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Characterization of immune response in *Staphylococcus aureus* chronically infected bovine mammary glands during active involution

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ABSTRACT

The aim of this study was to characterize the immune response in *Staphylococcus aureus* chronically infected bovine mammary glands during active involution. Twenty-one Holstein non-pregnant cows in late lactation either uninfected or with chronic naturally acquired *S. aureus* intramammary infections (IMI) were included in this study. Cows were slaughtered at 7, 14 and 21 d after cessation of milking and samples for immunohistochemical analysis were taken. Protein expression of toll-like receptor 2 (TLR2) and TLR4 was significantly higher in *S. aureus*-infected quarters than in uninfected controls at the three involution stages studied. Protein expression of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1α and IL-17 was significantly affected by IMI; being higher in *S. aureus*-infected than uninfected quarters during all evaluated stages. In *S. aureus*-infected and uninfected quarters protein expression of lactoferrin increased from day 7–14 of involution, decreasing significantly to day 21 in mammary quarters with chronic infections. The number of monocytes-macrophages was significantly higher in *S. aureus*-infected than in uninfected control quarters at 7 and 21 d of involution. The number of T lymphocytes was significantly higher in *S. aureus*-infected than in uninfected quarters at 7 and 14 d of involution while the number of B lymphocytes was significantly higher in *S. aureus*-infected than in uninfected quarters during all evaluated stages, showing a progressive increase as involution advanced. These results demonstrated a sustained and exacerbated innate and adaptive immune response during chronic *S. aureus* IMI, playing a critical role in the infection control during active involution.

1. Introduction

The nonlactating period prior to parturition in dairy cows is commonly referred to as the dry period. Duration of the nonlactating interval is an important determinant of milk production in the subsequent lactation [1]. Without a dry period, milk production may be reduced by 20%; and there is general agreement that a dry period of 40–60 days is required for optimal production [2]. Mammary gland involution is a period of intensive tissue remodeling. Over the course of a relatively brief period, a large proportion of the mammary gland epithelium undergoes apoptosis and is removed by phagocytes. In addition, the gland is cleared of residual milk fat globules as well as milk, and adipocytes become the predominant cell type. Professional phagocytes derived from the immune system can participate in the clearance of apoptotic and autophagic cells, the removal of residual milk components and the prevention of mastitis during mammary gland involution [3]. However, the role of the immune system in this process in the bovine mammary gland has not been clearly defined [4,5].

The early dry period is a critical stage, since changes that lead to increased concentration of protective factors, compared with lactating mammary glands, occur gradually over several days [6]. Increased incidence of infection during the dry period results in an elevated number of infected quarters at calving and is responsible for the high level of intramammary infections (IMI) during lactation in many herds. Such infections cause inflammation and affect mammary cell differentiation prior to calving, resulting in decreased milk production in the

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subsequent lactation [7].

Staphylococcus aureus is one of the most prevalent contagious pathogenic bacteria causing IMI and most likely presents as chronic subclinical mastitis with a low cure rate after conventional antibiotic therapy [8,9]. *S. aureus* IMI persistence relies on this organism's capacity to produce several virulence factors for the colonization of the mammary gland and evasion of the host immune system leading to ineffective pathogen clearance [9,10].

The defense mechanisms of the mammary gland function optimally when invading bacteria are recognized promptly, the initial inflammatory response is adequate to rapidly eliminate the infection, and the mammary gland returns to normal function without any noticeable clinical sign. Suboptimal or dysfunctional mammary gland defenses, however, may contribute to the development of severe acute inflammation or chronic mastitis that adversely affects the quantity and quality of milk [10]. The mechanisms by which *S. aureus* persistent infections are maintained in dairy cows involve both bacterial escape strategies and modulation of the host immune response [11,12]. Differences in the magnitude and duration of host responses are determined, in part, from specific bacterial virulence factors [10].

Despite substantial progress made in understanding the mechanisms employed by *S. aureus* to persist within the host, very few studies have addressed the host immune mechanisms evoked during a persistent staphylococcal IMI [13]. Characterization of components of innate and adaptive immune response in mammary tissue of cows infected with *S. aureus* during active involution is necessary to understand the host defense capability against chronic *S. aureus* infection and the impact of this response in mammary functionality. Therefore, knowledge about the defense mechanisms triggered during involution would enable the development of new strategies to control or treat *S. aureus* mastitis. The aim of this study was to characterize the immune response in *S. aureus* chronically infected bovine mammary glands during active involution. Pattern recognition receptors, soluble factors, cytokines and immune cells were evaluated in mammary tissue.

2. Materials and methods

2.1. Animals and experimental design

Twenty-one Holstein non-pregnant cows in late lactation (weeks 31–36) from the Rafaela Experiment Station of INTA herd were included in the study. Cows were from parity 3–5, milked twice daily, produced and average of 25 kg milk/d during lactation and an average of 12 kg milk/d before interruption of lactation. Cows with similar lactation number were included in each experimental group (uninfected and *S. aureus*-infected). All procedures used in this study were approved by the Ethics and Security Committee of the Facultad de Ciencias Veterinarias, UNL and consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies (2010)).

The infection status of mammary quarters was determined within 6 months before initiation of the experiment and confirmed 20 d and 3 d before cessation of milking. Infections were naturally acquired either in the previous dry period or during the first 2 months of the lactation preceding initiation of the study. Cows with *S. aureus* mastitis were selected based on results of monthly somatic cell counts (SCC $> 250 \times 10^3$ cells/mL) and subsequent testing by bacteriological analysis of quarter milk. A quarter was considered to be infected if *S. aureus* was isolated from two consecutive samples. Infected quarters were randomly selected from cows showing at least two quarters infected with *S. aureus*. Only animals with subclinical IMI at the time of milking interruption were included. Uninfected quarters were selected from cows free of infection at the time of sampling with negative results of bacteriological analysis of quarters and SCC less than 250×10^3 cells/mL. Cows were slaughtered at 7, 14 and 21 d after cessation of milking at a local abattoir and samples for histological analysis were taken. The experimental unit of study was the mammary quarter. Uninfected (n = 8) and *S. aureus*-infected (n = 8) mammary quarters in each time of sampling (7, 14 and 21 d) were included. At every sampling time 7 animals were used (3 with uninfected quarters and 4 with two quarters infected with *S. aureus*). According to the eligibility criterion used for experimental units' selection, 21 cows were included in the study (12 cows with at least two quarters infected with *S. aureus* and 9 uninfected cows).

2.2. Bacteriological examination

Mammary secretion samples (10 μL) were streaked onto blood agar plates supplemented with 5% bovine blood and incubated for 48 h aerobically at 37 °C. Plates were examined for bacterial growth at 24 h and 48 h. *S. aureus* was identified based on the hemolytic pattern on blood agar, catalase and coagulase tests and differentiated from other coagulase-positive *Staphylococci*, by acetoin production and selective growth on P agar with 7 μg/mL acriflavine [14,15]. Other mastitis pathogens were identified based on standard methodology [15]. The presence of one colony of *S. aureus* on blood agar was considered as a positive identification; therefore, detection limit was 100 colony forming units (CFU/mL).

2.3. Tissue sample preparation

Immediately after cows were slaughtered, three tissue samples were taken from selected mammary quarters from three zones following previous descriptions [16]. Zone 1 upper limit of the gland cistern; zone 2 approximately midway between the upper limit of the gland cistern and the dorsal boundary of the mammary gland at a depth of 4 cm (lobulo-alveolar zone) and zone 3 near to the dorsal boundary of the mammary gland (adjacent to abdomen).Tissue samples were fixed in 4% neutral buffered formalin, for 8 h and then washed in phosphate-buffered saline (PBS). For light microscopy, fixed tissues were dehydrated and embedded in paraffin wax. Sections (5 μm) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis MO, USA) and assigned for use in immunohistochemistry (IHC) procedures. Additional sections of mammary tissues were transferred into a freezing vial, weighed and placed in liquid nitrogen for Western blot assays.

2.4. Antigen/antibody specificity

To test the specificity of the primary antibodies used in the present study (Table 1), a pool of mammary gland tissue sections from the different experimental groups were homogenized in a radio-immunoprecipitation assay lysis buffer and a protease inhibitor cocktail as previously described by Baravalle et al. [17]. The homogenate was then centrifuged at 12,000 × *g* at 4 °C for 30 min and the supernatant was frozen at −80 °C. Protein concentration in the supernatants was estimated using fluorescence methods (QubitTM, Invitrogen). For the Western blot analysis, 40 μg of protein, along with pre-stained molecular weight markers (Bio- Rad, Hercules, CA, USA), were separated in SDS-polyacrylamide gels [10% resolving gel for toll-like receptor 2 (TLR2), TLR4 and bovine lactoferrin (bLf), and 15% resolving gel for cytokines] and transferred electrophoretically to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blotting, the membranes were blocked for 1 h 30 min in 5% nonfat milk in TBS containing 0.05% Tween-20 (Sigma-Aldrich Corp., St. Louis, MO) and then incubated overnight at 4 °C with specific primary antibodies. Following washing, membranes were treated for 1 h 30 min at 25 °C with secondary peroxidase-conjugated antibody (Table 1). Immunopositive bands were visualized with a chemiluminescent detection kit (ECL-Plus; GE-Amersham, Buckinghamshire, UK).

Table 1
Antibodies, source and commercially purchased reagents used.

Antibodies	Source	Company/catalogue number	pAB/mAB	Dilution IHC/WB
Primary antibodies				
Anti-TLR2	Rabbit	GenWay Biotech (GWB-B78984)	pAB	1:200/1:400
Anti-TLR4	Rabbit	GenWay Biotech (GWB-5C8433)	pAB	1:200/1:400
Anti-bLf[a]	Rabbit		pAb	1:100/1:500
Anti-TNF-α	Rabbit	Chemicon (AB1842)	pAB	1:100/1:150
Anti-IL-1α	Rabbit	Endogen (P420A)	pAB	1:100/1:150
Anti-IL-6 (clone 77830)	Mouse	R & D Systems (MAB 686)	mAB	1:200/1:400
Anti-IL-17	Rabbit	Abcam (ab79056)	pAB	1:150/1:200
Anti-CD14 (clone RPA-M1)	Mouse	Zymed (18-0121)	mAB	1:30
Anti-CD2 (clone CC42)	Mouse	Serotec (MCA833)	mAB	1:50
Anti-CD79 (clone HM57)	Mouse	Abcam (ab62650)	mAB	1:200
Secondary antibodies				
Polyvalent Biotinylated link (anti-mouse/anti-rabbit IgG2a)	Goat	Cell Marque (961D-22)	pAB	1:200 (IHC)
Anti-rabbit IgG peroxidase	Goat	Santa Cruz Biotech. (sc-2030)	pAB	1:7500 (WB)
Anti-mouse IgG peroxidase	Goat	Santa Cruz Biotech. (sc-2005)	pAB	1:600 (WB)

[a] Antibody donated by Chaneton et al. [49].

2.5. Immunohistochemistry

For IHC, the streptavidin-biotin immunoperoxidase method was performed as described by Andreotti et al. [18]. Briefly, sections were dewaxed, hydrated and subjected to microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol and non-specific binding was blocked with 10% normal goat serum (Sigma-Aldrich Corp., St. Louis, MO). Tissue sections were incubated with the primary antibodies (Table 1) for 18 h at 4 °C. Each antibody was used in at least 5 sections per sample. Slides were washed with PBS and incubated with the biotinylated secondary antibody for 30 min at 25 °C (Table 1). Detection was by a streptavidin-peroxidase solution (CytoScan HRP Detection System, Cell Marque), with 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA, USA) as chromogen. The sections were then counterstained with Mayer hematoxylin, dehydrated, and mounted. Negative control sections were included in which the primary antibody was replaced by non-immune rabbit serum. To exclude the possibility that endogenous peroxidase activity was unsuccessfully blocked, some sections were incubated with DAB reagent alone. Serial sections of bovine mammary tissue similarly processed and with known reactivity for the antigen were used as positive controls in each assay to normalize the image analysis.

2.6. Image analysis

Image analysis was performed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA). Images were digitized with an Olympus C5060 digital camera mounted on a conventional light microscope (Olympus BH-2; Olympus Co., Tokyo, Japan) using objective magnification of 40x. Image resolution was set to 1200 × 1600 pixels. Each pixel of the image corresponded to 0.13 μm^2 at the respective magnification and each field represented a tissue area of 0.031 mm^2. The system captured each image and automatically corrected for background. This prevented differential readings due to different lighting conditions. No further image processing was done. Details of image analysis as a valid method for quantifying expression levels and the methodological features were previously described [18,19]. Briefly, the immunohistochemical stained area (IHCSA) for each antibody reaction was calculated as a percentage of total area evaluated through the color segmentation analysis that extracts objects by locating all objects of the specific color (brown stain). The brown stain was selected with a sensitivity of 4 (maximum 5) and a mask was next applied to make separation of colours permanent. The IHCSA (% of black area) was calculated from at least 50 images in each one of the following structures: alveoli, ducts and interstitial tissue. The expression of TLR2, TLR4, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1α, IL-6, IL-17 and bLf was evaluated by positive IHCSA. For measuring number of monocytes-macrophages (stained with anti-CD14), T lymphocytes (stained with anti-CD2) and B lymphocytes (stained with anti-CD79), 40 random images from each slide were digitized at 40× and number of cells per mm^2 was calculated.

2.7. Statistical analysis

A statistical software package (SPSS 11.0 for Windows; SPSS, Inc., Chicago, IL) was used to perform statistical analysis. Data were analyzed using the general linear model procedure. The model tested for main effects of infection status, time and the interaction between the infection status*time. Individual means were compared by *t*-test. The level of significance was set at $P < 0.05$. Data are presented as means of three mammary gland zones obtained from the same mammary quarter ± SEM (standard error of the mean).

3. Results

3.1. Antibody specificity

Western blot recognition of proteins in mammary homogenates from uninfected and *S. aureus*-infected quarters are summarized in Figs. 1 and 3. Western blot analysis revealed positive bands of appropriate sizes for each of the proteins studied. The TLR2 and TLR4 antibodies detected single bands 89.8 kDa and 95.7 kDa, respectively (Fig. 1). The TNF-α antibody detected two bands at 17 kDa (soluble TNF-α) and 26 kDa (transmembrane precursor) (Fig. 3). The IL-1α and IL-6 antibodies detected a single band at 17 and 26 kDa, respectively (Fig. 3). The IL-17 antibody detected a single band at 18 kDa (Fig. 3). The bLf antibody detected a single band at approximately 80 kDa (Fig. 3).

3.2. Immunohistochemistry of TLR2 and TLR4

To obtain quantitative data for immunohistochemical labeling of TLR receptors and information about the localization in the bovine mammary gland, brown cytoplasmic staining was detected and then evaluated by the IHCSA. Representative patterns of TLR2 and TLR4 immunostaining and quantitative analysis of the immunoexpression of these proteins in *S. aureus*-infected and uninfected quarters are shown in Figs. 1 and 2.

Immunostaining for TLR2 was present in all tissue sections from all evaluated groups and was primarily associated with the apical domain of the epithelial cells lining the alveoli and ducts (Fig. 1). Cells with

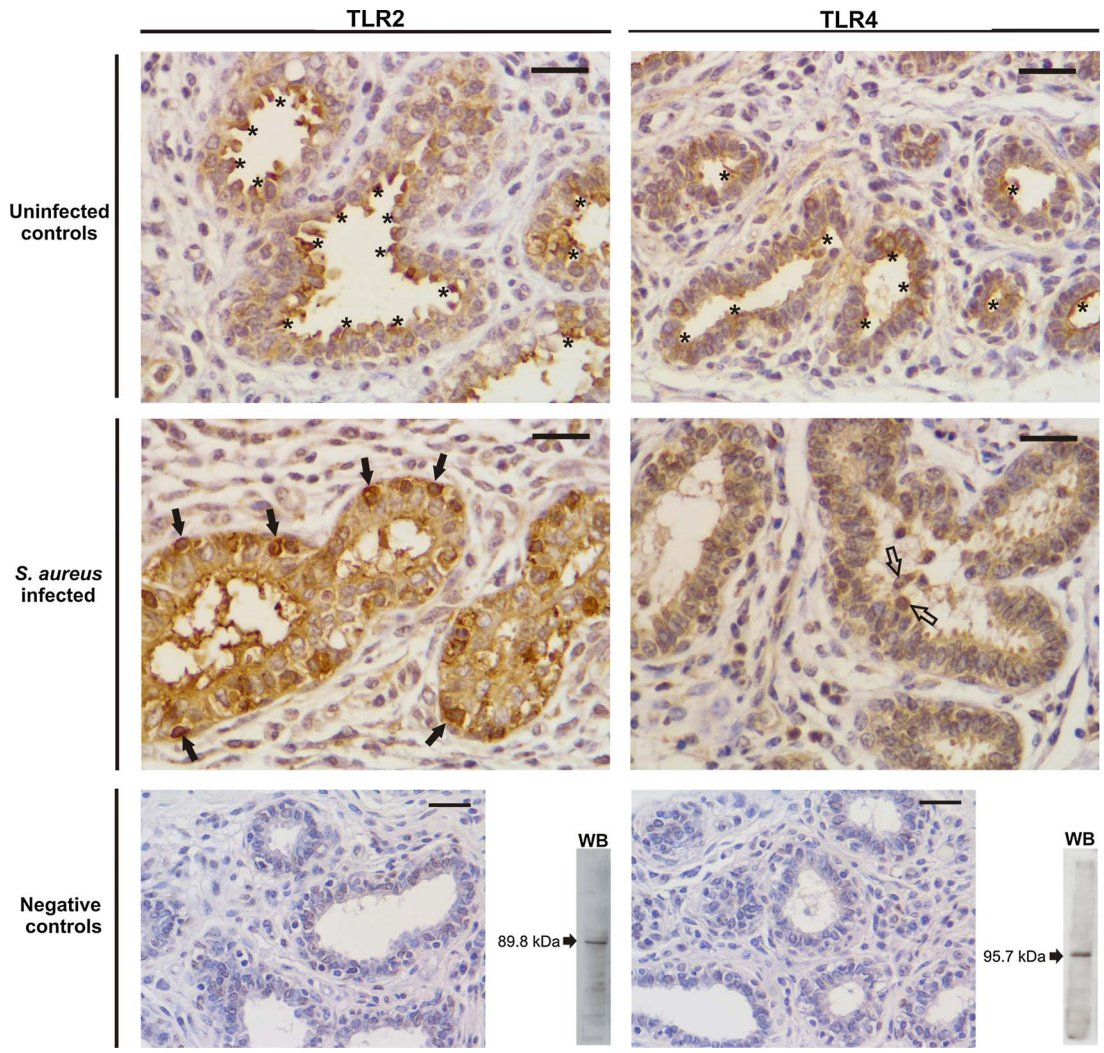

Fig. 1. Representative images of TLR2 and TLR4 immunostaining in uninfected and *S. aureus*-infected quarters at 14 d of involution are shown in the top panels. Immunoreactivity in the apical domain of epithelial cells to TLR2 and TLR4 antibodies are indicated with asterisks (*). Immunoreactivity of intraepithelial lymphocytes to TLR2 antibody is indicated with black arrows. Immunoreactivity of macrophages to TLR4 antibody is indicated with transparent arrows. Negative controls for immunostaining and verification of antibody specificity by Western blot analysis of mammary tissue homogenates are shown in the bottom panel. Magnification is the same for all panels and is indicated by 25 μm bars.

typical morphology compatible with macrophages in the lumen of the alveoli and in the stroma showed strong immunostaining. Furthermore, neutrophils, plasma cells and intraepithelial lymphocytes showed intense staining in their cytoplasm (Fig. 1). Protein expression of TLR2 in mammary tissue was significantly higher in *S. aureus*-infected than uninfected control quarters during all evaluated involution time and was affected by time of sampling (Table 2). In *S. aureus* infected quarters, the highest protein expression for TLR2 was observed at day 7 and gradually decreased towards 21 d of involution (Fig. 2A).

Immunostaining for TLR4 was present in all tissue sections at all evaluated groups and was associated with parenchymal and stromal mammary components. Intense immunoreaction was evident in the cytoplasm and at the luminal surface of alveolar and ductal epithelial cells (Fig. 1). Intense staining in the cytoplasm of cells with morphology compatible with macrophages, neutrophils, plasma cells and intraepithelial lymphocytes was observed. Protein expression of TLR4 in mammary tissue was significantly higher in *S. aureus*-infected than uninfected control quarters at 7, 14 and 21 days of involution and was affected by time of sampling (Table 2; Fig. 2B).

3.3. Immunohistochemistry of cytokines and lactoferrin

Representative patterns of TNF-α, IL-1α, IL-6, IL-17 and bLf immunostaining in *S. aureus*-infected and uninfected quarters are shown in Fig. 3, and a quantitative analysis of the expression of these proteins from immunohistochemical analysis is shown in Fig. 4.

Immunostaining for TNF-α was associated with mammary parenchymal and stromal structures in all studied quarters. Intense immunostaining in the cytoplasm of epithelial cells lining the alveoli and ducts was observed. Cells with typical morphology compatible with macrophages, neutrophils, lymphocytes and fibroblast, as well as endothelium and vascular smooth muscle cells showed intense immunostaining (Fig. 3). Protein expression of TNF-α in mammary tissue was significantly affected by *S. aureus* infection (Table 2); being the percentages of IHCSA higher in *S. aureus*-infected than uninfected quarters at all involution stages evaluated (Fig. 4A). Immunostaining for IL-1α was detected in *S. aureus*-infected and uninfected quarters at all involution stages evaluated and was associated with parenchymal and stromal structures. Intense immunostaining in the cytoplasm of epithelial cells lining the alveoli and ducts was observed. Cells with morphology compatible with macrophages in the lumen of the alveoli and in the stroma showed strong cytoplasm immunostaining. Moreover, neutrophils, intraepithelial lymphocytes and fibroblast, as well as endothelium and vascular smooth muscle cells, showed intense immunostaining. Protein expression of IL-1α was significantly affected by *S. aureus* infection (Table 2); being the percentages of IHCSA higher in

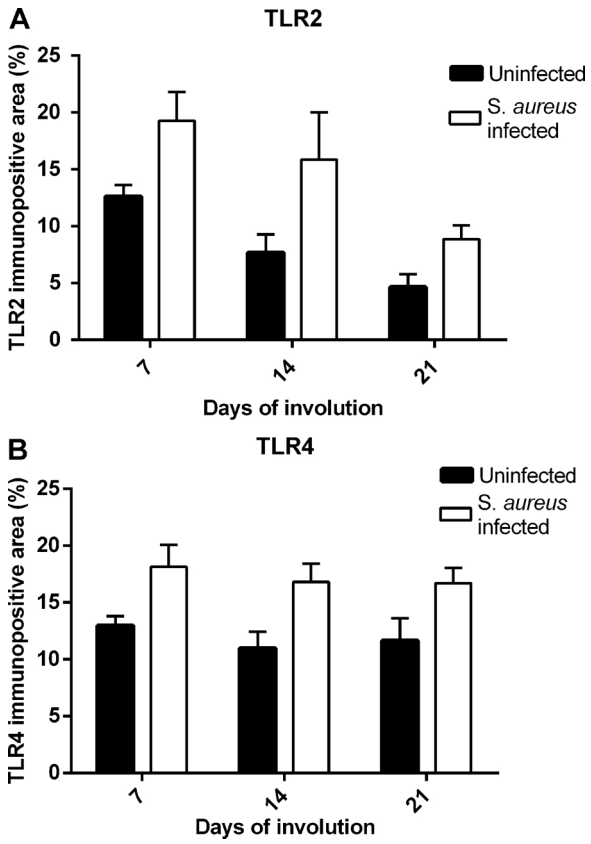

Fig. 2. Relative expression (measured as percentage of immunopositive area) of (A) TLR2 and (B) TLR4 in uninfected and *S. aureus*-infected quarters at 7, 14 and 21 days of involution. Values represent the mean ± standard error of the mean (SEM).

S. aureus-infected than in uninfected quarters at 7 and 14 d of involution ($P < 0.001$; Fig. 4B).

Immunostaining for IL-6 was observed in all tissue sections from the three evaluated groups. The pattern of localization for IL-6 was similar to IL-1α expression (Fig. 3). The percentages of immunostaining for IL-6 were not affected by infection status and time of involution without interaction among these factors (Table 2; Fig. 4C).

Immunostainig for IL-17 was present in all tissue sections at all involution stages evaluated and was associated with stromal and parenchymal components. Intense immunostaining in the cytoplasm of epithelial cells lining the alveoli and ducts was observed. Cells with typical morphology compatible with macrophages and neutrophils in the lumen of the alveoli and in the stroma showed strong immunostaining in their cytoplasm. IL-17 positive lymphocytes closely associated with the epithelium linin were found in the connective tissue (Fig. 3). Protein expression of IL-17 was significantly affected by *S. aureus* infection, time of sampling and interaction between these factors (Table 2); being percentages of immunopositive area for IL-17 higher in *S. aureus*-infected than in uninfected quarters at 14 ($P < 0.01$) and 21 d of involution ($P < 0.001$; Fig. 4D).

Bovine lactoferrin staining was present in all tissue sections at all involution stages evaluated and was associated with stromal and parenchymal components. A definite labeling of the apical part of the epithelial cells lining the alveoli and ducts was observed (Fig. 3). Cytoplasm of immune cells (macrophages, lymphocytes and neutrophils) showed intense staining (Fig. 3). The percentages of immunostaining for bLf were significantly affected by infection, time of involution and interaction between these factors (Table 2). In *S. aureus*-infected and uninfected quarters the percentages of immunostaining for bLf increased from day 7–14 of involution, decreasing significantly to day 21 in the mammary quarters with chronic infections ($P < 0.001$; Fig. 4E).

3.4. Monocytes-macrophages and lymphocytes quantification

Fig. 5 shows representative images of CD14, CD2, and CD79 immunopositive cells in uninfected and *S. aureus*-infected quarters. Monocytes-macrophages labeled with anti CD14 were localized in the stroma surrounding the alveolar epithelial cells. Some immunopositive cells were found within lining epithelial cells and in the lumina of alveoli and ducts. Mature macrophages were large, with pale nuclei and vacuolated cytoplasm and staining occurred strongly on their surfaces (Fig. 5). The number of monocytes-macrophages in mammary tissue was significantly influenced by infection, time of sampling and interaction between these factors (Table 2). The number of CD14+ cells was higher in *S. aureus*-infected than in uninfected quarters at 7 ($P = 0.004$) and 21 d ($P = 0.007$) of involution (Fig. 5A).

The CD2+ cells were located throughout the MEC, around the alveoli and within the connective tissue (Fig. 5). The number of CD2+ cells in mammary tissue was significantly affected by infection, time of sampling and interaction between these factors (Table 2); being the number of CD2+ cells higher in *S. aureus*-infected than in uninfected quarters at 7 and 14 d of involution ($P < 0.001$) (Fig. 5B).

The CD79+ cells were localized in the connective tissue surrounding the alveolar epithelial cells. In *S. aureus*-infected sections clusters of cells, mainly in areas with no alveoli, were observed (Fig. 5). The number of CD79+ cells in mammary tissue was significantly affected by infection, time of sampling and interaction between these factors (Table 2); being the number of CD79+ cells higher in *S. aureus*-infected than in uninfected quarters at 7, 14 and 21 d of involution ($P < 0.001$; Fig. 5C).

4. Discussion

The mechanisms by which *S. aureus* IMI are established and maintained in dairy cows involve both bacterial escape strategies and modulation of the host immune response [11,12]. However, little information is available about the immune response mechanisms that are triggered in S. *aureus* chronically infected mammary glands during the dry period. In this study we characterized different components of the immune response in *S. aureus* chronically infected bovine mammary glands during active involution.

Pathogens that enter the mammary gland lumen are sensed via TLRs that recognize pathogen associated molecular patterns (PAMPs) [20]. TLR2 and TLR4 gene expression by MEC, monocytes-macrophages and neutrophils has been reported [21,22]; however, expression at the protein level has been poorly documented. In uninfected and *S. aureus*-infected quarters, TLR2 and TLR4 were abundantly expressed and localized in the apical domain of ductal and alveolar epithelium. Moreover, we observed positive reaction in the cytoplasm of immune cells like macrophages, neutrophils, plasma cells and intraepithelial lymphocytes. These findings are consistent with previous reports in mammary tissue of healthy cows where TLR2 and TLR4 were detected in parenchymal and stromal cells by IHC after 7 d of drying off [17].

Previous studies in mammary gland tissues of cows with clinical mastitis demonstrated, that bovine TLR2 and TLR4, but not TLR9 are selectively up-regulated in both *Escherichia coli* and *S. aureus* infected animals [23], indicating the participation of both receptors in the recognition of these pathogens. Likewise, Yang et al. [24] observed that *S. aureus* and *E. coli* were able to induce a similar increase in genic expression of both TLR2 and TLR4 receptors in primary bovine mammary epithelial cells (pbMEC). In the present study, although the gene expression of these receptors was not evaluated, our results showed a significant effect of *S. aureus* IMI on protein expression of TLR2 and TLR4 in mammary tissue, as indicated by higher percentages of immnostaining in infected than uninfected quarters at all involution stages studied. Although PAMP recognition and triggering of an innate immune response to an invading pathogen is a crucial feature of TLR; these receptors also recognize endogenous mediators that are released

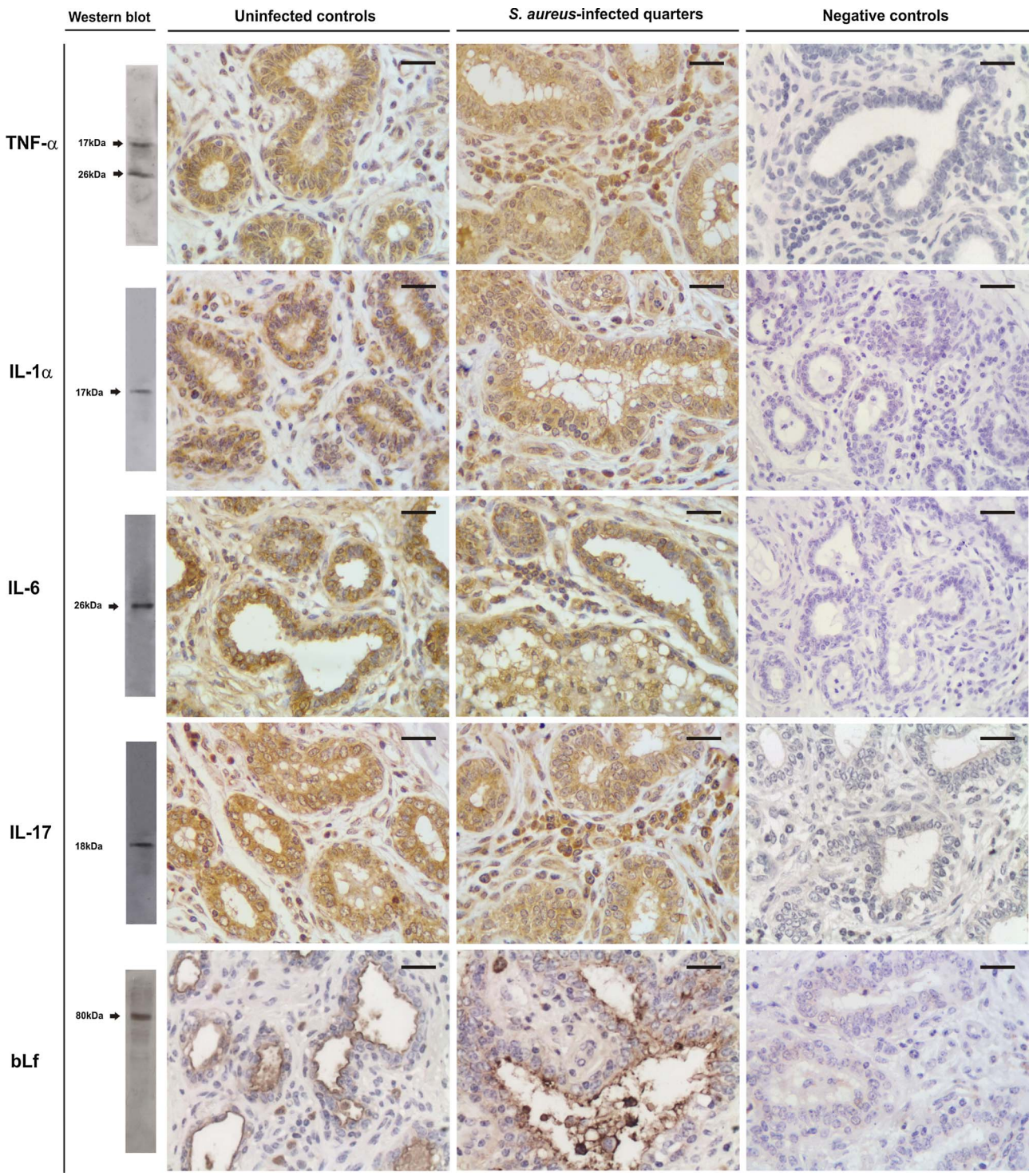

Fig. 3. Representative images of TNF-α, IL-1α, IL-6, IL-17 and bLf immunostaining in uninfected and *S. aureus*-infected quarters at 14 d of involution for cytokines and 21 d of involution for bLf. Negative controls for immunostaining are shown in the right panels. Verification of antibody specificity by Western blot analysis of mammary tissue homogenates are shown in the left panels. Magnification is the same for all panels and is indicated by 25 μm bars.

during infection by injured tissues and necrotic cells (damage-associated molecular patterns molecules or DAMPs) to warn the host of danger [25] inducing the activation of a strong pro-inflammatory response [26]. Increased TLR2 and TLR4 protein expression observed in *S. aureus*-infected quarters compared with uninfected quarters could also be associated with the recognition of damage molecules released by injured mammary tissue during chronic infection.

Activation of the TLRs initiates signal transduction pathways that culminate in the transcription of a wide range of immune genes including cytokines, which are synthesized by infiltrating cells [27] as well as resident cells in response to *S. aureus* infection [28]. TNF-α, IL1-β and IL-6 are the master pro-inflammatory cytokines that induce the production of other immune factors [29]. Transcripts for pro-inflammatory cytokines have been detected in milk somatic cells from cows both following experimentally induced *S. aureus* IMI [27] and in chronic IMI [30]; being this response characterized by a relatively low and short-lasting increase in these cytokines [24]. However, both *S. aureus* and host-factors contributing to this outcome have not been

Table 2
Two-way ANOVA analysis. The main effects of infection status (Is), time of involution (t) and infection status * time interaction (Is*t) for each variable studied are shown.

Parameter	P-values Is	P-values t	P-values Is*t
TLR2	P < 0.001	P < 0.001	n.s
TLR4	P < 0.001	P = 0.034	n.s.
TNF-α	P < 0.001	n.s.	n.s.
IL-1α	P < 0.001	n.s.	P < 0.001
IL-6	n.s.	n.s.	n.s.
IL-17	P < 0.001	P = 0.020	P = 0.001
bLF	P = 0.003	P < 0.001	P < 0.001
CD14	P < 0.001	P = 0.001	P = 0.038
CD2	P < 0.001	P = 0.004	P < 0.001
CD79	P < 0.001	P = 0.001	P < 0.001

n.s.: Not significant.

completely elucidated [31]. In the present study, all cytokines evaluated were expressed in mammary parenchyma and stroma structures both in uninfected and *S. aureus*-infected quarters, showing that these cytokines are constitutively produced in mammary glands of healthy cows at drying off. The biological significance of such a constitutive secretion is still unclear, although there is no doubt that the process of tissue-remodeling that occur during mammary gland involution is associated with activation of the immune system that include elements of acute inflammation [32,5].

In a previous study Dallard et al. [33], using a LPS-based biological response modifier or placebo (vehicle alone) infused in *S. aureus* chronically infected and uninfected bovine mammary glands during involution, observed an increase of immune expression for TNF-α in *S. aureus* chronically infected quarters during the first three weeks after drying off, regardless of the treatment received. Accordingly, in the present study, we observed a significant effect of IMI on TNF-α expression in mammary tissue, as indicated by higher immunostaining percentage in *S. aureus*-infected than uninfected quarters at all involution stages studied. This higher TNF-α expression coincided with a significant increase of the number of monocytes-macrophages in mammary glands infected with *S. aureus* compared with uninfected glands at 7 and 21 d of involution. These finding denote the importance of monocytes-macrophages present in bovine mammary gland as a possible source of pro-inflammatory cytokines in chronic *S. aureus* infections during mammary tissue-remodeling, associated with changes directed to limit the infection process and repair the injury to the host.

The highest number of monocytes-macrophages in *S. aureus*-infected quarters was observed at 7 d of involution with gradual decrease towards day 21, suggesting a lower influx of phagocytic cells to the infected mammary gland as involution progressed. These findings were associated with the expression of TLR2 immunopositive area observed in *S. aureus*-infected quarters, which showed the same pattern. The presence of viable *S. aureus* within macrophages and MEC from milk of infected cows [34], could favor evasion of the host defenses by the bacterium avoiding being recognized by TLR contributing to its persistence within mammary gland. The progressive decrease in the number of monocytes-macrophages in *S. aureus*-infected quarters observed in this study, could be related to bacterial intracellular persistence that hinder recognition by the immune system leading to lesser innate immune response stimulation and lower attraction of inflammatory cells to mammary gland.

Interleukin-1α is not usually secreted and remains localized intracellularly [29]; however, it can be released by injury or cell death [35]. In the present study, the immune expression of IL-1α was higher in *S. aureus*-infected than in uninfected quarters at 7 and 14 d of involution, but no differences were observed at day 21. These findings are connected to previous observations of increased percentages of apoptosis in mammary tissue of cows chronically infected with *S. aureus* during the first three weeks of involution [36,37]. The high IL-1α immunoexpression observed in *S. aureus* infected quarters could be induced by the extensive tissue damage and increment of apoptosis caused by chronic infection and taken together may represent a mechanism to counteract the effects induced by IMI to maintain integrity of tissue affected by the inflammatory reaction.

Interleukin-6 is one of the key mediators of the "acute-phase response" in inflammation [29]. Günther et al. [13] demonstrated that a dominant IL-6 gene expression is induced in response to *S. aureus* infection of pbMEC. Interleukin-6 mRNA transcription has been demonstrated to be greater in milk cells isolated from cows with naturally acquired [30] or experimentally induced mastitis [27]. Moreover, increased concentrations of IL-6 have been detected in milk of cows with naturally acquired [38] and experimentally induced mastitis [39]. In the present study, the protein expression of IL-6 in mammary tissue was not affected by IMI showing similar results between *S. aureus*-infected

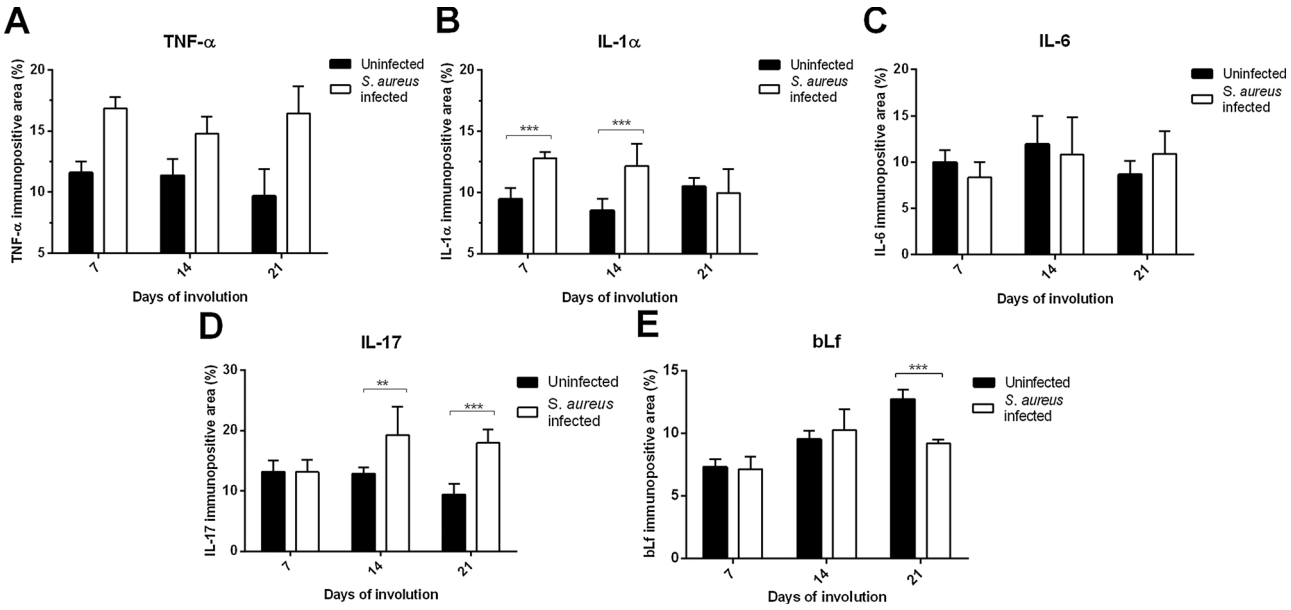

Fig. 4. Relative expression (measured as percentage of immunopositive area) of (A) TNF-α, (B) IL-1α, (C) IL-6, (D) IL-17 and (E) bLf in uninfected and *S. aureus*-infected quarters at 7, 14 and 21 d of involution. Values represent the mean ± standard error of the mean (SEM). At each sampling time, asterisks represent statistically significant differences between uninfected and S. aureus-infected quarters (**P < 0.01; ***P < 0.001).

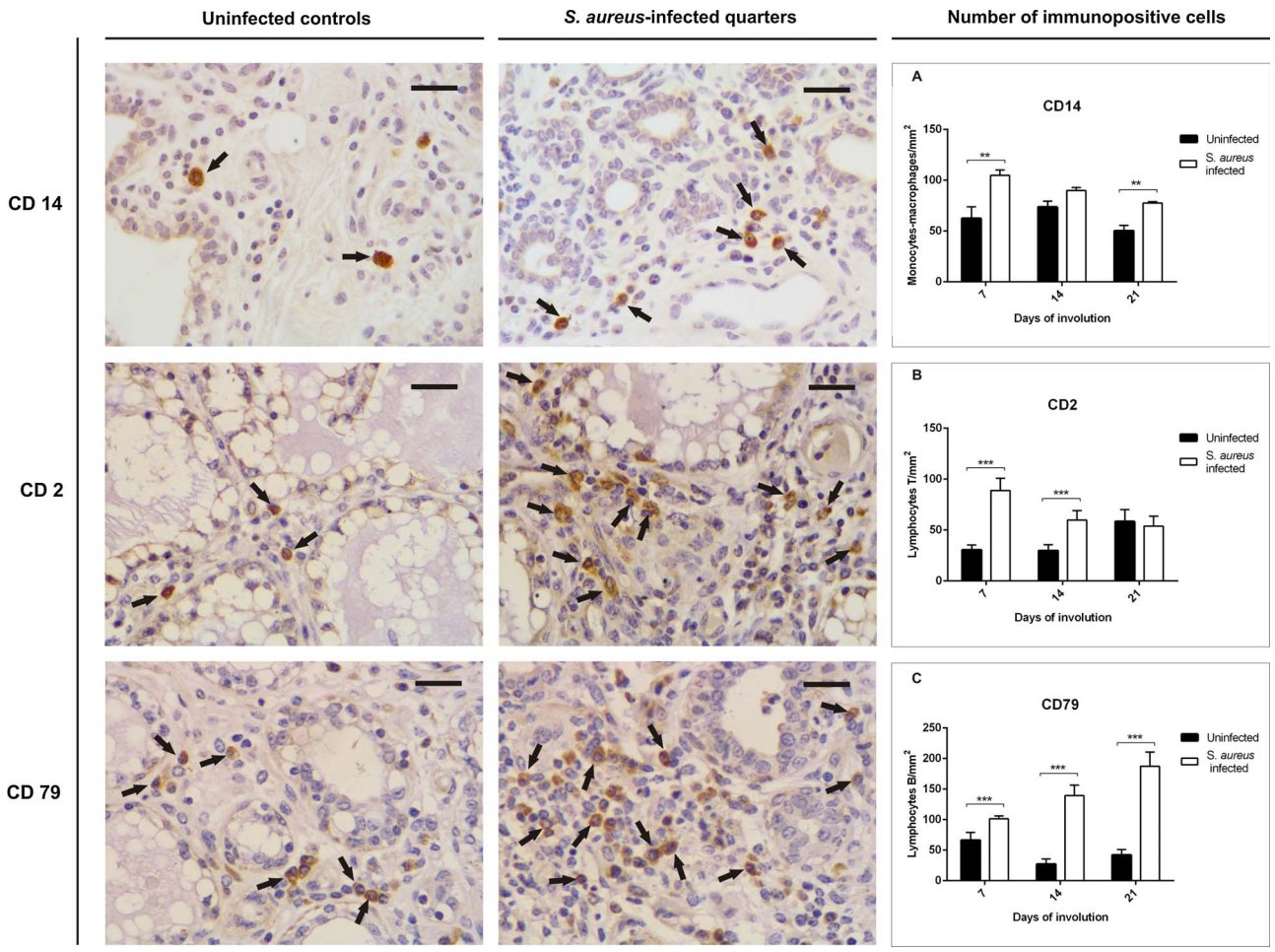

Fig. 5. Representative images of CD14, CD2 and CD79 immunopositive cells in uninfected and *S. aureus*-infected quarters at 7 d of involution are shown in the left panels. Immunoreactivity of monocytes-macrophages (labeled with anti-CD14), T-cells (labeled with anti-CD2) and B lymphocytes (labeled with anti-CD79) are shown as a brown staining of cytoplasm of cells (black arrows). Magnification is the same for all panels and is indicated by 25 μm bars. In the right panels, number of CD14+ monocytes-macrophages (A), CD2+ T cells (B) and CD79+ B cells in uninfected and *S. aureus*-infected quarters at 7, 14 and 21 d of involution are shown. Values represent mean numbers of immune cells/mm^2 ± standard error of the mean (SEM). At each sampling time, asterisks represent statistically significant differences between uninfected and *S. aureus*-infected quarters (** $P < 0.01$; *** $P < 0.001$).

and uninfected quarters in all involution stages evaluated. Considering that IL-6 is an acute phase cytokine, the nature of the discrepancies may rely in the time at which evaluation of IL-6 was performed in the different studies (acute vs chronic; kinetic studies vs fixed time studies). Moreover, most studies have quantified the concentration of this cytokine in milk and in cellular cultures and not its protein expression in mammary tissue.

The immunological axis IL-23/IL-17 and the associated newly recognized IL-17-producing Th lymphocytes (Th17 lineage) are considered major actors of the mobilization of neutrophils and modulators of innate and Ag-specific inflammation, both acute and chronic [40]. Th17 cytokines (IL-17A, IL-17F, IL-22) have been considered critical to mucosal immunity in primary challenge with different pathogens [41], but its role on the control of *S. aureus* IMI has not been fully clarified. Rainard et al. [42] detected IL-17A in milk and mammary tissue during antigen-specific inflammation in bovine mammary gland. In accord with the results obtained by these authors by IHC, in the present study IL-17 was located in the cytoplasm of epithelial cells lining alveoli and ducts, in connective tissue and epithelium-associated leukocytes, and in migrated alveolar leukocytes in *S. aureus*-infected and uninfected quarters. The strong labeling of MEC by antibodies to IL-17, suggests that these cells can be a source of this cytokine and contribute to its secretion in milk during *S. aureus* mastitis. Although immunostained area for this cytokine was significantly increased in *S. aureus*-infected quarters, immunostaining observed in parenchymal cells and immune cells in uninfected quarters suggests a role of this cytokine during mammary gland remodeling at early involution.

Whelehan et al. [43] evaluated the regional variation in the expression of gene encoding IL-17A in bovine mammary gland following intramammary infection with *S. aureus*, and demonstrated an increased expression of this cytokine in alveolar, ductal, gland cistern and teat canal regions compared with uninfected controls. Although the gene expression of IL-17 was not evaluated in the present study, the fact that labeled percentages of this cytokine were significantly influenced by the *S. aureus* infection is consistent with the results reported by Whelehan et al. [43]. Immunoexpression of IL-17 was influenced by week of involution showing higher percentages of labeling in *S. aureus*-infected than in uninfected quarters at 14 and 21 d of involution, which could indicate that production of this cytokine increased as involution progressed in an attempt to counteract the infection by stimulating chemotaxis of neutrophils to the site of infection.

An early study carried out during bovine mammary gland involution reported significantly lower concentration of bLf in milk of major pathogens-infected quarters, suggesting that decreased levels of this antibacterial component could contribute to lower natural defense against these pathogens, ultimately resulting in infection [44]. In the present study, in both *S. aureus*-infected and uninfected quarters the percentages of immunostaining for bLf were increased from day 7 to day 14 of involution showing similar percentages of labeling. At 21 d of involution, the protein expression of bLf in uninfected quarters was the

highest, whereas in *S. aureus*-infected quarters these percentages decreased significantly compared with uninfected quarters. Mehrzad et al. [45] demonstrated that milk proteases released by neutrophils during endotoxin-induced mastitis in dairy cows, were able to hydrolyzed different milk proteins, including Lf. Accordingly, the significant decrease in bLf immunoexpression observed in *S. aureus*-infected quarter at day 21 of involution, could be related to the high number of neutrophils migrating to the mammary gland triggering the proteases release and bLf degradation; contributing to generate an appropriate microenvironment for the persistence of *S. aureus* in mammary gland.

Lymphocytes are an important part of the adaptive immune defense against IMI, but traffic from different subsets of lymphocytes during *S. aureus* mastitis has not been fully elucidated [11]. In the present study, in accordance with increases in T lymphocytes reported by other authors in mammary secretion from *S. aureus*–infected animals [30,46], we observed a significant effect of *S. aureus* IMI on the number of T lymphocytes in mammary tissue, as indicated by higher number of cells/area in *S. aureus*-infected quarters compared with uninfected quarters. In *S. aureus*-infected quarters, the highest number of T lymphocytes was observed at 7 and 14 d of involution, decreasing progressively towards day 21. The increase of CD2+ cells in *S. aureus*-infected quarters strongly suggests both the migration of T lymphocytes into the infected mammary gland and development of a specific memory immune response against *S. aureus* during the first two weeks of involution.

Grönlund et al. [46] observed increased proportion of B lymphocytes in blood and milk during chronic sub-clinical mastitis. In the present study, the number of B lymphocytes was higher in *S. aureus*-infected than in uninfected quarters at 7, 14 and 21 d of involution, showing a progressive increase as involution advanced. This finding may be related to local lymphocyte proliferation and the development of a humoral response. In earlier studies, activation of B lymphocytes and development of an antibody-mediated immune response during chronic subclinical *S. aureus* mastitis was demonstrated [30,46]. However, this humoral immune response is not sufficient to limit persistence of *S. aureus* IMI [12]. It is known that CD21+ B lymphocytes are increased in milk samples with high SCC and major pathogens positive isolation, whereas the proportions of CD2+ and CD3+ T lymphocytes are decreased [47,48]. In the present study, in *S. aureus*-infected quarters, the number of B lymphocytes/area was greater than the number of T lymphocytes/area in all involution stages studied. This finding highlights the functional importance of B cells in chronic *S. aureus* IMI during early involution.

5. Conclusion

Increased protein expression of TLR2 and TLR4, pro-inflammatory cytokines, bLf and immune cells like monocytes-macrophages, T and B lymphocytes in *S. aureus*-infected quarters compared with uninfected quarters were indicative of a sustained and exacerbated host innate and adaptive immune system during active involution, playing a critical role in the infection control. The constitutive presence of innate and adaptive immune components in uninfected quarters appears to be an essential response to the process of intense tissue-remodeling that occurs during bovine mammary gland involution. Overall, results of this study provide new insights about the mechanisms of immune response triggered during chronic *S. aureus* IMI and the relevance of immune system in mammary gland remodeling during active involution.

Declaration of interests

Authors have no conflicts of interest.

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