harboring the control plasmid pTXΔ16 to those of corresponding deletion mutants as well as genetically complemented strains, which are described in Material 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: IL-32 expression level was down- while IL-6 and IL-8 expressions levels was up-regulated (Fig. 6). Interleukins expression levels in cells exposed to a wild type LAC (WT) pTXΔ16 strain was, similar to those induced by LAC wt (USA 300).

Exposure to psmα deletion mutant LAC ΔpsmA pTXΔ16 resulted in increased expression of all tested interleukins, similar to the increase of expression induced by the deletion mutant LAC ΔpsmA, while exposure to the complemented strain LAC ΔpsmA pTXΔα1-4 was resulted in a decrease of expression levels. Comparison of expression levels in cells exposed to LAC (WT) pTXΔ16, to corresponding deletion and complemented mutants showed that interleukin expression levels were significantly higher in cells exposed to complemented strain LAC ΔpsmA pTXΔα1-4.
compared to those in cells infected with LAC (WT) pTXΔ16 strain, but lower
compared to those from cells infected with a deletion mutant LAC Δpsma pTXΔ16.
To examine the role of other PSMs, interleukin expression levels in cells exposed to
LAC (WT) pTXΔ16 were compared to expression levels in cells exposed to the PSM-
deficient deletion mutant LAC Δpsmaβhld pTXΔ16 and to the complemented mutants
LAC Δ psmaβhld pTXΔ1α-4, LAC Δ psmaβhld pTXΔ1β1-2 and LAC Δ psmaβhld
pTXΔhld. As shown in Fig. 6, exposure to deletion mutant LAC Δpsmaβhld pTXΔ16
increased interleukin expression as compared to the wild type LAC (WT) pTXΔ16
strain expressing PSMs. Exposure to the complemented strains LAC Δ psmaβhld
pTXΔ1α-4 and LAC Δ psmaβhld pTXΔ1hld resulted in lower interleukin expression as
compared to the PSM-deficient LAC Δpsmaβhld pTXΔ16 strain, while no significant
difference was observed when complementing the psmβ locus (with plasmid
pTXΔpsmβ1-2) (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 PSMα3, 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 µg/ml of PSMα3.

Discussion
Mammary epithelial cells (MEC) represent the first line of contact with micro-
organisms and MEC are crucial for the early defense against intra-mammary
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 (31-33). Using live *E. coli* and *S. aureus* bacteria we set up *in-vitro* infection models that assess the effect 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 (32).

The role of the newly discovered pro-inflammatory IL-32 during inflammation and in several types of infections is well documented (34,35). However, to our knowledge there is no information about the involvement of IL-32 during the exposure to live *S. aureus* and there is 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*- as compared to *E. coli*-infected cells. This is important new information contributing to our understanding of the molecular mechanisms that underlay 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 (36-38). In contrast, expression of IL-32 by *S. aureus*-infected cells was decreased. Taking into account that IL-32α plays an intracellular mediatory role in IL-6 production in pro-monocytic cells (35) and considering the capacity of human IL-32γ to induce the maturation of dendritic cells...
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 (40). Several factors contribute to the pathogenicity of S. aureus including well-established virulence determinants accessory gene regulator and alpha-hemolysin (Hla) (41,42). The immunomodulatory actions of staphylococcal enterotoxins family are also reported (43,44). Additionally, PSMs have recently emerged as a novel toxin family that contributes to increased virulence and the spread of S. aureus infection (12,45). PSMs are divided into two groups, depending on their size: the short (20–25 amino acids) α-type peptides (PSMα1–PSMα4 and δ-toxin) and the long (44 amino acids) β-type peptides (PSMβ1 and PSMβ2) (13).

Prompted by those results, we here examined the role of PSMs in S. aureus-induced interleukin expression. Comparison of clinically related Lac wt strain to PSM deletion mutants allows to correlate the findings with those obtained by using of RF122 and NB305 strains isolated from cows with mastitis. The infection of cells with PSM deletion mutants resulted in an increase of IL-6, IL-8 and IL-32 expression, in contrast to the LAC wt (Fig. 5). Utilisation of wild type, deletion and complemented strains, harbouring plasmid let to comprehend the impact of different PSMs. Exposure of cells to a strain expressing the four PSMα peptides significantly decreased interleukin expression compared to a PSMα deletion mutant (Fig. 6), suggesting that PSMα expression by internalized S. aureus inhibits interleukin expression. Since the LAC∆psmαβhld mutant induced a higher level of interleukin
expression than the LACΔpsmα mutant we examined whether PSMβ peptides or, more likely, the highly-expressed δ-toxin may be involved in the inhibition of interleukin expression in addition to the PSMα peptides. Results from strains specifically expressing only the δ-toxin or the PSMβ peptides showed that also the δ-toxin has a significant effect, while no decrease of interleukin expression was observed with the PSMβ-expressing strain, a result that may be explained either by the different structure of the considerably longer PSMβ 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 (12,46). 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-1β 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 PSMα at 2 µg/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-α in contrast to the absence of IL-8 secretion in PSM-stimulated cell (47). Interleukin inhibition by internalized bacteria, observed in our experiments, are in accordance with data showing the inhibition of IL-6, IL-12 and TNF-α expression in dendritic cells by PSMα and data demonstrating the inhibition of TNF-α secretion by the USA300 wild-type strain harboring PSMs in contrast to the mutant strain deficient in all PSMs (18) and show that this effect extends to the "frontline" 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-α and IL-1β were secreted by the primary cultured rat epithelia infected with S. aureus (48). Human corneal epithelial cells react to S. aureus infection by enhancing the secretion of IL-6 (49).

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 the human medicine: it would be appropriate to determine the level of IL-32 in patients with mastitis.
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Figure legends.

Figure 1 Upregulation of IL-32, IL-6 and IL-8 expression in *E. coli*-infected cells.

BME-UV or PS cells (2x10^5) were grown in six well plates for 24 hours. The cells were then exposed to the *E. coli* K12 strain (MOI 30:1) for 8h, 24h and 48h. Isolation of total RNA, synthesis of cDNA and RT-qPCR were performed as described in Materials and Methods. After normalization using PPIA and RPL19 genes, interleukin expression at 8h, 24h and 48h PI was calculated relative to the values obtained from mock cells arbitrarily set to 1 at the indicated period. Data were calculated from three different experiments performed in triplicate. P-values < 0.05 (*), < 0.01 (**), were considered to be significant.

Figure 2 Analysis of IL-32, IL-6 and IL-8 expression in *S. aureus*-infected cells.

PS cells (2x10^5) were grown for 24 hours. The cells were then exposed to *S. aureus* NB305 or RF122 strains (MOI 80:1) for 8h, 24h and 48h. Isolation of total RNA, synthesis of cDNA and RT-qPCR were performed as described in Materials and Methods. After normalization using PPIA and RPL19 genes, interleukin
expression at 8h, 24h and 48h PI was calculated relative to the values obtained from mock cells arbitrarily set to 1 at the indicated period. When the expression was decreased compared to unstimulated mock cells, data were presented as a negative value. Data were calculated from three different experiments performed in triplicate. P-values < 0.05 (*), < 0.01 (**), were considered to be significant.

**Figure 3 Increased IL-32 expression during experimentally *E. coli*-induced mastitis.**

Cows were infected either with *E. coli* or *S. aureus* as described in Material and Methods. IL-32 specific RT-qPCR were performed with RNA extracted from tissue samples of udder quarters from uninfected cows or bacteria-infected cows. Data presented are values from five animals, statistical significance (P < 0.05) of *E. coli*-infected versus uninfected cows was observed using exact permutation tests after global comparison using a Kruskal and Wallis test.

**Figure 4 PSM production in bovine mastitis isolates.**

PSMs were analyzed in culture filtrates of cultures inoculated form pre-cultures and grown for 8 h in tryptic soy broth. HPLC/MS detection was performed essentially as described (23)(Joo & Otto Methods Mol Biol 2014). 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 deviation.
Figure 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 (2x10^5) were grown for 24 hours. The cells were then exposed to LAC wt, to the deletion mutants LACΔpsmα and LACΔpsmαβhld at MOI 80:1 for 8h, 24h and 48h.

An mRNA expression was measured in total RNA preparation by RT-qPCR. After normalization using PPIA and RPL19 genes, interleukin expression was calculated relative to the values obtained from mock cells arbitrarily set to 1 at the indicated period. According to ANOVA with post-hoc Tukey’s HSD test there was 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 interleukins expression levels in cells exposed to wild type LAC wt and corresponding deletion mutants as indicated with asterisks. Data were calculated from three different experiments performed in triplicate. P-values < 0.05 (*), < 0.01 (**), were considered to be significant.

Figure 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 (2x10^5) were grown for 24 hours. The cells were then exposed to LAC (WT) pTX16, to the deletion PSMα-deficient mutant LAC Δpsmα pTX16 and complemented LAC Δpsmα pTXα1-4 strain, as well as to the deletion PSM-deficient mutant LAC Δpsmαβhld pTX16 and complemented strains LAC Δpsmαβhld pTXα1-4, LAC Δpsmαβhld pTXβ1-2 and LAC Δpsmαβhld pTXηhld at MOI 80:1.
for 24 h. An mRNA expression was measured in total RNA preparation by RT-qPCR. After normalization using PPIA and RPL19 genes, interleukin expression was calculated relative to the values obtained from mock cells arbitrarily set to 1 at the indicated period.

According to ANOVA with post-hoc Tukey’s HSD test there was statistically significant difference between interleukin expression levels in cells exposed either to LAC (WT) pTX₁₁₆, or to 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) pTX₁₁₆ or complemented strains. Statistically significant differences between interleukins 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 < 0.05 (*), < 0.01 (**), were considered to be significant.

Figure 7 Analysis of the expression of IL-32, IL-6, and IL-8 in cells treated with synthetic PSM peptides

PS cells (2x10⁵) were grown for 24 hours, then cells were exposed to synthetic PSMα₃ ranging from 0.01 to 2 µg/ml for 8 h, 24 h or 48 h. An mRNA expression was measured in total RNA preparation by RT-qPCR. After normalization using PPIA and RPL19 genes, interleukin expression was calculated relative to the values obtained from mock cells arbitrarily set to 1 at the indicated period. According to ANOVA with post-hoc Tukey’s HSD test there were statistically significant differences between interleukin expressions levels in cells treated with 2 µg/ml of PSMα₃ for 8 h compared to those in mock cells. Data were calculated from three different
experiments performed in triplicate. P-values < 0.05 (*) were considered to be significant.

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Figure 1

Fold change of transcript level

E. coli 8h PI
E. coli 24h PI
E. coli 48h PI

BME-JV

PS
Figure 3
Fold change of transcript level

Figure 5
Figure 7