Original article

Staphylococcus aureus lipoteichoic acid triggers inflammation in the lactating bovine mammary gland

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Abstract – The response of the bovine mammary gland to lipoteichoic acid (LTA), which is a major pathogen-associated molecular pattern of Gram-positive bacteria, was investigated by infusing purified *Staphylococcus aureus* LTA in the lumen of the gland. LTA was able to induce clinical mastitis at the dose of 100 µg/quarter, and a subclinical inflammatory response at 10 µg/quarter. The induced inflammation was characterized by a prompt and massive influx of neutrophils in milk. LTA proved to induce strongly the secretion of the chemokines CXCL1, CXCL2, CXCL3 and CXCL8, which target mainly neutrophils. The complement-derived chemoattractant C5a was generated in milk only with the highest dose of LTA (100 µg). The pro-inflammatory cytokine IL-1β was induced in milk, but there was very little if any TNF-α and no IFN-γ. The re-assessment of CXCL8 concentrations in milk whey of quarters previously challenged with *S. aureus*, by using an ELISA designed for bovine CXCL8, showed that this chemokine was induced in milk, contradicting previous reports. Overall, *S. aureus* LTA elicited mammary inflammatory responses that shared several attributes with *S. aureus* mastitis. Purified LTA looks promising as a convenient tool to investigate the inflammatory and immune responses of the mammary gland to *S. aureus*.

1. INTRODUCTION

Staphylococcus aureus mastitis remains a major pathology of dairy ruminants. It poses unsolved problems owing to the long persistence of infections and the poor success rate of treatments with antibiotics [6]. A number of studies have resorted to intramammary infections with *S. aureus* to explore the host/pathogen interactions in the mammary gland, and a great deal of information has accumulated on the immune and inflammatory responses of ruminants and on the virulence factors of *S. aureus* [8, 9, 52]. In spite of their usefulness, experimental infections have two major drawbacks: they are costly because they last long and may result in the culling of the experimental animals, and the complexity of

the interactions between host defences and the array of the bacterial virulence factors and modulins defies the analysis [54]. Also, the infection foci usually involve small and scattered portions of the mammary tissue. This complicates investigations based on the analysis of tissue samples (PCR and immuno-histochemistry). A more controlled bacterial stimulus than live bacteria would be of help. In the case of *Escherichia coli* mastitis, the lipopolysaccharide (LPS) is used as a surrogate for infection. This Gram-negative bacteria outer-membrane component has been extensively used to mimic mastitis due to *E. coli*, and it has been shown that the responses it provokes are in many respects representative of those induced by the infection [10]. This makes LPS a relevant substitute for *E. coli*

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mastitis. A surrogate has yet to be defined for *S. aureus* mastitis. Progress in the knowledge and the availability of pathogen-associated molecular patterns (PAMP) for Gram positive bacteria, and in particular *S. aureus* [13], makes it feasible to research the role of defined molecules in the early inflammatory response induced in the mammary gland through interaction with the innate immune system. One of these molecules is lipoteichoic acid (LTA) of *S. aureus*, which is anchored to the cytoplasmic membrane and is exposed at the surface of the bacteria: LTA has been shown to be an important pattern for immune recognition of *S. aureus* [54].

Until recently, the effects of LTA on immune and inflammatory cells have been controversial, because most preparations of LTA were contaminated with endotoxin and other immunostimulatory components [14, 30]. Moreover, the isolation procedure yielded partially degraded LTA with much reduced immunostimulatory properties [29]. A new procedure was developed to obtain pure and active LTA, which proved to be a potent stimulus for cytokine release and neutrophil recruitment [29, 31, 55]. The availability of a commercial preparation based on this improved isolation procedure makes it possible to investigate the effects of *S. aureus* LTA in the mammary gland. One of the advantages of LTA as a tool to model inflammation is that it is a defined bacterial PAMP which targets identified pattern recognition receptors (PRR) and increasingly defined accessory molecules for recognition and for the signaling cascade. The recognition complex includes TLR2, TLR6 and CD14 [22, 45]. Other receptors such as the platelet activating factor (PAF) receptor or the scavenger receptor CD36 are also involved, maybe in a tissue-specific way, as well as the adaptor molecule LPS binding protein (LBP).

In the following investigations, we used a purified commercial preparation of *S. aureus* LTA to determine whether the bovine mammary gland responds to LTA, to determine the dose-response effects, and to begin to characterize the induced inflammatory response. The results will contribute to the development of a convenient and relevant procedure to better understand the interactions between *S. aureus* and the mammary gland.

2. MATERIALS AND METHODS

2.1. Experimental design

Twelve healthy Holstein cows from the experimental herd of the Institute at Nouzilly (INRA, France) that had no detectable bacterial growth and cell counts less than 100 000 cells/mL in milk samples from at least three quarters per cow in two consecutive weeks were used. These cows had no record of clinical mastitis in the period from calving to intramammary challenge. They were in their second or third lactation, and between two to six months in lactation. The use and care of the cows in this study were approved by the Regional Committee of Ethics for Animal Experimentation (CREEA).

Owing to the absence of published data, a pilot experiment was designed to assess the doses of LTA active in the mammary gland. The staphylococcal LTA used in this study was purified *S. aureus* lipoteichoic acid (PSLTA; InvivoGen, San Diego, CA, USA), which was prepared according to a new procedure [29]. This commercial preparation contains < 1.24 EU endotoxin/mg LTA. The lyophilized LTA was made soluble in sterile ultrapure water, then diluted in cell culture medium RPMI 1640 (Sigma , St. Louis, MO, USA). Doses of 0.1 µg, 1 µg, 10 µg and 100 µg in 0.5 mL RPMI were infused in the mammary glands of six cows through the teat canal with aseptic precautions. Three cows received RPMI in one gland, 0.1 µg in another gland, and 10 µg in a third gland. Three other cows were infused with RPMI in one gland, 1 µg in another gland, and 100 µg in a third gland. All infused glands were devoid of infection and shed less than 100 000 cells/mL milk. Infused quarters were sampled just before infusion, then at each milking. The cows were milked twice a day, at 8:00 and 16:00.

For the second experiment, six cows were used. Each one received at time zero RPMI in one quarter, 10 µg LTA in another one, and 100 µg LTA in a third quarter. The infused quarters were aseptically sampled just before infusion after the morning milking, then 4, 8, 12, 16, 24, 32, 48, 72 and 96 h post-infusion. At each sampling time up to 24 h post-infusion, the rectal temperature was taken and

a clinical examination was performed (appearance of gland and milk).

A portion of the milk samples was used for bacteriological examination (by plating over a blood-esculin-agar plate), somatic cell count (SCC) and cytospin slides. The remainder was centrifuged at $1\,000\times g$ for 30 min at 4 °C. Skimmed milk was stored in portions at –18 °C. One part was stored half-diluted in glycerol at –18 °C.

The chemokine CXCL8 was quantified in milk whey samples from a previous experiment [43]. In this experiment, six cows had been inoculated in one quarter with 50–100 colony-forming units of *S. aureus* strain 107.59, and foremilk samples had been collected once a day for five days, then at 7, 11, 14, and 21 days post-inoculation. Milk whey samples had been prepared by centrifugation at $90\,000\times g$ for 30 min, and stored frozen in portions at –20 °C.

2.2. Determination of milk SCC and proportion of neutrophils

Milk somatic cells were counted on an automated cell counter (Fossomatic model 90; Foss Food Technology, Hillerod, Denmark) as described [4]. For the determination of the proportion of neutrophils among milk somatic cells, cytospin slides were prepared with a cytocentrifuge (Shandon Southern centrifuge, Shandon Inc, Pittsburgh, PA, USA). Aliquots (50 to 100 μL) of undiluted milk or milk diluted with an equal volume of phosphate-buffered saline (as a function of the magnitude of the inflammatory modifications of mammary secretion) were mixed with 50 μL of foetal calf serum and centrifuged at $100\times g$ for 10 min. A differential cellular count was performed under a microscope after May-Grünwald staining, by counting at least 100 cells per slide.

2.3. ELISA for bovine serum albumin (BSA) and lactoferrin quantification

Concentrations of bovine serum albumin (BSA) were measured in milk by competitive enzyme-linked immunosorbent assay (ELISA). Prior to analysis, skim milk samples were diluted 1:10 000 with ELISA buffer (phosphate buffered saline supplemented with 0.5% gelatin). The ELISA was carried out with two 96-well flat-bottomed plates (Nunc Immunoplate Maxisorp, Roskilde, Denmark), referred to as mixing plates and ELISA plates. To each well of the mixing plate, 50-μL skim milk sample or BSA standards (Uptima Interchim, Montluçon, France) were incubated overnight at 4 °C with 50 μL of 1:100 000 dilution of rabbit anti-BSA prepared as described [35]. To prepare the ELISA plate, 100 μL of BSA solution (1 μg/mL) in sodium carbonate-bicarbonate buffer 0.1 M pH 9.6 were distributed in the wells and the plate was incubated overnight at 4 °C. Then the plate was washed with deionized water and blocked with gelatin (100 μL of ELISA diluent) for 30 min at 37 °C. Meanwhile, the mixing plate was warmed at 37 °C. Then the ELISA plate was washed and the content of the mixing plate was transferred in the ELISA plate with a multichanneled pipette. The plate was incubated for 60 min at 37 °C. Washing was performed with phosphate buffered saline (PBS) supplemented with 0.05% Tween 20, using a MW 96/384 microplate washer (Beckman Coulter Inc., Fullerton, CA, USA). The plate was then incubated with 100 μL of peroxidase-conjugated goat antibody to rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted 1:10 000 for 30 min. After washing, the wells received 100 μL of a 2% dilution of 2, 2′-azino-bis(3-ethylbenzthiazoline-sulfonate) (ABTS; Sigma) in 0.1 M citrate buffer pH 4.2, with the addition of 0.075% hydrogen peroxide just before use. The optical density was read at 414 nm on a microplate reader (BioKinetics reader; BioTek Instruments, Winooski, VT, USA). The values expressed in μg/mL were extrapolated using linear regression from the standard curve obtained with the known concentrations of BSA.

To measure the concentrations of lactoferrin in milk, a competitive ELISA was used as for BSA, except that rabbit anti-lactoferrin [36] was used, at the 1:100 000 dilution, and the standards were prepared with bovine lactoferrin prepared as described [36]. Samples were diluted 1:10 000 or more in ELISA diluent.

2.4. Quantification of IL-1β, TNF-α, C5a, CXCL1, CXCL2, CXCL3, and CXCL8

Milk IFN-γ concentrations were determined with a commercially available bovine IFN-γ ELISA kit (Mabtech AB, Nacka Strand, Sweden). Pilot experiments indicated that milk did not interfere with the detection of IFN-γ. The lowest level of quantification in milk was 0.016 ng/mL.

To determine the concentrations of IL-1β, a sandwich ELISA making use of mouse anti-IL-1β

monoclonal antibody (MAb 1D4; AbDSerotec, Oxford, UK), recombinant bovine IL-1β (AbD-Serotec) and rabbit anti-bovine IL-1β (AbDSerotec) was performed as described [4, 43]. The lowest level of quantification in milk was 0.10 ng/mL.

ELISA were performed as described elsewhere for TNF-α [37], C5a [38], and for CXCL8 (IL-8), CXCL1 (GRO-α), CXCL2 (GRO-β) and CXCL3 (GRO-γ) [40]. The lowest levels of quantification in milk were 0.2, 0.1, 0.15, 0.01, and 0.1 ng/mL for CXCL1, CXCL2, CXCL3, CXCL8, and TNF-α, respectively.

2.5. Statistical analysis

Statistical analyses of the variations in concentrations of analytes in milk were performed with the non-parametric exact Friedman test (StatXact 5; Cytel software Corp., Cambridge, MA, USA). Concentration comparisons as a function of LTA concentrations were carried out with the same test, considering the data as related series (three doses, 0, 10 and 100 μg LTA) at each time post-infusion. When variations were significant ($p < 0.05$), the significance of variations relative to time zero (infusion time) was analyzed by multiple comparisons between concentrations. Multiple comparisons were done with Georgin and Gouet software [15], who applied Siegel and Castellan's non parametric solution [49], with a Bonferroni correction. A probability level $p < 0.05$ was considered significant, taking into account one-sided p-values for components not present in milk before challenge (concentrations can only increase), and two-sided p-values for components already present in milk at the time of challenge (concentrations could vary both ways).

3. RESULTS

3.1. Cell concentrations in milk and clinical signs

A preliminary experiment was designed to evaluate the doses of LTA that trigger an inflammatory response when infused in the mammary gland through the teat canal. Cell concentrations did not increase in control quarters that received the vehicle (RPMI 1640) or in quarters infused with 0.1 or 1 μg of LTA (Fig. 1). An inflammatory response was obvious with 10 μg LTA, and the response
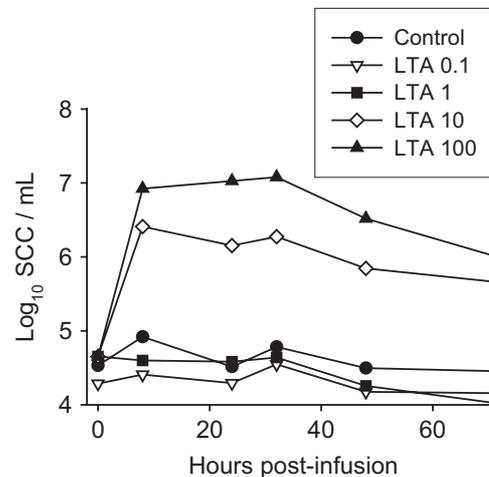

Figure 1. Concentrations of somatic cells in the milk of quarters infused at 0 h with different amounts (0.1, 1, 10 or 100 μg) of *S. aureus* lipoteichoic acid. Each value is the mean of three quarters of three different cows. Three cows had a control quarter that received the vehicle only (RPMI 1640). Samples were taken just before infusion, and 8, 24, 32, 48 and 72 h post-infusion.

provoked by 100 μg was somewhat higher (Fig. 1). Consequently, the two highest doses were retained for the following experiment that involved six other cows.

During the main experiment, a transient fever episode followed the intramammary infusion of LTA, with a significant ($p < 0.001$) increase in rectal temperature that peaked at 8 h post-infusion (hpi) (supplemental data available online only at www.vetres.org ; Fig. A). Local clinical signs of mastitis, including udder swelling and abnormal milk (clots and flakes), were evident in five out of six cows within 8 h of infusion of LTA at the dose of 100 μg, with a maximum at 16 hpi. With 10 μg LTA, only slight and irregular local clinical signs were detected between 8 and 16 hpi.

The inflammatory reaction to the two higher LTA doses was confirmed. An increase in cell concentrations was evident by 4 hpi for the two LTA doses (Fig. 2A). Cell concentrations in milk peaked at 6.7×10^6/mL with
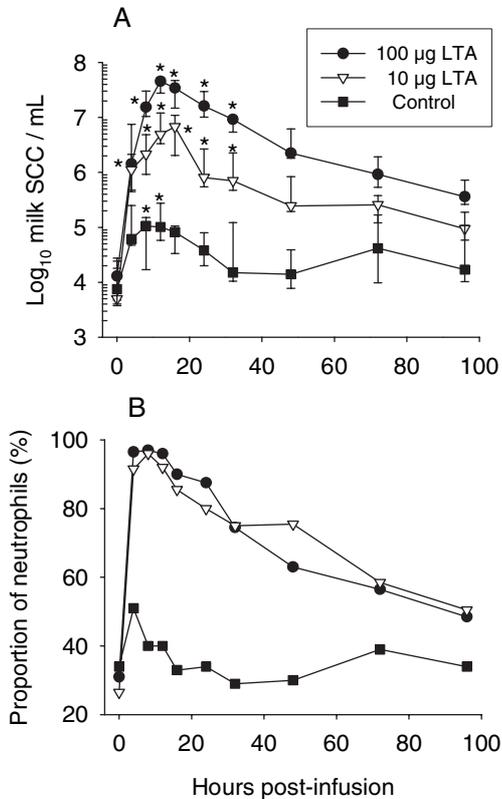
Figure 2. Effect of intramammary infusion with LTA on milk SCC (A) and proportion of neutrophils among recruited cells (B). Milk SCC were quantified in foremilk samples throughout the study, and the proportion of neutrophils determined after cytocentrifugation and May-Grünwald-Giemsa staining. The results are from six cows that received 10 μg LTA in one quarter, 100 μg LTA in another quarter, and vehicle only in a third quarter. Data are median values (and interquartiles in A).
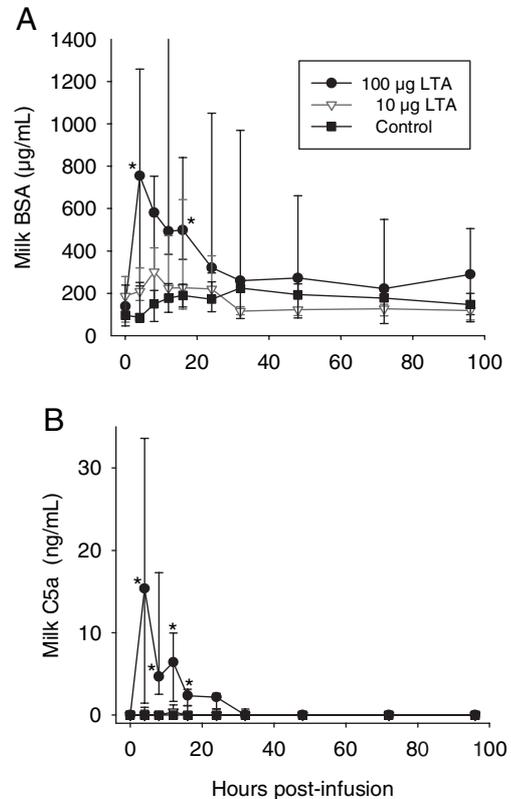

Figure 3. Concentrations of BSA (A) and of the complement-derived chemoattractant C5a (B) in skimmed milk samples of the quarters infused with *S. aureus* LTA from the time of infusion (time zero) to 96 hpi. Data are from six cows (median values and interquartiles). C5a values in control quarters and in quarters challenged with 10 μg LTA are superimposed. * Significantly increased concentration relative to time zero ($p < 0.05$).

10 μg LTA at 16 hpi, and at 45.7×10^6/mL at 12 hpi with 100 μg LTA (median values). The decrease in SCC was marked at 24 hpi. Cell concentrations after infusion of 10 μg and 100 μg LTA were not statistically different. Before challenge, mononuclear cells dominated in milk, but the cells that were recruited at the beginning of the inflammatory response were mainly neutrophils ($> 90\%$) from 4 to 12 hpi (Fig. 2B). Then, the proportions of neutrophils decreased to about 50% by 96 hpi (Fig. 2B).

Bacteriological examination of milk samples showed that the infused quarters did not shed bacteria, thus had remained uninfected throughout the experiment.

3.2. Milk concentrations of serum albumin and complement fragment C5a

Quarters infused with 100 μg LTA demonstrated an increase in BSA concentrations that peaked at 4 hpi and had almost returned to baseline values at 24 hpi (Fig. 3A).

Only a slight but significant elevation of BSA concentration ($p = 0.027$) was induced by 10 μg LTA with a maximum at 8 hpi. The generation of the chemotactic complement fragment C5a is favored by exudation of plasma that results in increases in availability of complement factors in milk, and also requires the activation of complement. Before challenge, C5a was not detected in milk. A significant generation of C5a occurred only with the highest dose of LTA (100 μg), in all the six challenged cows: concentrations peaked at 4 hpi and returned to normal (absence) by 32 hpi (Fig. 3B). With 10 μg LTA, five of the six cows demonstrated erratic and slight increases (< 4 ng/mL) between 4 and 24 hpi, whereas C5a remained undetected in milk samples of control quarters. C5a concentrations were significantly higher ($p < 0.05$) in 100 μg than in 10 μg LTA-infused quarters at 4 and 8 hpi.

3.3. Milk concentrations of ELR + CXC chemokines

The chemokines that have the trio of amino acids glutamate-leucine-arginine (ELR) before the CXC motif are specific neutrophil chemoattractants. Variations in concentrations of four of these chemokines were monitored in milk during the inflammatory response: CXCL1 (also known as GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ or ECIP-1), and CXCL8 (IL-8).

Before challenge, CXCL8 was undetectable in milk. The median concentration of CXCL1 was very low (0.15 ng/mL), and below the detection threshold of ELISA in several quarters. Median concentrations of CXCL2 and CXCL3 were 12 and 357 ng/mL, respectively. These two chemokines are constitutively expressed in bovine milk [40].

Intramammary infusion of LTA induced the secretion of the two chemokines CXCL2 and CXCL8 in milk as early as 4 hpi. (Fig. 4). For CXCL8, concentrations peaked at 33 ng/mL at 4 hpi with 100 μg LTA, versus 7.6 ng/mL with 10 μg LTA (Fig. 4D). The CXCL8 response was of short duration, the median

concentration being only 1.8 ng/mL at 16 hpi. CXCL8 concentrations were significantly higher ($p < 0.05$) in 100 μg than in 10 μg LTA-infused quarters at 8 hpi. The CXCL2 response was comparable to the CXCL8 response, but of lower magnitude, and of slightly longer duration (Fig. 4B). As for the two constitutive milk chemokines, the responses were less marked. The only distinct increase was for CXCL1 at 4 and 8 hpi with 100 μg LTA (Fig. 4A). With 10 μg LTA, neither CXCL1 (except at 4 hpi) nor CXCL3 concentrations increased significantly relative to 0 hpi (Figs. 4A and 4C). An increase in CXCL3 concentrations was induced by 100 μg LTA, from 373 ng/mL at 0 hpi to 584 ng/mL at 8 hpi (Fig. 4C), but this increase was statistically significant only at 4 and 8 hpi. The low concentrations of CXCL3 in the 16 hpi milk samples may have resulted from the marked alteration of milk in these samples (clots and flakes).

3.4. Milk concentrations of inflammatory cytokines

The cytokine TNF-α was not found in milk before challenge. It was not detected at any time in milk of control quarters or of quarters challenged with 10 μg LTA. It was found only in five of the six quarters challenged with 100 μg LTA, at 4 and 8 hpi only (0.52 ng/mL at both times, median values). The cytokine IFN-γ was detected in only one quarter that had received 100 μg LTA, at 12 and 16 hpi (21 and 12 pg/mL, respectively).

Intramammary infusion of LTA induced increases in IL-1β concentrations in the milk of challenged quarters. Initial increases in IL-1β levels were apparent at 4 hpi in the milk of quarters challenged with 100 μg LTA, and at 8 hpi in quarters challenged with 10 μg LTA (Fig. 5). Values peaked at 12 hpi or 16 hpi as a function of the LTA dose, and were higher with the 100 μg than with the 10 μg LTA challenge. Return to baseline (undetectable concentration) occurred at 24 hpi or 72 hpi in quarters challenged with 10 μg or 100 μg LTA, respectively (Fig. 5).
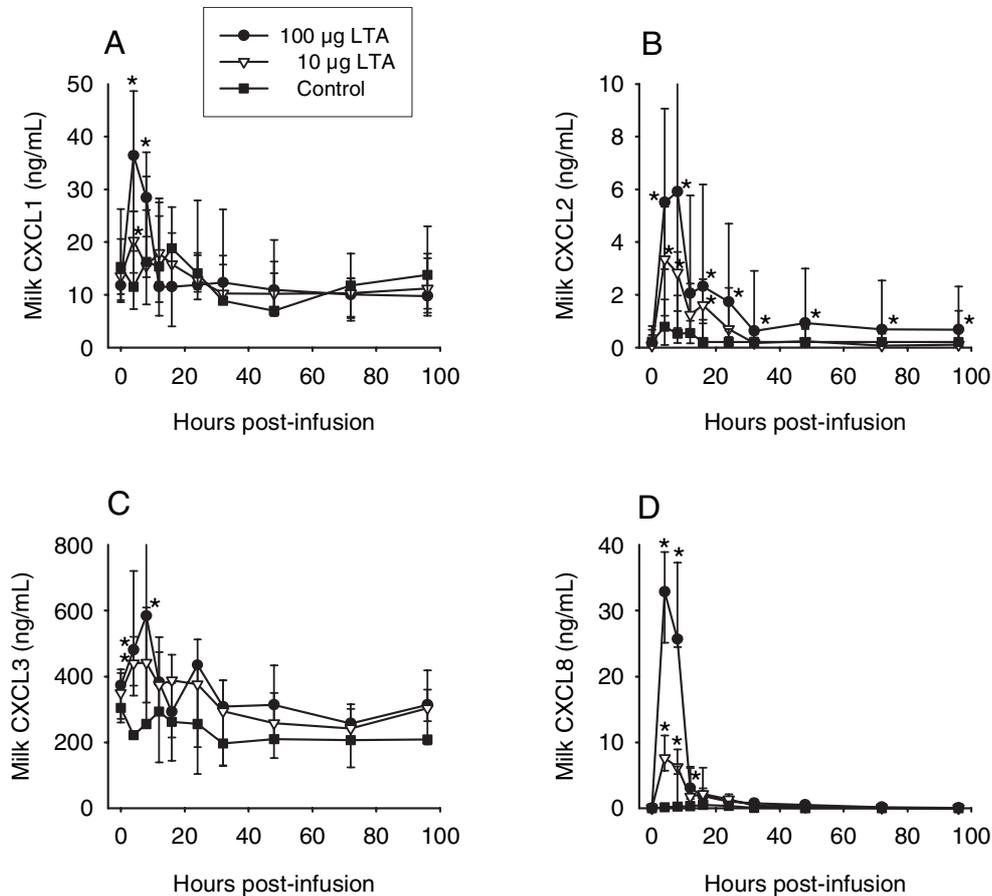
Figure 4. Concentrations of the ELR + CXC, neutrophil-oriented chemokines CXCL1 (A), CXCL2 (B), CXCL3 (C) and CXCL8 (D) in skimmed milk samples of the quarters infused with *S. aureus* LTA from the time of infusion (time zero) to 96 hpi. Data are from six cows (median values and interquartiles). * Significantly increased concentration relative to time zero ($p < 0.05$).

3.5. Milk concentrations of lactoferrin

Under basal conditions, lactoferrin was detected in normal milk at the concentrations of 250 to 450 μg/mL (Fig. 6). Milk concentrations increased rather steadily from 4 hpi onwards, to reach a plateau of high values at 48 hpi and onwards, in all quarters. Increases from time zero onward were significantly higher in quarters challenged with 100 μg LTA ($p < 0.001$), and tended to be higher than in other quarters, but differences with values in quarters challenged with 10 μg LTA were not significant (Fig. 6).

3.6. Concentration of CXCL8 in the milk of quarters challenged with *S. aureus*

The use of the newly developed ELISA allowed us to detect CXCL8 in the milk of five out of six challenged cows (one quarter per cow). The results of the individual animals are presented, because the infection process, and consequently the inflammatory response, of the cows was not synchronous (Fig. 7). Peak values ranged from 0.33 to 2.57 ng/mL among the five animals that responded to the challenge by an increase in CXCL8 concentration.
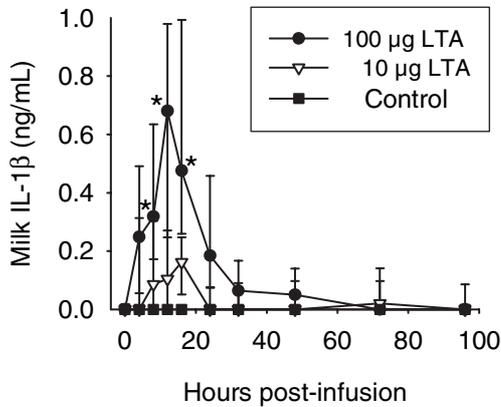
Figure 5. Concentrations of the inflammatory cytokine IL-1β in skimmed milk samples of the quarters infused with *S. aureus* LTA from the time of infusion (time zero) to 96 hpi. Data are from six cows (median values and interquartiles). * Significantly increased concentration relative to time zero ($p < 0.05$).

Figure 6. Concentrations of lactoferrin in skimmed milk samples of the quarters infused with *S. aureus* LTA from the time of infusion (time zero) to 96 hpi. Data are from six cows (median values and interquartiles). * Significantly increased concentration relative to time zero ($p < 0.05$).

4. DISCUSSION

These experiments showed that infusion of LTA alone in the mammary gland was sufficient to elicit a marked inflammatory response, characterized by a massive influx of neutrophils into milk. This suggests that during infection, LTA contributes to the recruitment of neutrophils, a reaction that is regularly induced by natural or experimentally induced *S. aureus* mastitis [1, 34].

The resident cells which in the mammary gland are likely to respond to LTA include the few macrophages and neutrophils of milk, but mammary epithelial cells (MEC) may be of prime importance owing to their exposed situation and overwhelming number compared to leucocytes in healthy lactating mammary glands [17, 39]. It has been shown that cultures of bovine MEC respond to *S. aureus* LTA [51]. The receptor complex involved in the recognition of LTA by MEC has not been characterized. It has been demonstrated that TLR2 is a major transducer of *S. aureus* LTA signaling for leucocytes [45], and that TLR6 was found to be an essential component of the receptor for *S. aureus* LTA, probably
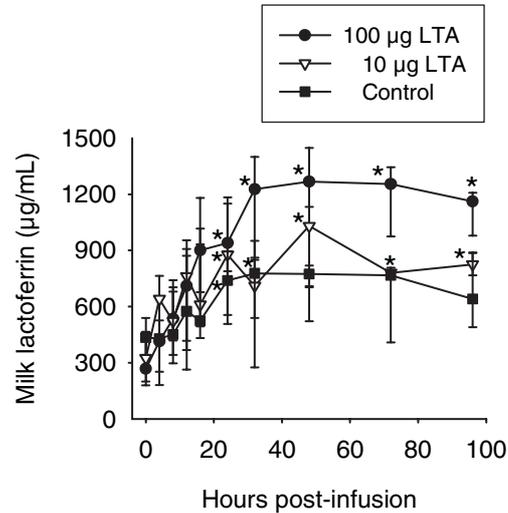
as a constitutive member of the heterodimer TLR2/TLR6 [22]. The recognition of LTA by leucocytes requires TLR2 and is improved by interaction with CD14 [45]. Bovine mammary tissue and bovine MEC express transcripts of the gene coding TLR2 [16, 51], and the protein is expressed on bovine MEC [24]. Bovine MEC express transcripts for CD14 [51], and the protein is found on the milk fat globule membrane [42], which derives from the apical face of MEC. The scavenger receptor CD36 has been reported to facilitate the response of monocytes and γδ T lymphocytes to LTA [23, 28]. Since CD36 is expressed at the apical face of bovine mammary epithelial cells [18], it is possible that CD36 contributes to the response of the mammary gland to LTA. All this circumstantial evidence suggests that the bovine mammary gland is well equipped to respond to LTA.

The influx of neutrophils in milk, which was the hallmark of the inflammatory response to LTA, may have been linked to increases in ELR + CXC chemokine concentrations.
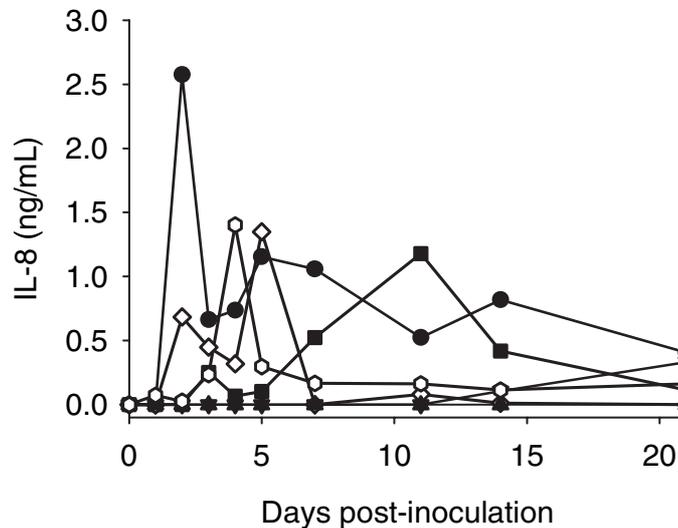
Figure 7. Concentrations of CXCL8 (IL-8) in milk whey of six cows challenged with *S. aureus* (one quarter per cow) from the day of inoculation (day 0) to 21 days post-inoculation [43]. Milk samples were collected at days 1, 2, 3, 4, 5, 7, 11, 14, and 21 post-inoculation. The values of the six individual cows are presented.

Intramammary infusion of LTA induced the secretion of CXCL8 in milk (Fig. 4). This observation diverges from previous findings obtained by infecting bovine mammary glands with *S. aureus* [4, 43] but is in keeping with the finding of IL-8 chemotactic activity in *S. aureus* mastitic milk [5]. Also, IL-8 has been detected in mastitic milk after infection of ovine udders with *S. aureus* [32]. Since the ELISA kit previously used to monitor the IL-8 concentrations in bovine milk, developed for human IL-8, had proved to be poorly adapted to bovine IL-8 [25], we used our newly developed ELISA to measure IL-8 concentrations in the milk whey samples used in a previous experiment [43]. IL-8 was detected in these samples, at concentrations slightly lower than those found in milk samples of quarters infused with 10 μg LTA (Fig. 7). It can be noted that very high concentrations of IL-8 (> 70 ng/mL) were found in bovine milk with ELISA reagents developed for ovine IL-8 after infusion with *E. coli* endotoxin [33], whereas much lower concentrations (> 1 ng/mL) were found in milk with an ELISA developed for human IL-8 during *E. coli* mastitis [4, 48]. It thus appears

that reagents of human IL-8 quantifying assays were not suitable for bovine IL-8. Our results indicate that, as for *S. aureus* mastitis, LTA induced the secretion of IL-8 in milk, although at moderate concentrations when compared to concentrations induced by *E. coli* LPS [33].

CXCL8 was not the only chemokine present in inflammatory milk after infusion of LTA. At the peak of the inflammatory response, at least four ELR + CXC chemokines were present in milk at rather high concentrations. One of them, CXCL3, is already in high concentrations (100–500 ng/mL) in normal milk, whereas CXCL1 is also present in normal milk, but at much lower concentrations (2–20 ng/mL) [40]. After infusion of 100 μg LTA, the concentrations of all four chemokines increased, whereas only the concentrations of the two chemokines CXCL2 and CXCL8, which were not detected in normal milk, increased after infusion of 10 μg LTA (Fig. 4). These chemokines target neutrophils by interacting with one (CXCL1, CXCL2, CXCL3) or two (CXCL8) receptors, CXCR1 and CXCR2, which are expressed by neutrophils of several species including humans, guinea pigs and cattle [26, 27, 53].

The chemokines CXCL1, CXCL2 and CXCL8 reached maximum concentrations very early (4 hpi, Fig. 4) in milk, which illustrates the prompt response of the mammary gland to LTA. The respective roles of the ELR + CXC chemokines in the recruitment of neutrophils in the mammary gland remain to be determined.

The increases in milk BSA concentrations, which mainly reflect increased permeability of the endothelial and epithelial barriers, were of low amplitude compared to endotoxin-induced mastitis: 3 to 10 mg/mL after intrammammary infusion of *E. coli* endotoxin [2, 46], compared to less than 1 mg/mL in this study. It is noteworthy that 10 μg LTA induced a change in BSA similar to that seen in experimental subclinical mastitis [43] and 100 μg LTA induced a change in BSA similar to that seen in clinical *S. aureus* mastitis [4]. Overall, these observations suggest that *S. aureus* LTA is a weaker inducer of vascular permeability than is *E. coli* LPS in the mammary gland.

The mammary gland reacted to infusion of LTA by a sizeable secretion of lactoferrin (Fig. 6). Increases in lactoferrin concentrations were significant with 10 μg and 100 μg LTA, but also in the control quarters. The appearance of inflammation in quarters adjacent to infected quarters has already been described [21], illustrating the fact that the udder quarters are not completely independent at the inflammatory and immune levels. On the contrary to the release of the other inflammatory mediators monitored in the study, the release of lactoferrin was progressive, reaching a maximum at 32–48 hpi when other mediators had returned to normal levels, and it was protracted, remaining at a plateau of elevated values up to the end of the experiment. Increased concentrations of lactoferrin have been reported in milk of chronic *S. aureus* mastitis [20]. The main source of lactoferrin is likely to be mammary epithelial cells, which constitutively secrete lactoferrin but are also able to upregulate its synthesis [44], which has been demonstrated in vitro by co-culture with *S. aureus* [56]. Since lactoferrin is endowed with many activities related to the defence of the

mammary gland [12, 39, 50], the response of mammary epithelial cells to LTA is likely to be of biological significance.

The cytokine pattern elicited by LTA mimicked that elicited by live *S. aureus*. Inflammation induced by 100 μg LTA was comparable to that seen during clinical *S. aureus* mastitis, whereas 10 μg LTA induced an inflammatory response resembling subclinical *S. aureus* mastitis [43]. LTA induced the release of chemoattractants such as CXCL8, as did *S. aureus* infection (Fig. 7). It also induced the release of IL-1β, in concentrations less than 1 ng/mL, comparable to those induced by experimental *S. aureus* mastitis [4]. At the dose of 10 μg, LTA did not induce increases in C5a and TNF-α, and only a slight increase in BSA. This profile resembles that seen following experimental infection with *S. aureus*.

In a mastitis model with another Gram-positive pathogen, *Streptococcus uberis*, IL-1β and TNF-α were found in milk, but only when clinical signs had developed, at least 48 h after the initial recruitment of leucocytes in milk [3, 41]. The low and delayed TNF-α and IL-1β responses may contribute to the apparent inefficiency of neutrophils to control the initial stages of intramammary infections by Gram-positive bacteria.

The purified LTA used in our experiments proved to be bioactive. This most likely results from the procedure used for its isolation, which retains the biological activity of the molecule, on the contrary to the previous phenol extraction procedure [30]. Nevertheless, our study determined that at least 10 μg of LTA was necessary to induce an inflammatory response in the mammary gland (Fig. 1). Assuming that LTA makes up about 2% of the dry cell weight [19], 20 μg LTA would equate to several billion staphylocci, numbers well above the numbers found in mastitic udders. Nevertheless, LTA is likely to be of biological significance because (i) recent data indicate that the physical presentation of LTA (such as immobilization on a solid surface) increases LTA activity by several orders of magnitude [11]; and (ii) LTA can act synergistically with other PAMP, which

could still lower the amount required to induce inflammation by an order of magnitude.

The absence or very low concentrations of TNF-α in mammary secretions after challenge strongly supports the absence of contaminating endotoxin in the commercial preparation, a problem with previous commercial preparations of LTA, because endotoxin is a strong inducer of TNF-α in the bovine mammary gland [7, 37, 47]. Despite the intense influx of neutrophils, the release of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ) was limited. Our results showed that a rather high amount of LTA was necessary to induce TNF-α, suggesting that the bovine mammary gland is only moderately responsive to LTA, as it is in general to *S. aureus*. It has been shown that, although MEC express TLR2, NF-κB is not activated by LTA in bovine MEC [57], which could contribute to the limited inflammatory reaction to LTA. Further studies are necessary to determine the roles of staphylococcal PAMP in the initiation and orientation of the inflammatory and immune responses of the mammary gland to *S. aureus* infection.

In conclusion, the bovine mammary gland responded to *S. aureus* LTA by a prompt and intense recruitment of neutrophils. This neutrophilic inflammation was concomitant with the release into milk of several ELR + CXC chemokines, which target primarily neutrophils, but it was not accompanied by a sizeable induction of pro-inflammatory cytokines. In that respect, the inflammatory responses to LTA resembled the initial response induced by *S. aureus* infection. LTA is likely to be one of the bacterial inducers of the response of the mammary gland to infection by *S. aureus*, and as such purified LTA seems to be a suitable and relevant tool to investigate the interactions of *S. aureus* with the mammary gland.

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