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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

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Abstract – The outcome of an udder infection is influenced by the pathogen species. We established a strictly defined infection model to better analyze the unknown molecular causes for these pathogen-specific effects, using *Escherichia coli* and *Staphylococcus aureus* strains previously asseverated from field cases of mastitis. Inoculation of quarters with 500 CFU of *E. coli* ($n = 4$) was performed 6 h, 12 h, and 24 h before culling. All animals showed signs of acute clinical mastitis 12 h after challenge: increased somatic cell count (SCC), decreased milk yield, leukopenia, fever, and udder swelling. Animals inoculated with 10 000 CFU of *S. aureus* for 24 h ($n = 4$) showed no or only modest clinical signs of mastitis. However, *S. aureus* caused clinical signs in animals, inoculated for 72 h–84 h. Real-time PCR proved that *E. coli* inoculation strongly and significantly upregulated the expression of β-defensins, TLR2 and TLR4 in the pathogen inoculated udder quarters as well as in mammary lymph nodes. TLR3 and TLR6 were not significantly regulated by the infections. Immuno-histochemistry identified mammary epithelial cells as sites for the upregulated TLR2 and β-defensin expression. *S. aureus*, in contrast, did not significantly regulate the expression of any of these genes during the first 24 h after pathogen inoculation. Only 84 h after inoculation, the expression of β-defensins, but not of TLRs was significantly (> 20 fold) upregulated in five out of six pathogen inoculated quarters. Using the established mastitis model, the data clearly demonstrate a pathogen-dependent difference in the time kinetics of induced pathogen receptors and defense molecules.

toll-like receptor / mastitis / beta-defensin / mammary epithelial cell

1. INTRODUCTION

Mastitis still represents one of the major diseases in dairy cattle [27] causing high economic losses. Bacteria are the main mastitis pathogens, followed in relevance by yeasts and fungi.

Two of the most important bacterial mastitis pathogens are *Staphylococcus aureus* and *Escherichia coli* [4]. The courses of the udder inflammations may vary significantly between these pathogens. Intramammary infections with *E. coli* very often result in an acute mastitis with severe clinical consequences [3]. Infected animals may die or recover from the disease within days [47]. In contrast, mastitis caused by *S. aureus* is mostly less severe but can eventually turn into a chronic infection with a lifelong persistence of the bacteria [34, 49]. Some *S. aureus* infections may also cause subclinical mastitis with no apparent clinical symptoms (e.g. swelling of the udder, milk flakes, fever) and only intermittent detection of bacteria in milk paired with elevated somatic cell counts (SCC) [33].

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A significant number of studies used experimentally infected udders to study the events after pathogen contact [23, 24, 31].

However, these studies varied considerably in the applied standards regarding the animals used, their history of mastitis, and status of SCC at the time of infection. SCC values ranged from < 20 000 [16] to < 600 000 [12] before infection. The time of the experimental infection differed between 72 h [26] and 48 days [39] for both bacterial species, sometimes with repeated infections of the animals [26]. The infection dose varied for both *S. aureus* and *E. coli* between 50 and about 10 000 colony forming units (CFU per udder quarter) [23, 55]. In addition, animals were not always prescreened for a prolonged absence of pathogenic bacteria within the udder prior to experimental infections [40]. Thus, results of the different model systems are difficult to compare.

The host responses induced early during infection may be crucial determinants for the different pathogenesis after infection with *E. coli* or *S. aureu*s. Knowledge about these responses is still rather limited [3, 33]. Proper recognition of the pathogen by the host represents quite likely a key event in the pathogenesis of infectious diseases [32].

Recognition of pathogens is mediated by cellular pattern recognition receptors recognizing pathogen-associated molecular pattern (PAMPs) such as lipopolysaccharide (LPS) from *E. coli* or lipoteichoic acid (LTA) from *S. aureus*. The most important PRR group is represented by the transmembrane toll-like receptors (TLRs), which are specialized for the recognition of distinct PAMPs (e.g. LPS by TLR4 or LTA by TLR2). Overall, a total of 13 different TLRs are described for mouse and man [22]. In cattle, currently sequences for ten TLRs are described [59]. We have previously demonstrated in udder tissues of cows with clinical mastitis, that bovine TLR2 and TLR4, but not TLR9 are selectively up-regulated in both *E. coli* and *S. aureus*-infected animals [13].

Ligand binding activates TLRs and triggers the expression of regulatory cytokines and other mediators [52] which orchestrate the subsequent immune responses as well as the modulation of recruited effector cells.

Bacterial infections elicit in the host the synthesis of bactericidal and fungicidal factors, like β-defensins. Expression of β-defensins can be induced by pathogens [10, 11] and it has been shown that expression of human β-defensin-2 depends on TLR2- and TLR4-mediated nuclear factor kappa B (NF-κB)-activation [5, 57]. In cattle, 16 β-defensin genes and two pseudogenes are described [35]. Defensins are cysteine- and arginine-rich peptides (cationic amphiphilic 38–42 amino acids) which can act in an oxygen-independent way [39, 63]. Especially, because of low oxygen pressure in infected tissues, oxygen-independent mechanisms leading to bacteriolysis seem to be very important [30]. Defensins are synthesized predominantly in epithelial cells and neutrophilic granulocytes where they make up to 15% of total cytoplasmatic protein [6]. Previously, we demonstrated by in situ hybridization that alveolar epithelial cells in the udder are the dominant location of β-defensin-5 (BNBD5) expression in the inflamed udder [13]. We also showed that NF-κB, eventually as delivered by activated TLR-receptors, is a necessary, but not the sufficient component for β-defensin expression in the bovine MEC [61].

Molecular mechanisms underpinning the escape of subclinical mastitis causing pathogens, like *S. aureus*, from the host's immune defense are basically unknown. They might include a pathogen species related inefficiency to mount an adequate immune response in the udder, indicating an inappropriate alert of the innate immune system. The infection-associated regulation of factors contributing to the innate immune defense in the udder is not well analyzed.

It is largely unknown, how rapid the expression of bovine TLRs and bactericidal β-defensin peptides is modulated after an experimental udder infection with either *E. coli* or *S. aureus*, and if the time course and the extent of this induction is influenced in a pathogen-specific fashion.

Thus, this study had two goals. On the one hand, we wanted to establish a standardized

and quite stringent animal model for acute and subclinical mastitis, and characterize clinical parameters elicited by our model pathogens. This model should be useful for subsequent systematic experimental analyses of pathogen-specific differential activation of the immune defense in the udder. On the other hand, we wanted to compare the impact of an *E. coli*-versus *S. aureus*-caused mastitis on the time course and the extent of activation of factors contributing to the innate immune defense in the udder. Here, we focused on β-defensin and TLR expression.

2. MATERIAL AND METHODS

2.1. Animals

The trial included 14 German Holstein cows in the middle of their first lactation (aged between 26–30 months, 3 to 5 months post partum) and was conducted under the approval of the ethics committee of the regional government in Hannover, Germany (No. 509.6-42502-03/678). Animals were kept at the University of Veterinary Medicine (Hannover, Germany), and never had clinical or subclinical mastitis before. Quarter milk samples of these animals were tested weekly six times pre-trial and on the day of trial to be free from mastitis pathogens and to have less than 100 000 somatic cells/mL. Daily milk yields were between 15 and 25 L. The trial was conducted in four rounds, each including four animals (trials 1-3) or two animals (trial 4). In the first two rounds, two animals were arbitrarily assigned to become inoculated with either *E. coli* or *S. aureus*. In the third round, four animals were inoculated with *S. aureus* only.

2.2. Bacteria

Strain *Escherichia coli* (*E. coli* 1303) and *Staphylococcus aureus* (*S. aureus* 1027) were isolated from udder secretions of cows with clinical mastitis. Cultivation of bacteria, storage in glycerol stocks, as well as their biochemical characterization was performed in the Institute of Microbiology, University of Veterinary Medicine (Hannover, Germany). *E. coli* 1303 was positive for indol generation, lysine decarboxylase, methyl red, glucose, gas generation, lactose, adonite, rhamnose, oxidative, and fermentative glucose degradation. *S. aureus* 1027 was positive for mannite cleavage, clumping factor, and free coagulase. The characterization

was performed according to Burkhardt [7]. Bacteria were kept cryo conserved (Mikrobank-system Cryobank™, Mast Diagnostika, Reinfeld, Germany) for subsequent infections. Inoculum doses were generated as described by Van Oostveldt et al. [54]. Briefly, bacteria were plated on trypticase soy agar and incubated (37 °C) overnight. Several colonies were transferred to a tube of brain heart infusion broth and the tube was incubated for 6 h (37 °C). A 100 μL sample was then transferred to a tube, containing 9.9 mL trypticase soy broth. After 24 h the inoculum was prepared using serial dilutions. The desired inoculum was achieved by determination of the OD related to the amount of colony forming units.

2.3. Experimental pathogen inoculation, milk and blood sampling, tissue sampling

Cows were synchronized by two injections of Cloprostenol-sodium salt (Estrumate®, Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$)-analogue (Essex, Munich, Germany)) in a 12 day interval, to avoid hormonal influences depending on the sexual cycle. Animals were inoculated with bacteria three days after the second $PGF_{2\alpha}$-injection during estrus.

The 14 cows were split into three groups of four animals and one group of two animals each, and inoculated intramammary with either 500 CFU *E. coli* 1303 over 24 h or 10 000 CFU *S. aureus* 1027. After application of 20 I.E. oxytocin (i.v.) the udder was entirely milked out. Teats were cleaned and disinfected with ethanol (70%). Bacteria were suspended in 2 mL 0.9% sterile pyrogen free saline, and administered intracisternally through the teat canal.

Animals challenged over 24 h (trials 1, 2) were inoculated in the front right, hind right, and hind left quarter at 0 h (T0), 12 h (T12), and 18 h (T18) respectively after starting the trial. The front left quarter received 2 mL sterile saline at T0. Animals challenged over 72 h or 84 h were inoculated in both right quarters at 0 h (T0) after starting the trial. The left quarters received 2 mL of sterile saline at T0. Every 12 h (trials 1 to 4), the udder secretion was sampled before regular milking. The first 5 mL were discarded; the following 10 mL were used for SCC determination and bacteriology. Another 10 mL were milked into a tube containing 40 mL sterile PBS for the flow cytometric cell characterization (see below). Cows were milked at T0 and T12 just prior to the inoculation, and 24 h after the trial start (T24). Finally, cows were killed at T24 (trials 1, 2), at T72 (trial 3), or T84 (trial 4) with

a penetrating captive bolt gun followed by exsanguination.

Udder tissue samples and lymph node samples were collected aseptically from slaughtered cows within 5 to 10 min after killing as described [56].

2.4. Clinical score and laboratory parameters

Three weeks before pathogen inoculation, milk yield, California mastitis test (CMT), food intake, and body temperature of each animal were documented twice daily. Quarter milk samples for determination of the SCC and for bacteriological examination were collected weekly. The somatic cell count was determined using the Fossomatic 360 (FOSS Electric, Hillerod, Denmark) cell counter as described [37]. Bacterial levels were determined by the Institute for Animal Health (Ahlem, Germany) by plating 10 μL of quartermilk samples on Columbia sheep blood agar. After incubating overnight at 37 °C, the plates were analyzed and the number of bacteria was counted semiquantitatively: 1–20 CFU were classified as low grade (+), 21–200 CFU as middle grade (++), and > 200 CFU as high grade (+++). No animal showed fever, increased SCC, or alteration in milk during this pre-infection period, and the milk was free from mastitis pathogens. The clinical status was obtained after measuring and scoring body temperature, milk yield, milk appearance, udder consistency after palpation, and the general condition of the animals. Changes in those parameters were classified on a scale from 0 to 3. Red and white blood cell counts as well as differential counts were determined before and at every time point during experimental infection. Blood was taken from the *vena jugularis* aseptically using EDTA- vacutainers (Becton-Dickinson, Heidelberg, Germany).

2.5. Flow cytometric milk cell analysis

Milk samples (10 mL in 40 mL PBS) were kept on ice before processing. After centrifugation (400 × *g*, 10 min, 4 °C), fat and skimmed milk were removed. The cell rich sediment was suspended in 50 mL PBS, centrifuged (400 × *g*, 10 min, 4 °C) and finally the cells were suspended in 1 mL PBS. After adding propidium iodide (2 μg/mL final) and acridine orange solution (2.5 pg/mL final), the cells were acquired with a FACScan® (Becton Dickinson) cytometer after setting a live gate on green fluorescing particles. The relative proportions of viable cellular subpopulations were determined after gating on propidium iodide-negative events.

Neutrophils, large cells and lymphoid cells were identified in forward versus side scatter dotplots based on their morphological characteristics. Flow cytometric data were analyzed with the software WinMDI (version 2.8)[1].

2.6. Pathology and immunohistology

After slaughtering, udder tissue, regional and peripheral lymph nodes were macroscopically checked for prominent alterations and tissue samples were analyzed by histopathology after H&E staining. Tissue samples were collected aseptically from slaughtered cows within 5 to 10 min after killing. A piece of tissue (5 × 5 × 5 cm) was removed from a deeper area of the udder quarter, 10 cm dorsal of the milk cistern. After changing instruments, a smaller tissue piece (2 × 2 × 2 cm) was gained out of the center of the first one and served as original material for all further analyses. Immuno-histochemistry was used to visualize the expression of lingual antimicrobial peptide (LAP) and TLR2 in serial sections (7 μm) from formalin fixed and paraffin-embedded blocks of uninfected control or 24 h infected quarters. After dewaxing and deparaffinisation and rehydration the sections were taken through a heat induced epitope retrieval (HIER) procedure to reverse the formalin caused cross linking of epitopes. The slides were immersed into 0.1 M sodium citrate (pH 6.0) and heated to 125 °C for 5 min in a steam pot. After slowly (~50 min) cooling to room temperature the slides were rinsed in water (3 × 5 min, each) and subsequently immersed into PBS. The polyclonal anti-LAP antiserum was kindly provided by Drs Molenaar and Wheeler (AgResearch, Hamilton, New Zealand). In rabbits, we established an antiserum against a recombinant expressed truncated bovine TLR2. Briefly, a segment of the cDNA encoding the N-terminal 581 amino acid residues of the bovine TLR2 receptor was cloned into the prokaryotic expression vectors pASK-IBA3puls (IBA, Göttingen, Germany) The construction involved the PCR amplification of the respective area from a full length cDNA clone of the bovine TLR2 factor (kindly provided by Thomas Jungi and Dirk Werling, Institute for Immunology, University of Bern, Switzerland). We used the primers TLR2_IBA3f (5'- TCCC-GAATTCCCACGTGCTTTGTGGACAGCATG)

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[1] Trotter, J., free software for the analysis of flow cytometric data, available at ftp://facs.scripps.edu/ [consulted on November 2007].

and TLR2_IBA3r (GTCGAC<u>CTCGAG</u>GGACC-TTATTGCAGCTCTCAAATTTAAC) to initially amplify almost the entire coding sequence of TLR2, from codon 2 down to the codon of the c-terminal amino acid residue, introducing an E*co*RI and a X*ho*I site at the 5', and 3'termini, respectively. The PCR amplificate comprising 2359 bp of the bovine TLR2-encoding cDNA was cloned into pGEM-Tesay (Promega, Mannheim, Germany). The insert was retrieved via E*co*RI and XhoI digestion and cloned into an appropriately digested pASK-IBA3 plus vector, in front of the vector-encoded Streptactin-binding domain. The carboxy-terminally located TIR domain was deleted from the construct, since this domain is highly conserved among TLR-receptors and hence might elicit undesired cross-reacting antibodies. To this end, the construct was S*al*I digested and religated, shortening the encoded reading frame down to the desired N-terminal 581 aa residues. The expression vector was transfected into *E. coli* XL-1 blue and the ~55 kDA recombinant TLR2 protein was purified from bacterial lysates via affinity chromatography, using a Streptactin domain binding column (IBA), as prescribed by the manufacturer.

Further purification of this crude antigen preparation using preparative Laemmli gel-electrophoresis, antibody production in rabbits and affinity purification and concentration of the TLR2 specific antibodies on antigen-coupled Sepharose columns was essentially as described [42].

Antibodies were applied in blocking solution (1× Roti-Block (Roth, Karlsruhe, Germany); in PBS/T (PBS containing 0.1% of each, Triton X-100, and Tween 20 (Sigma-Aldrich, St. Louis, Mo, USA)). The LAP-antiserum was diluted 1:100, while 15 μg/mL of the affinity column purified TLR2 specific antibody were applied. Bound primary antibodies were visualized with anti-rabbit IgG (whole molecule)–FITC antibody produced in goat (Sigma; diluted 1:160 in PBS/T). Nuclei were counterstained with propidium iodide (2 μg/mL PBS/T) and labeled sections were examined under a fluorescence microscope (Leica DMRE microscope, Leica, Wetzlar, Germany).

2.7. Real-time PCR quantization of mRNA-copy numbers

We used TRIZOL (Invitrogen, Karlsruhe, Germany) for RNA extraction and the LightCycler instrument with the Syber Green plus reagent kit (both from Roche, Basel, Switzerland) for Real-time-PCR quantization of mRNA copy numbers, essentially as described [13]. Oligonucleotide and fluorescence acquisition temperatures during Light-Cycler measurements are given in Table I. We used an equimolar mixture of two different forward primers for TLR2 measurements, binding to two different 5'-terminal exons of the bovine TLR2. We had found in 5'-RACE amplifications that these may alternatively be spliced to exon 2 (unpublished). The conserved primers referred to as 'multiple-defensins' amplify several of the bovine β-defensin encoding genes. Cloning and sequencing of amplificates using RNA from infected udders revealed that they amplify mRNAs from the genes encoding BNBD4, BNBD5, another BNBD5-like gene (divergent from BNBD5 by one amino acid substitution), EBD and LAP and another, LAP like gene (data not shown). LAP messages account for ~80% of all β-defensin encoding messages in the infected udder. We used our own complete gene characterizations to derive primers for TLR3 and TLR6 measurements (accession numbers AJ812027 and AJ618974 for TLR3 and TLR6, respectively).

2.8. Statistical analyses

The clinical scoring of samples was analyzed by a nonparametric paired samples test (Friedman's test) in SPSS 12.0. The quantitative RT-PCR data on the mRNA concentrations for β-defensins and TLRs were modeled by a repeated measurement model using the procedure 'mixed' of the statistical software package SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Duration of pathogen presence was considered as a repeated factor. The posthoc tests between different levels of 'duration of pathogen presence' were done by t-tests with a Tukey correction to ensure an experiment wise error rate $\alpha = 0.05$. *P*-values of $\leqslant 0.05$ were considered significant. Significances of alterations in milk cell numbers following pathogen inoculation were assessed with an analysis of variance, applying a twofold cross classification. As fixed factors we considered pathogen inoculation (a), time of repeated sampling (b), and the interaction of both (a with b).

3. RESULTS

3.1. Establishment of infection conditions to elicit *E. coli* or *S. aureus* induced mastitis

A first key objective of this study was to establish clinical parameters occurring after

Table I. Oligonucleotide primer sequences and fluorescence acquisition temperatures for TLR and β-defensin measurements.

| Gene	Forward	Reverse	cDNA primer	Measuring temperature (°C)
TLR2	a) TGCGTTGGTTTGGATAGTGA & b) TCACTCGAAGCGGCTGTGAC	AGACCAGAGGGGATGGAGTT	CCTCAGAGTCTTCAGGTTCAC	83
TLR3	TTGACTGAACTCCATCTAATGTC	CTCTTTGATTTGATTTGATGACAAC	AAGTCCAGGCGTCTCAAGTTG	73
TLR4	TAGCATGAATTCAAGCTTATTATTTTT CGAACT	GCCCTGAAATGTGTCGTCTT	CCACGGCCACCAGCTTCTG	80
TLR6	CTTTAGAAGTTATTTTCTCTCCTTC	TCATCAGAGAACTGGATTATGGT	CTAAGAAATTCAGTTGGGTCAAG	78
TLR9	ACTGGCTGTTCCTGAAGTCTGT	GAGATTAAGGAGAGGCTGGTGA	GCAAGTGGTGGATGCGGTTG	82
'Multiple defensins'	AGGCTCCATCACCTGCTCCT	GAAACAGGTGCCAATCTGTCTC	dT(20)N	80
BNBD5	CTGGATCACCTGCTCCTCGT	GGTGCCAATCTGTCTCATGTTG	CGTGTGTTTGCCTTCTTTTACCA	80
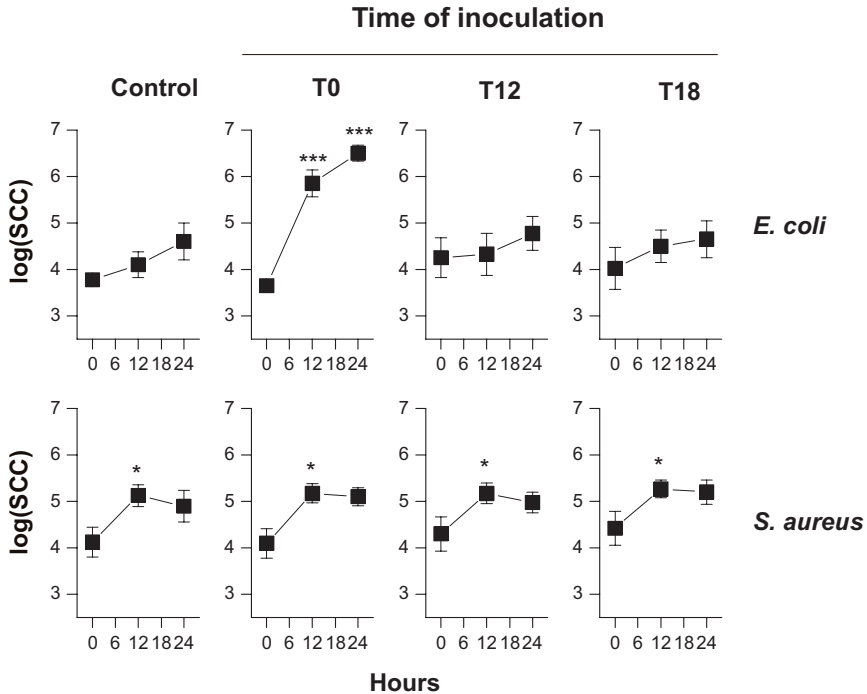
Figure 1. Somatic cell counts in milk of udder quarters after sequential inoculation with *E. coli* or *S. aureus* Udder quarters were mock inoculated (control), inoculated at T0 for 24 h, at T12 for 12 h or at T18 for 6 h (means ± SEM, 4 cows per group). * $P < 0.05$; *** $P < 0.001$.

experimentally inoculated udders of healthy lactating cows with the selected model strains for acute (*E. coli*) or subclinical mastitis (*S. aureus*). We established first in small experiments the suitable inoculation doses and relevant time courses for the experimental follow up of the infected animals. Regarding *E. coli* we consistently observed that as little as 500 CFU, administered in 2 mL of sterile saline was enough to elicit a strong cellular response in the inoculated udder quarter within 12 h. Major clinical symptoms, such as udder swelling and elevated counts of somatic cells in milk were restricted to pathogen inoculated udder quarters. All animals inoculated with the *E. coli* strain 1303 suffered from acute mastitis within 12 h post inoculation. The *S. aureus* strain 1027, on the contrary, required the administration of 10 000 CFU to cause increased cell counts in the milk, in most of the inoculated quarters (Figs. 1 and 2). It became clear that infections caused by this strain can

have a longer time lag before they become manifest and cause clear cut clinical signs of infection (Fig. 2). At 72 h after intra-cisternal application of *S. aureus*, the bacteria could be reisolated from 8 of 12 quarters (Fig. 2). By corollary this implies that the pathogen is not washed (or diluted) out in the course of the regular milkings every 12 h subsequent to pathogen inoculation. Interestingly, in quarters of cows showing a sharp increase in somatic cell counts 12 h after inoculation of *S. aureus*, the strain could be reisolated rather rarely (Fig. 2), suggesting that bacteria are eliminated very fast in case of a strong influx of neutrophilic granulocytes.

These trials showed very clearly that the *E. coli* infections may only be observed for a period of 24 h, because at that point in time after infection the animals will invariably suffer from acute mastitis. The *S. aureus* strain, on the contrary, will require longer times to firmly establish a successful infection
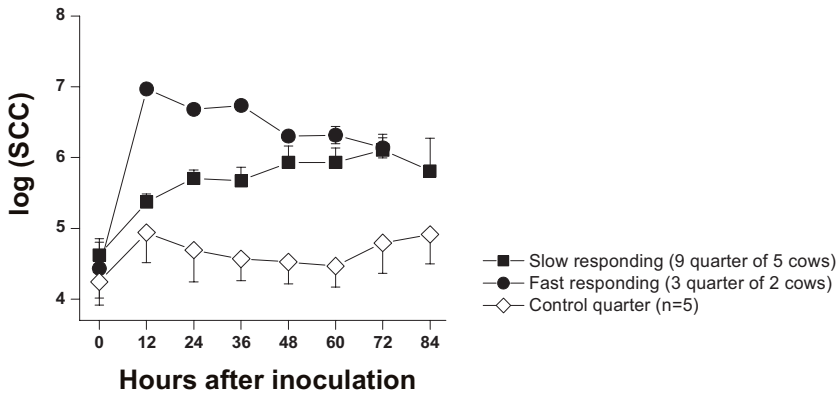
Re-isolation of *S. aureus* from milk of inoculated quarters

		Hours after inoculation						
Quarter	0	12	24	36	48	60	72	84
Fast responding	0/3	0/3	0/3	0/3	0/3	0/3	1/3	n.d.
Slow responding	0/9	5/9	5/9	6/9	6/9	8/9	7/9	4/9

Figure 2. Somatic cell counts in milk of udder quarters after inoculation with *S. aureus* for up to 84 h. Quarters of cows with less than 100 000 cells/mL were inoculated with *S. aureus* 1027 for 72 h (4 cows, 8 quarter) or 84 h (2 cows, 4 quarter). Somatic cell counts were determined in 12 h intervals. Based on the development of SCC values, the quarters were classified as fast (3 quarter, filled circles) or slow responding (filled squares). Numbers of quarters from which bacteria could be re-isolated at the indicated time points are given below the graph.

and reveal manifestation of mastitis. Thus, the *S. aureus* infections were conducted in four different trials. Two trials were short term inoculations, spanning an observation period of 24 h. In each of these trials two by two cows were arbitrarily assigned to become inoculated with *E. coli* or *S. aureus*. In other two trials observed over longer periods (72 h or 84 h) cows were inoculated with *S. aureus* only.

3.2. Body temperature, milk yield, differential blood cell numbers

All cows inoculated with *E. coli* developed fever (> 39.2 °C) within the first 12 h after infection with maximal values (40.4 °C) between 12 h and 18 h after inoculation (Fig. 3). This was accompanied by a significant leukopenia affecting both neutrophils, lymphoid cells (Fig. 3) and monocytes (data not shown). Leukopenia reached its maximum 18 h after pathogen inoculation. No increase in body temperature and no changes of blood

leukocyte numbers could be seen in *S. aureus*-inoculated animals (Fig. 3) after 24 h.

In animals infected for longer times (72 h–84 h) with *S. aureus*, a slight transient neutropenia could be seen after 48 h (not significant, data not shown). Milk yield dropped always within the first 12 h (Fig. 3), but differentially. The milk production almost ceased to only 16% of initial values in the *E. coli*-inoculated animals 24 h after infection. Casein synthesis, indeed, was blunted in the 24 h inoculated quarters, to less than 5% of the control quarters [56]. In contrast, milk yield decreased significantly to only about 50% in *S. aureus*-inoculated cows 12 h after pathogen inoculation and stabilized then at around 70% of the initial yields. Casein synthesis was not impaired in the *S. aureus* inoculated quarters.

3.3. Bacteriology

Quarter secretions obtained after pathogen inoculation contained only the pathogens used for infection whereas control quarters

E. coli **S. aureus**

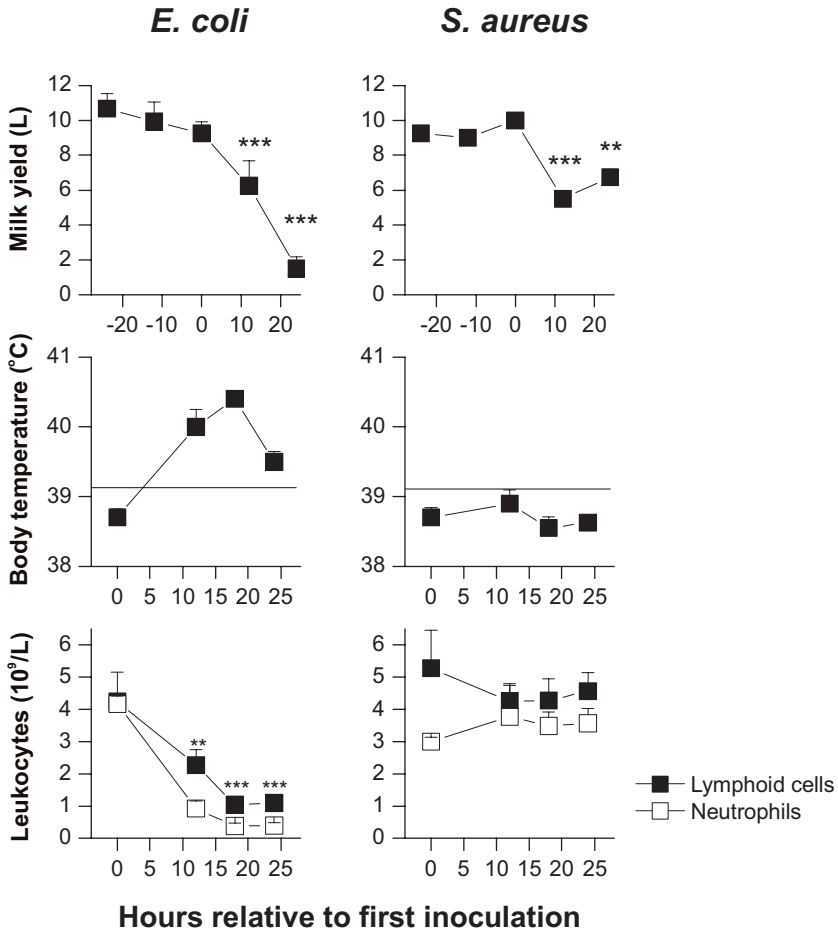**Hours relative to first inoculation**

Figure 3. Milk yield, body temperature, and blood leukocyte counts after experimental inoculation with *S. aureus* or *E. coli*. Three quarters per animal (4 per group) were sequentially inoculated at 0 h, 12 h, and 18 h. Milk yield (means of total milk yield ± SEM), body temperature (means ± SEM), and blood leukocyte counts (means ± SEM) were measured before and after experimental inoculation. ** $P < 0.01$; *** $P < 0.001$.

remained bacteriologically negative during the observation period (Tab. II). The infection strains and the reisolated strains of *E. coli*- and *S. aureus*-inoculated animals showed identical plasmid pattern (data not shown, analyzed by Prof. S. Schwarz, FAL, Mariensee, Germany). In quarters of *E. coli*-inoculated animals which were infected at T0 and T12, a high-grade content of *E. coli* could be determined 12 h after infection, whereas quarters infected for 6 h contained fewer bacteria (Tab. II). Numbers of reisolated bacteria from *S. aureus*-inoculated

quarters were heterogeneous among the animals. While early after inoculation (< 24 h) we only could reisolate *S. aureus* pathogens from ~60% of the inoculated udder quarters (Tab. II), this percentage increased with time. However, extending the duration of the observation of *S. aureus*-inoculated animals allowed to consistently reisolate the bacteria from most of the inoculated quarters (Fig. 2). Indeed all four evaluated quarters from two cows having been inoculated for 84 h were eventually *S. aureus*-positive (Fig. 2).

Table II. Reisolation of *E. coli* and *S. aureus* from milk secretions after experimental inoculation.

Hours of inoculation of individual quarters[1]	Hours after inoculation[2]	*E. coli*-inoculated animals				*S. aureus*- inoculated animals			
		1054	1055	1161	1162	1056	1057	1159	1160
6 h	–18	–	–	–	–	–	–	–	–
	–6	–	–	–	–	–	–	–	–
	6	++	+	+ + +	+	++	+	+ + +	+
12 h	–12	–	–	–	–	–	–	–	–
	0	–	–	–	–	–	–	–	–
	12	+ + +	+ + +	+ + +	++	+ + +	–	++	–
24 h	0	–	–	–	–	–	–	–	–
	12	+ + +	+ + +	+ + +	+ + +	++	+	–	–
	24	+ + +	+ + +	+ + +	+ + +	+ + +	+	–	+

[1] Quarters of individuals were consecutively inoculated for 24 h, 12 h, and 6 h before culling at 24 h after the first inoculation.
[2] During the 24 h period, milk samples were taken before (negative values) and after inoculation (positive values) of individual quarters.
Semiquantitative results of foremilk samples; +: low grade (0-20 CFU/10μL milk); ++: middle grade (21–200 CFU/10μL milk); +++: high grade (> 200 CFU/10μL milk). Control quarters without inoculated bacteria remained negative with only one quarter of animal 1054 at 24 h after the first inoculation (low grade positive, data not shown).

Table III. Clinical signs during experimental inoculation with *E. coli* or *S. aureus*.

Parameter	*E. coli*- inoculated animals				*S. aureus*- inoculated animals			
	1054	1055	1161	1162	1056	1057	1159	1160
Body temperature	1	1	2	1	0	0	1	0
Udder secretion	3	3	3	3	1	0	0	0
Udder palpation	3	3	3	3	1	0	0	1
Milk yield	3	2	2	3	2	1	2	2
General condition	1	1	1	1	0	0	0	0
Clinical score	11	10	11	10	4	1	3	3
Mean ± s	10.8 ± 0.5				2.8 ± 1.3			

Each parameter was graded on a scale from 0 (no change) to 3 (high grade change). The clinical score is the sum of all parameter scores (0–3: no clinical changes; 4–7: low grade clinical changes; 8–11: middle grade clinical changes; 12–15: high grade clinical changes).

3.4. Clinical score

The development of clinical signs during the first 24 h after inoculation is documented in Table III. *E. coli*-infected animals showed mainly high-grade changes in udder secretions as well as a high grade inflammatory swelling of the infected udder quarters resulting in a mean clinical score of 10.8 ± 0.5. *S. aureus*-inoculated animals showed less clinical signs and especially no signs of udder inflammation (clinical score: 2.8 ± 1.3, Tab. III).

3.5. Pathogen-specific effect upon somatic cell recruitment into milk

3.5.1. Quantitative aspects

The different pathogens exerted a differential effect upon cell recruitment after pathogen inoculation. *E. coli* caused in the quarters inoculated at T0 a massive and significant rise of SCC already 12 h after pathogen inoculation (Fig. 1, T0, from 8 000 to 1 Mio cells/mL). Interestingly, quarters infected at T12 did not respond with a significant rise in SCC 12 h
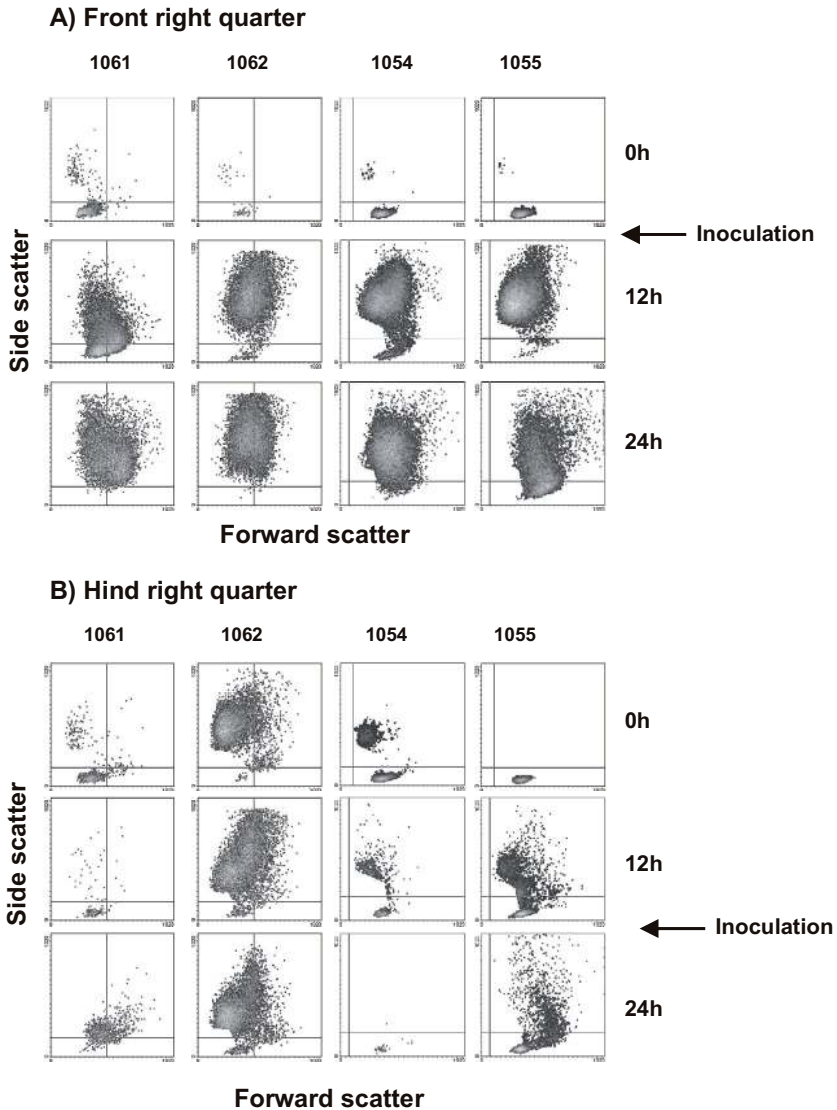
Figure 4. Flow cytometric analysis of milk cells from *E. coli* -inoculated udder quarters. The animal number is noted on top of the columns. Shown are density plots (forward vs side scatter) of cells after gating on nucleated (acridin orange-positive) and viable (propidium iodide-negative) milk cells. (A) Front right quarter inoculated for 24 h; (B) Hind right quarter inoculated at T12 for 12 h.

later (Fig. 1) and also their relative cellular composition remained unchanged (Fig. 4B, compare second with third row of dotplots).

S. aureus-inoculation caused quantitatively a different pattern of increase in milk cell numbers. In our first two *S. aureus* infection trials, we recorded the changes over 24 h

only. The cell numbers increased significantly within 12 h after the first infection in all quarters by about ~8 fold and, remarkably, also in the control quarters. However, subsequently further increased SCC values differed only insignificantly from the 12 h values (Fig. 1). The increased SCC values in pathogen inoculated

Table IV. Pathological and histological characterization of udder tissues after experimental inoculation with *E. coli* or *S. aureus*.

	Acute purulent processes		Lymphocytic interstitial processes		Interstitial edema	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
Control quarter	0.0	0.3	0.3	0.8	0.0	0.0
6 h inoculation	0.0	1.0	0.5	1.0	0.0	0.0
12 h inoculation	0.5	0.5	0.8	1.1	0.3	0.0
24 h inoculation	2.6	1.0	0.0	0.8	1.9	0.8

Table V. Pathological and histological characterization of lymph nodes after experimental inoculation with *E. coli* or *S. aureus*.

	Follicular hyperplasia		Acute purulent lymphadenitis	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
Right udder[1]	1.0	2.5	3.0	0.3
Left udder	1.8	2.4	1.3	0.0
Right superficial cervical	2.6	2.0	0.0	0.0
Left superficial cervical	2.1	2.3	0.0	0.0

[1] The right udder lymph node drained the quarter which was inoculated for 12 h or 24 h; the left udder lymph node drained the side of the control quarter and the quarter which was inoculated for 6 h. Values are presented of means ($n = 4$ cows per group) of scores from 0 (absent) to 3 (high grade).

quarters were statistically not different from control quarters ($p > 0.1$). The quarters of cows inoculated with *S. aureus* for 72 h or 84 h were either fast (three quarter) or slow (nine quarter) responding with elevated SCC values (Fig. 2).

3.5.2. *Qualitative aspects*

Flow cytometric analysis showed that all animals started with milk containing very few neutrophils, and a significant population of cells with lymphoid morphology (shown for *E. coli*-inoculated animals in Fig. 4A, first row of dotplots). However, different quarters of the same individual eventually differed vastly in the content of somatic cells (Fig. 4, compare animal 1062 at time 0).

This was mainly caused by immigrating cells with morphological characteristics of neutrophils (Fig. 4A, compare second with third row of dotplots). *S. aureus* expositions for 24 h, on the contrary, neither influenced the total cell number (Fig. 1, *S. aureus*) nor the relative proportions of the various cell types found in the milk (not shown). Taken together, the data show that both pathogens

exert a profoundly different, pathogen-specific effect upon the inoculation-caused recruitment of cells.

3.6. *E. coli* inoculation caused clear mastitis pathology

Acute purulent processes dominated in udder tissues inoculated with *E. coli* for 24 h (Tab. IV), but also interstitial edemas were seen, especially in 24 h- inoculated quarters. Lymphocytic interstitial processes were only infrequently seen. Tissues of individual *S. aureus*-inoculated animals showed some signs of acute purulent processes, however with no apparent correlation to the duration of the infection per quarter (Tab. IV). All lymph nodes examined (regional udder lymph nodes and distant control lymph nodes) showed signs of follicular hyperplasia both in *E. coli*- and *S. aureus*-inoculated animals, but only udder lymph nodes draining the 24 h and 12 h inoculated quarters in *E. coli*-inoculated animals, showed an acute purulent lymphadenitis (Tab. V). In summary, *E. coli* inoculation resulted in a much more severe pathology than caused by *S. aureus*.

Table VI. Absolute copy numbers of defensins (universal defensins (uDEF))-, BNBD5-, and TLR-mRNA in udder tissues of mock-inoculated quarters.

	BNBD5	uDEF	TLR2	TLR3	TLR4	TLR6
Mean	189	11 921	4 894	242	7 776	1 623
SEM	58	5 985	1 013	67	1 713	451
CV	30.7	50.2	20.7	27.5	22.0	27.8
n	8	8	8	8	8	8
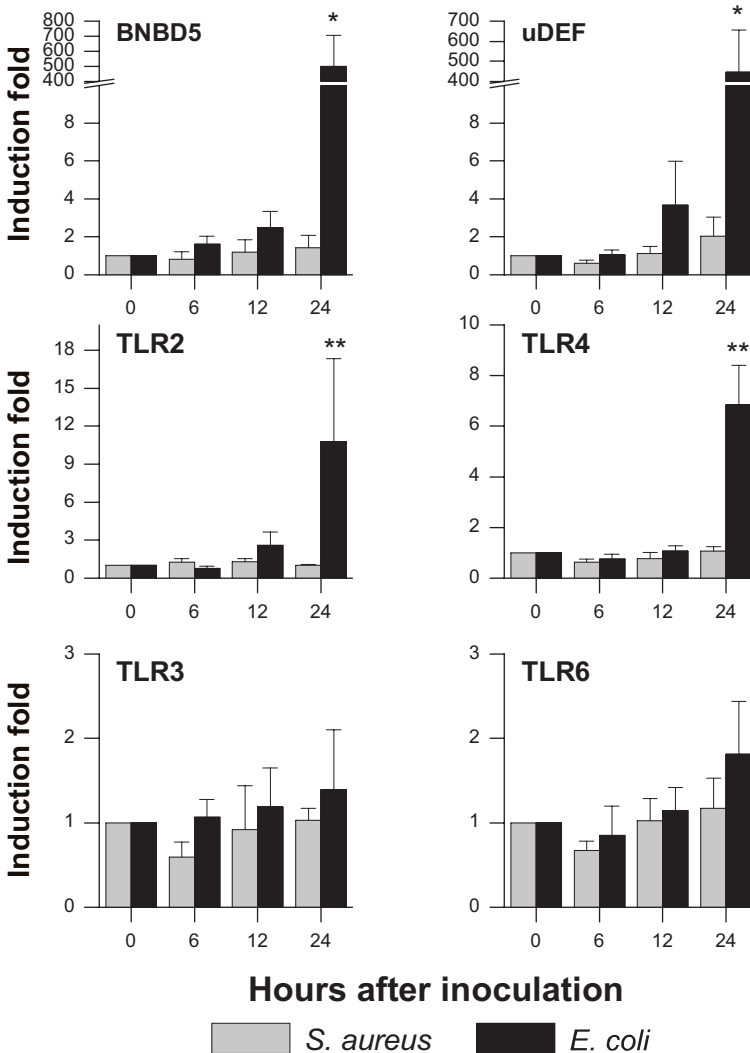
Figure 5. Expression of TLR and β-defensin genes in udder tissues after experimental inoculation with *S. aureus* or *E. coli.* mRNA for the indicated TLRs, BNBD5, and β-defensins (uDEF) was quantified in tissue samples of udder quarters inoculated for 24 h, 12 h and 6 h. The expression levels of the genes were calculated as x-fold induction compared to the expression level in the control quarters (bars time 0 h = 1) ($n = 4$ per quarter, means ± SEM). * P < 0.05; ** P < 0.01.
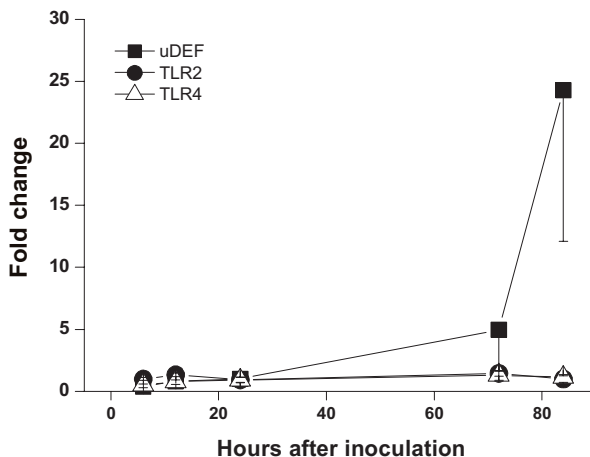
Figure 6. β-defensin and TLR copies following intramammary inoculation with *S. aureus*. mRNA for TLR2, TLR4, and β-defensins (universal defensin (uDEF)) was quantified in tissue samples of udder quarters inoculated for 12 h (4 quarters), 24 h (4 quarters), 72 h (8 quarters), and 84 h (6 quarters). The expression levels of the genes (means ± SEM) were calculated as x-fold induction compared to the expression level in the control quarters (averaged mRNA copies of control quarters were set as 1). The 84 h values of uDEF are statistically significant different from the controls (*, $p < 0.05$).

3.7. *E. coli* **inoculation, but not** *S. aureus* **increased TLR2, TLR4, and β-defensin expression in udder tissues within 24 h after pathogen inoculation**

The absolute mRNA copy numbers, as measured in the uninoculated control quarters are given in Table VI. The variance was very high for both β-defensin parameters, due to one of four *E. coli*-inoculated cows and one of the eight *S. aureus*-inoculated cows.

E. coli-inoculation caused a significant ($p < 0.001$) and massive upregulation of TLR2 and TLR4 expression 24 h after inoculation, compared to the uninoculated control quarters (Fig. 5). The expression remained low in all control quarters, at values typical for healthy, uninoculated animals. Similarly, BNBD5, as well as the other β-defensin-encoding genes described as 'universal defensin' (uDEF), were dramatically upregulated (> 400 fold) in udder quarters *E. coli*-inoculated for 24 h, but remained low in the control quarters. The level of significance is lower here ($p < 0.05$), due to the large variation. The mean expression levels of the other TLR receptors remained almost unchanged, and small changes were all statistically in-

significant. In no case occurred a significant down-regulation of either TLR gene expression or defensin gene expression compared to control quarters.

S. aureus inoculations, in contrast, did not significantly modulate the expression of any of these genes within 24 h after exposition (Fig. 5). Hence, it was necessary to run additional inoculation trials, to eventually verify that this *S. aureus* strain is indeed capable at all to elicit an immune response in mammary tissue. We found that β-defensin expression may eventually be strongly upregulated at later times after inoculation with *S. aureus* (Fig. 6). Two from among the six animals infected for longer periods (72 h or 84 h) revealed in one quarter only a doubled β-defensin mRNA concentration, while the other pathogen inoculated quarter did not react. The other four animals revealed 5–500 fold increased β-defensin copy numbers, in at least one quarter. This large quarter and animal individual influence precludes a meaningful statistical analysis of these data. Regarding the expression of TLR-encoding genes, we found that inoculation with *S. aureus*, unlike with *E. coli*, do not regulate the expression of any of these genes
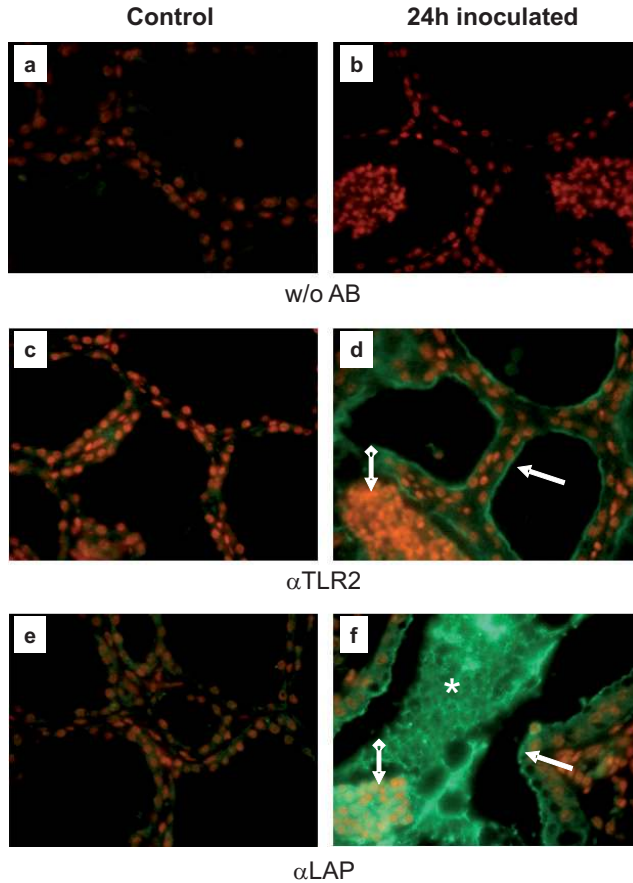
Control   **24h inoculated**

w/o AB

αTLR2

αLAP

Figure 7. Bacterial inoculation stimulates the expression of TLR2 and LAP at the apical membrane of MEC. Sections from uninoculated control quarters (control) or from quarters inoculated for 24 h with *E. coli* were either incubated without primary antibody (a, b) or with rabbit antibodies against bovine TLR2 (αTLR2, c, d) or bovine LAP (αLAP, e, f). Bound primary antibodies were visualized with a FITC-labeled secondary antibody. Nuclei were stained with propidium iodide (100× magnification) (a color version of this figure is available on www.vetres.org).

under investigation (Fig. 6). We observed at maximum a doubled TLR4 concentration in a quarter with a > 250 fold increased β-defensin mRNA concentration. These data show that the *S. aureus* 1027 strain may eventually provoke a defense reaction in the host tissue after extended times of pathogen presence in the alveoli. However, the host reaction is different from that elicited by *E. coli*, since the *S. aureus* pathogen does not alter TLR2 or TLR4 expression.

Immunohistological analysis proved that inoculation with *E. coli* induced the expres-

sion of the TLR2 protein and the lingual antimicrobial peptide (LAP) β-defensin in the MEC, lining the alveoli (Figs. 7d, 7f, arrow). The TLR2 antibody reveals a spatially very restricted localization of the protein, at the apical membrane, while the LAP is distributed across the entire cell body, polarized though towards the apical membrane. Immigrating leukocytes (mainly PMN, Figs. 7d, 7f, knobbed arrow) were largely negative for TLR2, but stained massively with the LAP specific antibody. LAP decorated also heavily clumps of precipitated milk (Fig. 7f, aster-
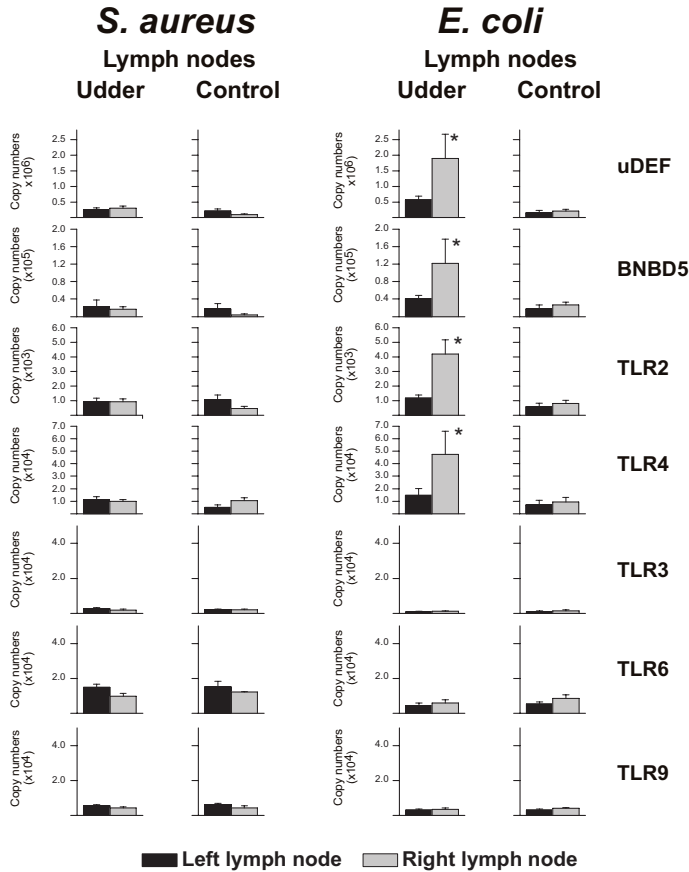
Figure 8. TLR and β-defensin expression in regional udder lymph nodes and peripheral lymph nodes 24 h after experimental inoculation with *E. coli* or *S. aureus*. The left udder lymph nodes drained the control quarter and the 6 h inoculated quarter; the right udder lymph node drained quarters inoculated for 12 h and 24 h. Control lymph nodes refer to left and right peripheral lymph nodes. Means ± SEM of mRNA copy numbers. * *P* < 0.05.

isk), in sections from *E. coli*-inoculated udder quarters after 24 h. This staining appears to be specific, since in serial sections used for immune histochemistry the TLR2 specific antibody never stained these clumps of precipitated milk. These results prove for TLR2 and LAP that increased mRNA abundance of these factors results in increased protein abundance. It may be added that the poor decoration of immigrating leucocytes with the TLR2 antibody is not a general feature, since positive TLR2 staining of such cells was frequently seen in other experiments, using in freeze-cut sections from infected udders (not shown).

3.8. TLR and defensin expression in lymph nodes

TLR- and β-defensin expression was examined in local mammary lymph nodes and peripheral lymph nodes (superficial cervical lymph nodes). In *E. coli*-inoculated animals, the right mammary lymph nodes draining the quarters inoculated for 24 h and 12 h featured significantly increased TLR2 and TLR4 expression. In contrast, the left mammary lymph node, draining the control quarter and the quarter inoculated for only 6 h, showed no such modulated expression (Fig. 8). The

same pattern was seen for uDEF and BNBD5 mRNA which were significantly upregulated in the right mammary lymph nodes of *E. coli*-inoculated animals. Control lymph nodes in the periphery showed no modulated expression of either TLR or defensin genes. *S. aureus* inoculation did not cause any infection-related change in the expression of these genes, in none of the lymph node analyzed.

4. DISCUSSION

We previously described the jointly upregulated expression of pattern recognition receptor genes TLR2 and TLR4 as well as the enhanced expression of the β-defensin gene BNBD5 in naturally occurring clinical cases of *S. aureus* and *E. coli*-mastitis, based on samples collected in a field study [13]. Hence, those samples were derived from animals in different, uncontrolled stages of lactation, and uncontrolled durations of infections.

Controlled, experimental infections of the udder were performed during the last 30 years to study various aspects of host-pathogen interactions. These previous studies focused generally on changes of milk composition during the course of an induced mastitis, the time course of effector-cell appearance in the milk, their functional capabilities, and the expression of soluble immune mediators [3, 12, 41, 43].

Here, we addressed the sequence of events occurring during the first 24 h after inoculation. We established an experimental model system based on animals of the same age and stage of lactation to insure comparable physiological situations in the host. We used asseverated strains of pathogens to provide comparable conditions regarding the pathogens. We analyzed the early events after host-pathogen contact, since conceivably these are decisive for the subsequent pathogenesis of mastitis in cattle. It is known since long that *E. coli* pathogens are prone to result in acute infections and inflammations of the udder, while *S. aureus* pathogens very frequently cause subclinical, chronic infections, but the molecular mechanisms underpinning this difference are largely unknown.

The bacteria strains used in our study were not characterized in depth regarding their virulence factors, so far. However, they are relevant since they were isolated from cows suffering from cases of clinical mastitis. We verified in pilot trials that 500 CFU of the *E. coli* strain consistently cause acute mastitis within 12 h, while 10 000 CFU of the *S. aureus* strain reliably causes subclinical mastitis, eventually after longer incubation times (> 40 h). Hence, while our main interest in this study was on the analysis of early stage (< 24 h) of host-pathogen interaction, we include also the results from the long time infection trials 3 and 4 (72 h and 84 h, respectively) verifying that our strain, as used here, establishes subclinical infections, indeed.

Regarding the inoculation doses used, it should be noted that others achieved clinical mastitis with as little as 72–74 CFU/quarter of either bacteria species [3]. However, it is well known that different strains of the same bacterial pathogen species may differ vastly in their genome [20, 53]. Indeed, we wanted to establish a model for subclinical mastitis. Such subclinical infections are characterized by very mild or no clinical signs of inflammation, often associated with individual responses. While milk cell counts rose very quickly within the first 12 h in two cows of trial 3 (Fig. 1), it took almost 60 h in other cows to react with levels > 1 Mio cells/mL (Fig. 2). These individual and even udder quarter dependent differential reactions upon inoculations with this strain of *S. aureus* were also reflected in the selective and variable activation of β-defensin expression (see below). In summary, our isolated *S. aureus* strains seems to represent a quite relevant model for such subclinical infections [34].

Standardization of animals is a major issue in experimental infection models. In addition to a hormonal synchronization we selected animals which never had mastitis before to avoid influences of adaptive immune mechanisms. Although bacteria-specific antibodies in serum or milk were not determined, the proven absence of previous history of mastitis made it unlikely that the animals responded in our model with components (antibodies,

memory T-cells) of the adaptive immune system. Since high SCC values can ameliorate or alter the course of a natural or experimental infection [28, 45], we only inoculated animals having consistently low SCC values (< 100 000 cells/mL) in their quarter milk. This was monitored over a period of three weeks prior to challenge and assured that the local immune system was not alerted or altered. At the time of inoculation, the leukocyte populations in milk consisted mainly of lymphoid cells with variant numbers of neutrophilic granulocytes (Fig. 4).

The homogeneous responses of *E. coli*-inoculated animals support the assumption of proper standardization. Their clinical symptoms, obvious 24 h after pathogen inoculation, resulted in similar clinical scores (Tab. III) and are characteristic for an acute inflammatory response. They are in line with observations made by Burvenich et al. [8]. Also, we could re-isolate the same strain of *E. coli* bacteria from inoculated quarters as used for the infections (Tab. II). Our *E. coli* infections caused a much stronger depletion of white blood cells from the blood stream than what was recorded after LPS infusions into the udder [38].

In contrast, the response to *S. aureus* inoculations was more heterogeneous. All animals lacked any signs of an acute inflammation. The clear proof that *S. aureus* and the host were in contact and exchanged information during the first 24 h of infection was the significant drop of milk yield (Fig. 3) which, however, was not paralleled by a clear pathogen-induced SCC increase (Fig. 1). Moreover, the highly significant up-regulation of TGF-β in control quarter tissues 24 h after inoculation with *S. aureus* strongly indicates that the animals responded systemically after perceiving the presence of the *S. aureus* pathogens [62].

The infections with this *S. aureus* strain obviously need more than 24 h to establish. This could be seen from the elevated SCC values and increased β-defensin copy numbers, as recorded from the 72 h and 84 h *S. aureus*-challenge trials (Figs. 2 and 6).

The individually differing responses of the various host animals to the 10 000 CFU *S. aureus* per quarter were also reflected by the heterogeneous numbers of bacteria, which were re-isolated after inoculation (Tab. II). This is basically in line with observations by Schukken et al. [40], who noted that 18 out of 48 *S. aureus*-infected udder quarters remained bacteriologically negative throughout the study. The poorer qualitative and quantitative recovery of *S. aureus*, compared to *E. coli* pathogens after infection, conceivably indicates a slower multiplication rate of the *S. aureus* bacteria in the udder, albeit their generation time is similar to the *E. coli* pathogens under laboratory culture conditions.

E. coli, but not *S. aureus* infections resulted in a consistent and strong recruitment of somatic cells into the milk. The failure of *S. aureus* to significantly increase the cell counts in milk is surprising, since it was demonstrated that the major molecular patterns of *S. aureus* (LTA) and *E. coli* (LPS) can induce relevant immune mediators (CXCL8, TNF-α, IL-1β) after in vitro stimulation of primary MEC. However, the effect of LTA including the induction of the neutrophil-attractive chemokine CXCL8 was only transient [48].

This response, however, was not observed during the first 24 h after *S. aureus* inoculation in our model. In part, this is in line with findings of Bannerman et al. [3], who recognized in vivo significant less induction of CXCL8, C5a, and TNF-α by *S. aureus*, as compared to *E. coli*. The reduced induction of some pro-inflammatory cytokines may also explain why *S. aureus*-inoculated animals showed no signs of fever and leukopenia compared to *E. coli*-inoculated animals (Fig. 3). The lack of response towards *S. aureus* is unclear. It may reside in a differential presence of humoral milk factors which may alter the ability of bacteria to attach to MEC [1, 16], or to survive intracellular in macrophages [15] or epithelial cells [25].

TLR2, TLR4, as well as β-defensins were significantly upregulated 24 h after inoculation of udder quarters with *E. coli*, selectively in udder tissue and in local udder lymph nodes. Other TLRs (TLR3, TLR6, and TLR9) remained unaffected during this period (Figs. 5 and 8). These results show that the expression of TLR2 and TLR4 receptors is dynamically

regulated during infection, while the expression of TLR3 and TLR6 is only marginally influenced by ongoing mastitis. They also show that changes in the expression profiles of the udder quarters are spatially regulated and restricted to the infected quarter. They are correlated with the duration of pathogen presence within the quarter, not within the animal. Hence, the regulation of the expression of these genes is largely independent from systemic factors but rather controlled by factors inside the infected udder quarter.

These results are basically in line with our previous observations from cases of clinical mastitis where we recognized an upregulated expression of TLR2 and TLR4 but not of TLR9 [13]. However, whereas mastitis related β-defensins showed a 10–30 fold increase in long time infected udders from a field study [13], the experimental infection resulted in a much higher increase (about 500 fold, Fig. 5). This indicates that to date unidentified counter-regulatory mechanisms may be responsible for the less enhanced expression of these molecules in long-term infected udders.

We could also unequivocally demonstrate by immunohistology that MEC are the major cell type expressing both β-defensins and TLRs (Fig. 7). Regarding TLR2, these images prove first time, to the best of our knowledge, that this factor indeed is localized in the apical membrane of the MEC, which could only be assumed so far. Similarly, we show that the distribution of the LAP-peptide is polarized towards the apical membrane of the MEC. This, together with fact that this antibody stained heavily the milk of infected quarters only, proves for the cow that LAP serves as a secreted bactericidal peptide in milk. This had previously been concluded on the basis of MEC restricted localization of the LAP- [50] and BNBD5-mRNA [13]. The concentrations of β-defensins in milk have so far only been reported from women, amounting up to 40 μg/mL [2, 19].

The inflammatory PMN in the histological sections were considerably less stained and thus are unlikely to contribute to the strong TLR2 (and most likely also TLR4) RNA message which could be measured 24 h after *E. coli* inoculation.

The lack of TLR3, TLR6, and TLR9 modulation indicates that the expression of these PRRs is not regulated upon ligand binding. Their expression was also unchanged by the infection in local mammary lymph nodes as well as in udder-distant lymph nodes after experimental inoculation with *E. coli* or *S. aureus* (Fig. 8), albeit these immune cell rich tissues contain TLR9 expressing cell types, such as dendritic cells [9, 21].

The molecular controls determining an upregulation of TLR2, TLR4, and β-defensin expression are unknown. They may be associated with elevated levels of pro-inflammatory cytokines, since it occurred only in the *E. coli*-inoculated animals, which concomitantly showed cytokine-induced fever. Expression levels of TLR3, 6, and 9, on the contrary, remained unchanged, indicating that their expression is regulated differently from that of the former group of TLRs.

S. aureus-inoculated animals showed unchanged expression levels of all TLRs and β-defensin genes during the first 24 h after pathogen inoculation (Fig. 5). We recorded no signs of inflammation in these animals during the first 24 h of pathogen exposure. They developed no fever and did not reveal elevated SCC values. Hence, the *S. aureus*-induced mechanisms causing the consistent and statistically significant drop in milk yield are independent from immune mechanisms. It is unclear, why the host animals do not respond with a visible pro-inflammatory reaction early after *S. aureus*-inoculations. This may in part be specific for the particular *S. aureus* strain used for challenge, since others demonstrated a rise in SCC values within 24 h after a *S. aureus* challenge [3]. Striking differences between *S. aureus*- and *E. coli*-elicited gene expressions showed up when we analyzed mammary tissue samples at later stages of pathogen exposure (72 h to 84 h, *S. aureus* only). Here, only β-defensin genes were upregulated whereas TLR2 and TLR4 mRNA remained at the level of not inoculated quarters (Fig. 6). Although we could not compare *E. coli*-elicited responses at such stages,

this clearly points to bacterial species-specific modes of interactions with the host, and represents a correlate of different courses of the disease (clinical or subclinical).

TLR2 and TLR4 upregulation in the udder occurred much later than the rise in SSC numbers, fever, and drop in milk yield, parameters which changed already ~12 h earlier. Thus, expression modulation of PRRs and also the *de novo* synthesis of defense molecules are rather late events during the early phase of an infection. However, the time kinetic experiments using sequentially infected pathogen inoculated udder quarters must be interpreted with some care since the cellular response within 12 h of quarters inoculated at T12 was not comparable to those infected at T0 (Fig. 4). This indicates that the response in subsequently inoculated quarters is affected by systemic effects (e.g. cytokines, lipid mediators, neuromediators) induced in previously challenged quarters. This resembles somehow the findings of Shuster and Harmon [44], who noted that endotoxin infusion into udders quarters induced an acute response with systemic involvement, whereas subsequent infusions into the same quarters failed to induce systemic responses. They also noted a persistent mammary leukocytosis in repeatedly infused quarters. This could indicate that the systemic response in *E. coli*-inoculated animals results in a refractory state of the tissues, e.g. adjacent, not-inoculated quarters. An endotoxin-mediated desensitization of monocytes-macrophages resulting in a decreased production of pro-inflammatory cytokines after re-exposure to endotoxin has recently been validated in vitro [46].

Leukopenia and fever are usually attributed to the *E. coli* endotoxin LPS [18] which induces high levels of TNF-α within 8–16 h after infection [3, 17]. The pro-apoptotic effect of TNF-α for lymphocytes and neutrophils [29, 36, 51] may explain the leukopenia in our model. In contrast, the upregulation of TLR2, TLR4, and β-defensins occurs spatially restricted, in specific udder quarters, and hence are independent from systemic, circulating TNF-α and other inflammatory cytokines. Although it has been shown that IFN-γ and

TNF-α cause enhanced expression of TLR2 and TLR4 on renal epithelial cells during inflammation [60], we recognized upregulation only locally in udder tissue and regional lymph nodes (Figs. 5 and 8) but not in peripheral control lymph nodes (Fig. 8). Thus it is more likely that modulated expression of β-defensins and TLRs results from locally induced soluble mediators and signaling through baseline expressed PRRs [57, 58]. This might also explain why the systemic, cytokine-mediated effects leading to an altered responsiveness of adjacent quarters did not result in an upregulation of TLRs and β-defensins.

Whether an enhanced expression of TLRs following infection is beneficial for the animal (better recognition of bacteria) as stated by Hayashi et al. [14] or whether it results in a too strong and deleterious inflammatory response needs further investigations.

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