Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens

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ABSTRACT: Different mastitis pathogens induce different courses of infection, i.e. more or less severe. Mammary epithelial cells play an important role in the initial combat against microorganisms by expression of cytokines and acute phase proteins that regulate the immune response. The objective of the present study was to investigate the involvement of the epithelial cells into the outcome of mastitis induced by different pathogens. Primary epithelial cell cultures isolated from milk were used to test the immune response by measuring the mRNA expression of immunomodulators and their influence on polymorph nuclear chemotaxis. Because the cells showed different responses to isolated bacterial endotoxins (lipopolysaccharide, lipoteichoic acid, and peptidoglycans) compared to whole bacteria, they were treated with heat inactivated (10 MOI) gram-negative *Escherichia coli*, a very common pathogen causing acute intra-mammary infections, with *Staphylococcus aureus*, a prevalent cause of chronic, and, *Streptococcus uberis,* an inducer of acute and chronic mastitis. *E. coli* induced an increased mRNA expression of interleukin (IL)-8 within a 1 h treatment. A treatment for 6 h with *E. coli* and *S. aureus* induced increased mRNA expression of IL-6, IL-8, TNF-α and serum amyloid A (SAA). After a 24 h treatment the expression of these immunomodulators was still elevated, except in the *E. coli* treatment the SAA expression showed no differences to control cells anymore. Interestingly, *Str. uberis* in the same concentration did only induce the expression of IL-8 after a 6 h treatment but had no influence on other immunomodulator mRNA expression. Cell culture supernatants of *E. coli* and *S. aureus* treated cells for 12 h increased leukocyte chemotaxis in a 96-well MultiScreen[TM]MIC-plate. *S. aureus* seemed to induce increased chemotaxis after shorter treatments than *E. coli*. In conclusion, mammary epithelial cells are involved in the different immune response to various mastitis pathogens, and the induction of chemotaxis of leukocytes from blood to milk during mastitis. Therefore, most likely epithelial cells play a role in the differential pattern of immunomediators stimulated by different pathogens.

Keywords: infection; mammary gland; *Escherichia coli*; *Staphylococcus aureus*; *Streptococcus uberis*

The innate immune response of the mammary gland plays a pivotal role in the development of mastitis, induced by invading pathogens. Besides polymorphonuclear blood cells (PMN) entering the mammary gland (Burton and Erskine, 2003), mammary epithelial cells are involved in the initial combat against microorganisms by expression of cytokines and acute phase proteins that regulate the immune response (Wellnitz and Kerr, 2004). The course of mastitis is different depending on the cow (Detilleux, 2002; Burvenich et al., 2003), the lactational stage (Burton and Erskine, 2003), other environmental influences, and especially the pathogens involved (Zecconi et al., 2005). The gram-negative bacterium *Escherichia coli* is a very common pathogen causing mainly acute and severe intramammary infections. In contrast, the gram-positive *Staphylococcus aureus* is a prevalent cause of chronic and subclinical, and *Streptococcus uberis* of both, acute and severe, and chronic and sub-

clinical mastitis. The mammary gland secretes immune modulators if it is stimulated with endotoxins from mastitis pathogens (Schmitz et al., 2004), or infected with different pathogens like *E. coli* or *S. aureus* (Riollet et al., 2000). These immune modulators produced by already stimulated leukocytes in the mammary gland, and, additionally, by the mammary epithelial cells, are responsible for the recruitment of leukocytes from the blood. The leukocytes, mostly PMN, leave the blood and migrate into the tissue in the direction of increasing concentrations of specific chemoattractants. The recruitment of leukocytes into the mammary gland is crucial to eliminate invaded bacteria by phagocytosis. Depending on the progression of neutrophil diapedesis the mastitis becomes more or less severe. In *S. aureus* infection the increase of somatic cell count (SCC) is delayed compared to *E. coli* infection (Bannerman et al., 2004).

A detailed understanding of pathogen specific mastitis could be an important tool to be able to treat or prevent particularly the long lasting and often not recognized chronic infections. Here we describe a combined model, where the direct influence of different common mastitis pathogens on the expression of immunomodulators of epithelial cells, and, additionally, the direct involvement of the infected epithelial cells in leukocyte recruitment is investigated. An *in vitro* model, where primary mammary epithelial cell cultures isolated from the milk of dairy cows was used. The cells were treated with mastitis isolated and heat inactivated *E. coli, S. aureus* and *Str. uberis* bacteria. In addition, their secretions (i.e. cell culture supernatants) were tested for the influence on chemotactic activity of PMN.

MATERIAL AND METHODS

Primary mammary epithelial cell culture

Isolation and culturing of epithelial cells from milk. Milk (1.2 l) from 8 cows in late lactation with no clinical signs of mastitis and free of detectable bacteria in the milk was centrifuged in 500 ml-centrifugation tubes for 10 min at 500 × g. Cell pellets were resuspended in 25 ml hanks balanced salt solution (HBSS; SIGMA) per every 400 ml milk, transferred to a 50 ml centrifugation tube, and filtered through a 100 µm pore size filter membrane. Then cells were washed twice in HBSS by centrifu-

gation for 5 min at 500 × g and resuspension. After washing, cells were seeded in 7 ml DMEM/F12 containing 10% FBS, 100 µg/ml gentamicin, and 5 µg/ml amphotericin B (SIGMA) in a 25 cm²-tissue culture flask. Medium was changed every one or two days. After one week gentamicin was reduced to 50 µg/ml, amphotericin B to 2.5 µg/ml, and FBS to 5%. When cells reached confluence after about two weeks they were split by removing the medium, washing with 2 ml PBS, and addition of 2 ml Trypsin/EDTA. 1 ml Trypsin was removed after 1 min and cells incubated for 10 to 15 min at 37°C, 90% humidity, and 5% CO_2. Then 5 ml DMEM/F12 medium containing 20% FBS for inhibition of the enzyme where added. Cells were washed in 10 ml DMEM/F12 medium by centrifugation for 5 min at 500 × g, and seeded in 7 ml medium in new 25 cm² tissue culture flasks. Before the third passage all cells were stored at −80°C in DMEM/F12, 20% FBS and 10% DMSO. All experiments were performed with cells in the fourth passage.

To verify the epithelial origin, cells were tested cytochemically for cytokeratins (clone MNF 116, DakoCytomation). All cells were stained. To exclude leukocyte contamination RT-PCR for CD45, a leukocyte specific gene, was performed on total RNA of the cells and could not be detected.

Treatment of epithelial cells with bacteria. For bacterial treatment of epithelial cells, *E. coli*, isolated from a bovine mastitis and tested with standard protocols, *S. aureus* M 60 (Bramley et al., 1989) and *Str. uberis* (ATCC) from over night cultures in the stationary growth phase were diluted 1:1000 in Luria-Bertani-(*E. coli*) or Tryptone-Soya-Broth-(*S. aureus* and *Str. uberis*) medium, respectively. Bacteria were incubated at 37°C until an optical density at 600 nm of 0.4–0.8. Then concentrations were determined by plate dilution method, and heat inactivated at 63°C for 30 min in a water bath. After heat inactivation the plating on blood agar plates tested the effectiveness of killing. The heat inactivated bacteria were kept in aliquots at −80°C until experimental use.

Epithelial cells were thawed, seeded in 12-well plates (150 000 cells/well) and grown for two days. When cells reached approximately 70% confluence (~ 5×10^5 cells) medium was changed, and thawed heat inactivated bacteria added in different concentrations for different durations. Total RNA of cells was harvested by adding 0.5 ml Trifast (Peqlab) and stored at −80°C until RNA extraction according to manufacturers protocol. For chemotaxis assays

supernatants were collected, centrifuged to remove cell debris, and stored at −20°C.

qRT-PCR. Total RNA concentrations and integrity were detected with the Agilent Bioanalyzer (Agilent Technologies). For quantitative analysis of mRNA the QuantiTect SYBR Green RT-PCR kit (Qiagen) and the real-time thermocycler Rotor-Gene 3000 (Corbet Research[TM]) were used. Reactions were performed in 100 µl reaction tubes with 5 µl 2*QuantiTect[TM]SYBR®Green RT-PCR master-mix, 0.1 µl QuantiTect RT Mix, 0.5 µl forward primer (1µM), 0.5 µl reverse primer (1µM), and 200 ng total RNA in a volume of 3.9 µl. Forward and reverse primer sequences were: CCA CGT TGT AGC CGA CAT C and CCC TGA AGA GGA CCT GTG AG for TNF-α, CCT GGG CTG CTA AAG TGA TC and TAC TTG TCA GGC AGG CCA G for SAA, GCT GAA TCT TCC AAA AAT GGA GG and GCT TCA GGA TCT GGA TCA GTG for IL-6, ATG ACT TCC AAG CTG GCT GTT G and TTG ATA AAT TTG GGG TGG AAA G for IL-8, and, GTC TTC ACT ACC ATG GAG AAG G and TCA TGG ATG ACC TTG GCC AG for GAPDH mRNA detection. The following five-step program was used: reverse transcription for 20 min at 50°C, denaturation for 15 min at 95°C, the 35 cycles of amplification and quantification (15 s at 94°C, the primer-specific annealing temperature for 30 s, and extension at 72°C for 20 s), ending with a melting curve program (60–99°C with a heating rate of 0.1°C/s with continuous measurement) and cooling down to 40°C.

Crossing Point (CP) values were acquired by "competitive quantification" of the rotor-gene software 5.0 (Build 60). CP of the target gene was normalized to the housekeeping gene GAPDH (ΔCP).

Chemotaxis of leukocytes

Isolation of PMN from peripheral blood. Bovine PMN were isolated from 40 ml EDTA-stabilized blood as described by Carlson and Kaneko (1973) with slight modifications. 40 ml were divided into 10 ml fractions in 15 ml tubes, and centrifuged at 1 000 × g for 10 min at room temperature. Plasma, buffy coat, and the upper phase of the blood cell layer where aspirated and discarded. PMN where isolated by hypotonic lysis of erythrocytes with 20 ml deionized water. Then 10 ml of PBS with 2.7% NaCl where added to restore isotonicity. The cell suspension was centrifuged at 200 × g for 10 min

at room temperature. Cells where washed twice in 35 ml PBS with 10% glucose (200 × g, 10 min, room temperature) and resuspended in 4 ml PBS containing glucose. Cell concentration was determined using a Neubauer Chamber, and was adjusted to 10^7 cells/ml in DMEM/F12 containing 5% FBS, 100 µg/ml gentamicin, and 2.5 µg/ml amphotericin B.

Chemotaxis assay. PMN were assayed in a 96-well MultiScreen[TM]MIC-plate, equipped with 5.0 µm pore-sized hydrophilic polycarbonate filters (Millipore GmbH). 150 µl of cell culture supernatants were placed in the lower chambers. A 75 µl PMN suspension, containing $2 × 10^7$ cells/ml was then placed in the upper chambers. After 2 h incubation at 37°C, 90% humidity, and 5% CO_2, cells that had migrated into the lower chambers were fixed with 1% paraformaldehyde and counted with a fluorescence activated cell sorter (FACS, forward scatter/side scatter analysis) equipped with a BD Multiwell[TM] AutoSampler (BD Biosciences).

Statistical Analysis

Results are presented as means ± SEM. Quantitative RT-PCR results are represented as ΔCP values (CP of target mRNA minus CP of the house keeping gene GAPDH). All the statistical analyses, means, and standard errors of the mean were computed with SigmaStat 3.0 (SPSS Inc.). To determine the statistical significance of differences between the control and treatment groups a paired Student's *t*-test was used. For the chemotaxis assay, results of treatments were corrected by results of bacterial controls (heat inactivated bacteria in medium without epithelial cells). Statistical significance was determined at *P*-values of < 0.05.

RESULTS

Immune relevant gene expression of epithelial cells

Cytokine mRNA expression of mammary epithelial cells of one cow after the treatment of different concentrations of LTA (lipoteichoic acid) and/or PGN (peptidoglycans) are shown in Figure 1. Statistical analysis was not performed, because results were received from only two wells each of cells from one cow. The results show different responses to different
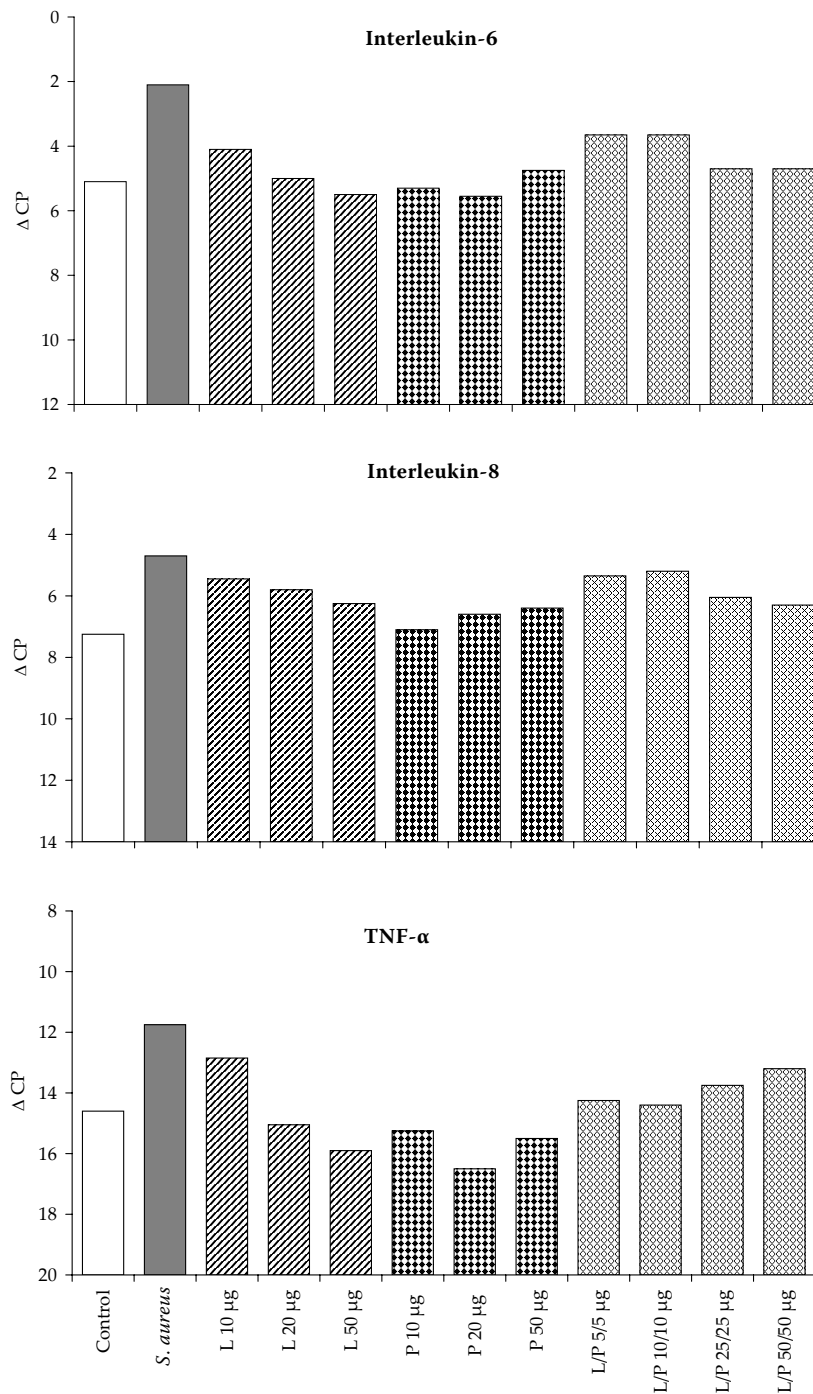
Figure 1. Il-6, Il-8, and TNF-α mRNA expression (ΔCP) of mammary epithelial cells from one cow treated with or without heat inactivated S. *aureus* (10 MOI), or with 10, 20, or 50 μg LTA (L) and/or 10, 20, or 50 μg PGN (P) for 6 hours. The results are presented as means of duplicates

concentrations of LTA and PGN compared to *S. aureus* treatment. Therefore, further experiments were performed with heat inactivated bacteria. This also provided a better comparison of infection pressures between the different bacteria.

Different concentrations (0.5, 2.5, 5, 10, and 50 MOI, respectively) of inactivated *E. coli* and *S. aureus* in the media of epithelial cells for 3 h did not increase cytokine mRNA expression of TNF-α and IL-6 with increasing concentrations (Table 1).

Table 1. mRNA expression (ΔCP) of TNF-α of mammary epithelial cells from four cows after treatment with different MOI of heat killed *E. coli* or *S. aureus* for 3 hours

Gene	Treatment	Control	0.5 MOI	2.5 MOI	5 MOI	10 MOI	50 MOI
TNF-α	*E. coli*	28.8 ± 1.4	24.5 ± 3.6	27.1 ± 1.0	22.5 ± 2.4	21.9 ± 5.1	25.9 ± 5.8
	S. aureus	26.8 ± 2.4	27.1 ± 3.1	28.6 ± 3.8	27.0 ± 2.2	29.7 ± 2.8	26.4 ± 2.6
IL-6	*E. coli*	24.9 ± 5.8	17.5 ± 1.3	12.2 ± 0.4	19.6 ± 6.9	16.3 ± 3.2	15.4 ± 3.6
	S. aureus	21.8 ± 5.1	20.6 ± 6.3	16.1 ± 2.5	18.5 ± 3.5	19.0 ± 6.8	13.0 ± 0.4

Therefore, further experiments were performed with concentrations of 10 MOI of inactivated *E. coli, S. aureus,* and *Str. uberis.*

Results of epithelial cell treatments with 10 MOI of *E. coli, S. aureus,* or *Str. uberis* are shown in Figure 2.

IL-8 mRNA expression of mammary epithelial cells was significantly increased after a 1 h *E. coli* treatment, and after 6 h in all treatments (*E. coli, S. aureus*, or *Str. uberis*, respectively). IL-6 and TNF-α mRNA expression did not change within 1 h of all treatments, and was elevated after a 6 and 24 h treatment with *E. coli* and *S. aureus*. SAA mRNA expression was not different after a 1 h treatment with all three pathogens, but was increased after 6 h *E. coli* and *S. aureus* treatment. After 24 h the mRNA expression of SAA was only elevated in *S. aureus* treatment.

Chemotaxis

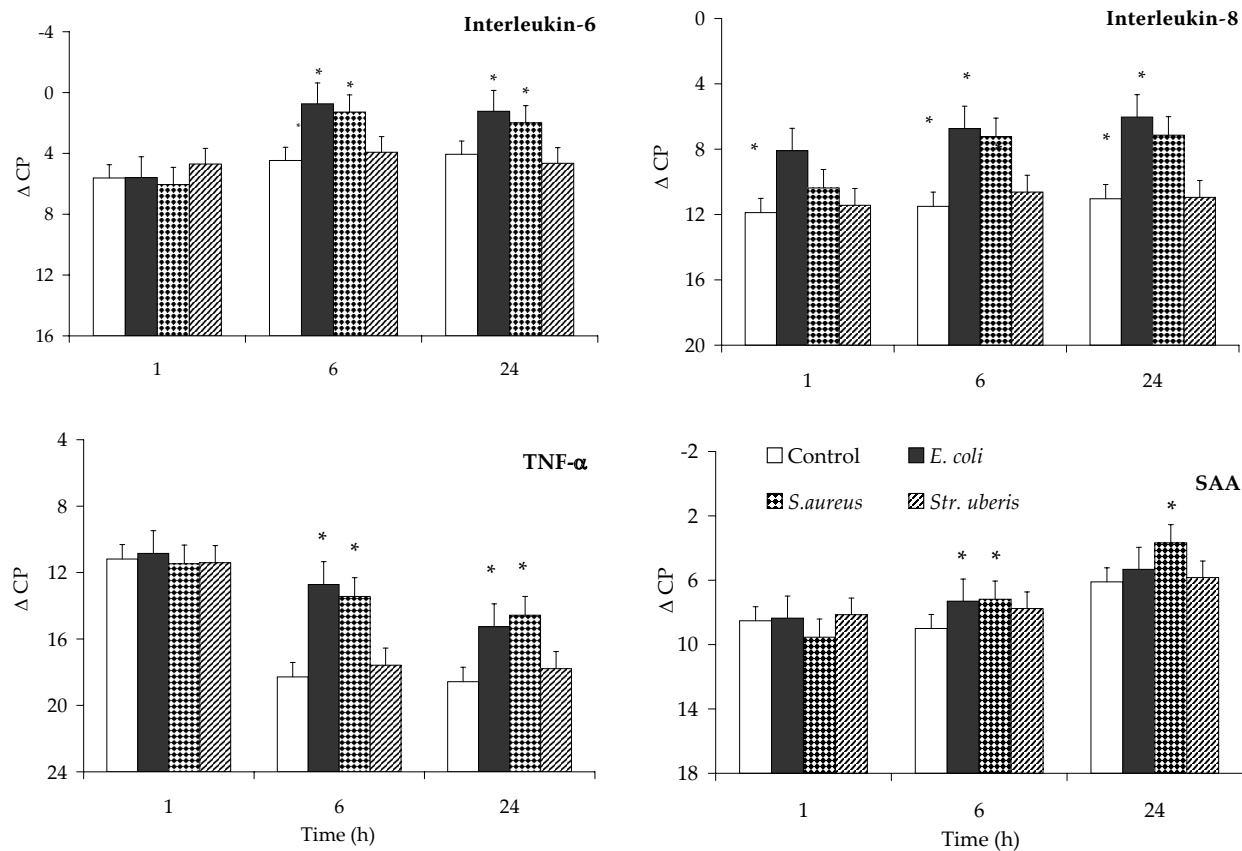The treatment of mammary epithelial cells with heat inactivated bacteria induced the chemotaxis

Figure 2. Il-6, Il-8, TNF-α, and SAA mRNA expression (ΔCP) of mammary epithelial cells from six cows treated with or without heat inactivated *E. coli, S. aureus,* or *Str. uberis* with an infection pressure of 10 MOI for 1, 6, or 24 h, respectively (*$P < 0.05$)
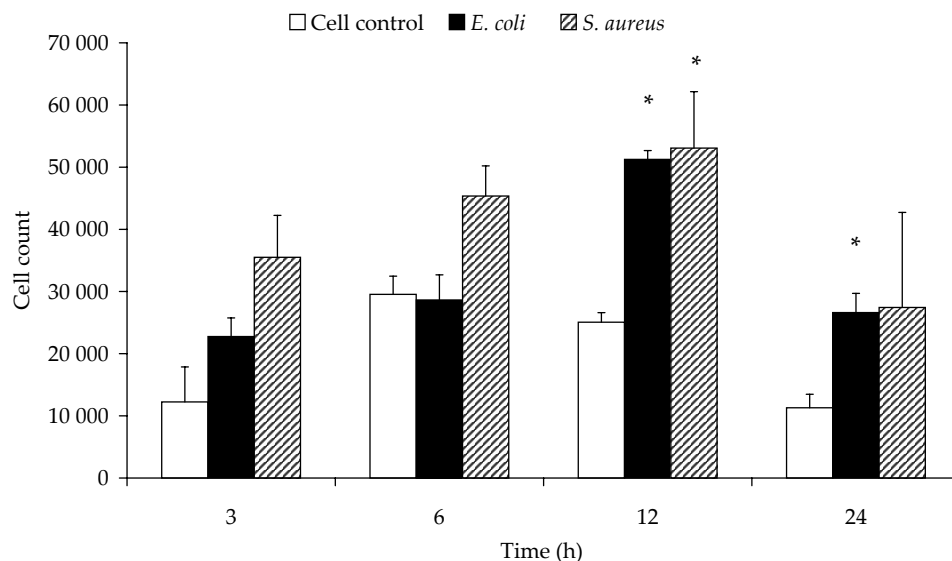
Figure 3. Chemotaxis assay: migrated leukocytes towards cell culture supernatants of mammary epithelial cells of four cows treated with or without 10 MOI heat inactivated *E. coli* or *S. aureus* for 3, 6, 12, 24 h, respectively. Results of treatment groups are corrected for controls of bacteria placed in cell culture media without epithelial cells (*$P < 0.05$)

of leukocytes towards the cell culture supernatants. The number of migrated cells increased significantly after an incubation time of 12 h (see Figure 3). The chemotactic effect seemed to be induced earlier, i.e. after 3, and 6 h, in *S. aureus* treatment ($P < 0.1$).

DISCUSSION

Mammary epithelial cells play an important role in the immune defense against invading pathogens to prevent the gland from severe inflammations. Besides activated leukocytes in the mammary gland (Prgomet et al., 2005) the epithelial cells produce several immunomodulators to initiate the immune response (Pareek et al., 2005). Primary mammary epithelial cell cultures from different cows were shown to be a useful method to study the immune response to different stimuli (Wellnitz and Kerr, 2004). Here we used primary cell cultures of epithelial cells isolated from milk. Cells from different cows were isolated and cultured separately, yet simultaneously. The culture of cells from different cows at the same time provides the biological replication.

We investigated their immune response to the mastitis pathogens *E. coli, S. aureus,* and *Str. uberis* that induce mainly different courses of inflammations, i.e. acute, chronic, or both types, respectively.

Surprisingly, an infection pressure of 0.5 to 50 MOI *E. coli* or *S. aureus* did not significantly increase TNF-α and IL-6 mRNA expression with increasing concentrations. Therefore, an infection pressure of 10 MOI was chosen for all experiments. This is a rather high dose compared to *in vivo* studies, were clinical mastitis could be induced with ~ 72–74 CFU bacteria in one quarter (Bannerman et al., 2004). However, due to the heat inactivation bacteria could not reproduce anymore, and it is difficult to compare this infection pressure to *in vivo* situations.

Heat inactivated bacteria were used for all the experiments, although the pathogenic components of the bacteria are known and commercially available in isolated forms. The major pathogenic component of gram-negative *E. coli* is the cell wall component lipopolysaccharide (LPS) and it is well described for use in experiments to mimic gram-negative infections. On the other hand, gram-positive bacteria have at least two different pathogenic components: lipoteichoic acid (LTA) and peptidoglycans (PGN). A preliminary experiment with different concentrations of LTA, PGN, and LTA plus PGN showed lower responses in cytokine expression compared to treatments with heat killed *S. aureus*. Therefore, using the entire bacteria seemed to be more useful to compare infection pressures between different pathogens, i.e. gram-negative and gram-positive bacteria. The inactivation of the bacteria was nec-

essary to provide a defined comparable infection pressure and to avoid an overgrowth and depletion of nutrients and to ensure the continuousness of bacteria concentrations throughout the entire experiments, where no change of the cell media was necessary. A degradation of immune important proteins due to the force of heat is possible. But, initial experiments with inactivated bacteria using a French press showed similar responses (data not shown), but the contamination of survived bacteria that replicated in the media during treatments posed difficulties.

After an incubation of the epithelial cells with heat inactivated bacteria for one hour the IL-8 mRNA expression in the *E. coli* treatment was significantly increased. This faster response of IL-8 mRNA expression to *E. coli* agrees with results from *in vivo* experiments, were IL-8 increases in milk in *E. coli* infections within 4 to 24 h p.i. (Riollet et al., 2000; Persson Waller et al., 2003) and not in *S. aureus* infections (Bannerman et al., 2004). Therefore, the origin of milk IL-8 is most likely not only the leukocytes, but also the epithelial cells. Because IL-8 has high chemotactic impacts on PMN (Barber and Yang, 1998) this may also explain a very rapid increase in somatic cell count in milk during *E. coli* infection compared to a slower but longer increase in *S. aureus* infections (De Haas et al., 2004). 6 h treatments of epithelial cells with *E. coli* and *S. aureus* increased IL-6, IL-8, TNF-α and SAA mRNA expression. It was still elevated after a 24 h treatment except for SAA mRNA expression in *E. coli* treatment. Most interestingly, the treatment with *Str. uberis* in the same concentrations as *E. coli* and *S. aureus* did not increase the mRNA expression of measured cytokines and chemokines. Merely, IL-8 mRNA expression was increased after a 6 h treatment. *In vivo* experiments of Rambeaud et al. (2003) did not show increased IL-8 and TNF-α levels in whey after an experimental infection with ~ 6–10 × 10^3 CFU *Str. uberis*/gland before 66 h p.i. If the epithelial cells are involved the treatment time of 24 h was maybe not sufficient to show measurable responses.

In addition to the measurements of cytokine mRNA expression, the involvement of the mammary epithelial cells in the chemotactic activity, and, therefore, recruitment of leukocytes into the mammary gland was tested. These migrating blood cells, especially the neutrophils, are the major contributors of the immune defense of the mammary gland. Their migration is enhanced by inflammatory mediators that are produced in the infected

tissue by immune cells and also by epithelial cells responding to bacterial toxins or metabolites. The supernatants of heat inactivated bacteria treated cells in chemotaxis chambers showed that epithelial cells produced chemoattractants that enhanced the chemotactic activity of PMN compared to the effects of the bacteria alone. After 12 h of treatment the chemotaxis was significantly increased by cell influences. This time frame is not surprising, because chemokines accumulate in the supernatants with time. No significant differences between *E. coli* and *S. aureus* treatments could be detected with this method. Although, it seems ($P < 0.1$) that *S. aureus* increased chemotaxis after a shorter incubation time than *E. coli*. Milk from cows with *E. coli* mastitis contains more cells than milk from *S. aureus* infected glands (Djabri et al., 2002). And the peak of SCC is reached earlier p.i. in *E. coli* than in *S. aureus* infections (Bannerman et al., 2004). Our results suggest that the epithelial cells can only play a tangential role in this effect and that this is maybe due to chemokine production of leukocytes. However, chemokines accumulate in the supernatant with time if they are not degraded. Therefore, it is possible that short-term strong stimulations of chemotactic effects are not recognized with this method. For technical reasons the influence of *Str. uberis* was not tested. For the chemotactic effect of cell culture supernatants chemokines like IL-8, which was also shown to be expressed on the protein level by mammary epithelial cells in culture (Wellnitz and Kerr, 2004), SAA (Badolato et al., 1994), and others like RANTES (Pareek et al., 2005) are most likely responsible. The induction of gene expression of cytokines is mediated by toll-like receptors (TLR). TLR-2 and –4 mRNA was shown to be expressed in mammary gland epithelial cells (Strandberg et al., 2005) and could also be detected in the epithelial cell isolated from milk (not shown).

In conclusion, mammary epithelial cells are involved in the different immune response to different mastitis pathogens. *E. coli* seems to induce an earlier immune response as IL-8 mRNA expression was already increased after a 1 h treatment with these bacteria. *Str. uberis* in the same concentrations induced only an increased IL-8 mRNA expression after a 6 h treatment. The epithelial cells are involved in the induction of chemotaxis of leukocytes from blood to milk during mastitis. However, in the chemotaxis assay there was no clear difference between *E. coli* and *S. aureus* treatment of epithelial cells measurable.

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