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Immune response of *Staphylococcus aureus* strains in a mouse mastitis model is linked to adaptive capacity and genotypic profiles

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ABSTRACT

Staphylococcus aureus is one of the most frequently isolated major pathogens from intramammary infections (IMI) worldwide. 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. Moreover, it was shown that different *S. aureus* strains have varying effects on the immune response. The aim of this study was to investigate the immune response in a mouse mastitis model of two *S. aureus* strains isolated from bovine IMI with different clinical manifestation (persistent-P or non-persistent-NP), phenotypic and genotypic profile. Both strains were capable of establishing an IMI after 264 h post inoculation (pi). Strain A (NP) showed a more aggressive behaviour than strain B (P) at early stages of IMI, while strain B multiplied initially at a lower rate but increased its replication capacity from 120 h pi to the end of the study (264 h pi). Strain A triggered a stronger initial inflammatory response compared with strain B inducing higher gene and protein expression of TLR2, NF-κB activation and higher gene expression of IL-1α at initial stage of IMI (6–12 h pi) but inducing extensive mammary tissue damage. Immune cells response was different for each *S. aureus* strain throughout the course of infection, showing mammary glands inoculated with strain A greater initial immune cells stimulation compared with strain B and then a second immune cells stimulation (from 120 to 264 h pi) represented by monocytes-macrophages, T and B lymphocytes, mainly stimulated by strain B, consistent with inflammatory process becoming chronic. Strain-specific pathogenicity observed underscores the importance of pathogen factors in the progression of the infectious process. These results contribute to increase the available information on host-pathogen interaction and point out for the need of further research to expand the knowledge about these interactions for developing new strategies to intervene in the IMI progress.

1. Introduction

Bovine mastitis is one of the most prevalent infectious diseases of cattle that results in major economic losses to producers and the global dairy industry (Hogeveen et al., 2011). While mastitis is caused by several etiologic agents, *Staphylococcus aureus* is the most frequently isolated major pathogen from intramammary infections (IMI) in Argentina (Dieser et al., 2014), as well as in other countries (Reyher et al., 2011). Although *S. aureus* can cause acute and clinical mastitis with macroscopic alteration of milk, such bacterial infections can evolve towards chronic and subclinical mastitis, without milk macroscopic changes but with high somatic cell counts and bacterial persistence in the mammary gland. Antibiotic treatment at this stage is often ineffective (Smith et al., 2006) and so far commercially available vaccines have shown limited success to prevent *S. aureus* IMI (Schukken et al., 2014). Poor response to antibiotic therapy has been attributed, among other factors, to *S. aureus* ability to evade the host immune response and survive within different mammary gland cell types for a long period of time (Craven and Anderson, 1979; Almeida et al., 1996; Hebert et al., 2000).

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Mastitis outcome is determined by pathogen virulence and cow's immune response (Le Maréchal et al., 2011). According to several *in vivo* and *in vitro* studies (Wellnitz and Bruckmaier, 2012), distinct bacterial species stimulate differential cow's innate immune responses. *Escherichia coli* or its cell wall lipopolysaccharide induces a stronger and qualitatively different immune reaction in the mammary gland and in bovine mammary epithelial cells than *S. aureus* or lipoteichoic acid (LTA) from *S. aureus* (Lee et al., 2006; Lahouassa et al., 2007). Moreover, it was shown that different *S. aureus* strains trigger varying effects on the immune response which can influence the course and severity of mastitis (Kim et al., 2011; Zbinden et al., 2014). To date the molecular basis underpinning the pathogen specific differences associated with the resolution of mammary gland infections are unclear.

In a previous study we evaluated the behavior of selected *S. aureus* isolates from bovine IMI categorized as persistent-P and non-persistent-NP, showing different genotypic profiles including genes involved in adherence and biofilm production, during early interactions in contact with bovine mammary epithelial cells (MAC-T) (Pereyra et al., 2016). *Staphylococcus aureus* internalization into MAC-T cells was shown to be strain-dependent and internalized bacteria overexpressed adherence and biofilm-forming genes compared with those that remained in co-cultures supernatants; particularly those genes encoding FnBPs and IcaD (Pereyra et al., 2016). These *in vitro* findings during early *S. aureus*-host interactions gave rise to the need to explore aspects of the immune response induced by distinct strains using *in vivo* experimental models. A better understanding both of the pathogen and the immune response aspects are crucial to propose alternatives to intervene in the disease progress.

The innate immune response is initiated when specific pattern recognition receptors (PRR) on the surface or within host cells bind particular bacterial molecules termed pathogen-associated molecular patterns (PAMP). Pathogens that enter the mammary gland lumen are sensed via toll-like receptors (TLR) that recognize PAMP (Petzl et al., 2008). Toll-like receptor 2 (TLR2), that is part of PRR, recognizes LTA and peptidoglycan from Gram positive bacteria and is a crucial recognizable receptor activated by *S. aureus* infection in mammary tissue (Yang et al., 2008; Wang et al., 2015). Activation of the PRR initiates signal transduction pathways that culminate in the transcription of a wide range of immune genes including cytokines, which are synthesized by infiltrating cells (Lee et al., 2006) as well as resident cells in response to *S. aureus* infection (Lahouassa et al., 2007). Milk macrophages and epithelial cells are the first cells to encounter and recognize bacterial pathogens entering the mammary gland. Pro-inflammatory cytokines secretion leads to neutrophil recruitment from the blood into the infected mammary gland, where they recognize, phagocytize and kill the invading pathogens at the early stage of infection (Wellnitz and Bruckmaier, 2012).

Pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β, are expressed during the mammary gland early immune response (Aitken et al., 2011). Transcripts for these cytokines have been detected in milk somatic cells from cows both following experimentally induced *S. aureus* IMI (Lee et al., 2006) and with chronic IMI (Riollet et al., 2001); being this response characterized by a relatively low and short-lasting increase in pro-inflammatory cytokines (Yang et al., 2008). Most of the changes seen in cows have been further studied at the molecular level through *in vivo* studies in mouse mastitis models (Notebaert and Meyer, 2006; Breyne et al., 2014). However, few studies have evaluated the persistence of *S. aureus* in murine mammary tissue and induced immune response beyond 72 h post challenge (Notebaert and Meyer, 2006).

Adaptive immunity plays an important role in immune clearance when innate defenses fail to completely eliminate mastitis-causing pathogens. B cell production of opsonizing antibodies that enhance neutrophil phagocytosis must be stimulated to establish a host adaptive immune response against pathogens. In addition to cytotoxic T lymphocytes (CD8+), large numbers of T helper (Th) lymphocytes (CD4+) migrate into the infected udder and orchestrate adaptive immune responses (Rivas et al., 2000; Nagahata et al., 2011). Traffic from different lymphocytes subsets during the acute and chronic phases of bovine *S. aureus* mastitis has not been fully elucidated (Nagahata et al., 2011) and there are few reports concerning changes of lymphocytes subsets in mouse mammary tissue following *S. aureus* experimental infection (Cao et al., 2012; Zhao et al., 2015). To investigate the innate and adaptive immune response in mastitis, we established a mouse model of *S. aureus* IMI, which has been used previously to study the pathogenesis and control of bovine mastitis (Brouillette and Malouin, 2005; Notebaert and Meyer, 2006). The aim of this study was to investigate the immune response in a mouse mastitis model of two *S. aureus* strains isolated from bovine IMI with different clinical manifestation (persistent-P or non-persistent-NP), phenotypic and genotypic profile. Furthermore, tissue damage and *in vivo* bacterial persistence were evaluated during the infectious process.

2. Materials and methods

2.1. Bacterial strains

Two different *S. aureus* strains (designated I17 and I5128) were selected from bovine IMI based on results obtained in a previous study (Pereyra et al., 2016). Both isolates were not clonally related and were represented by different pulse-field gel electrophoresis (PFGE) type. Strain A (I17) isolated from a milk sample taken from a cow with clinical mastitis previous to administering antibiotic treatment and not isolated from the same cow following antibiotic treatment was considered with low adaptation to bovine mammary gland and designated as non-persistent-NP. This strain came from a dairy farm with high prevalence of *S. aureus* (62%) IMI, with bulk somatic cells count ≥500.000 cells/mL at the time it was isolated, resistant to penicillin by *in vitro* antimicrobial susceptibility testing, β-lactamase and α-hemolysin producer, moderate biofilm producer, high invasion ability in MAC-T cells (7.45 ± 0.72%), high bacterial growth *in vitro*, presence of *cap8* and *blaZ* genes and *in vitro* overexpression of *clfA, fnbA, fnbB, icaA* and *icaD* genes following internalization in MAC-T. Strain B (I5128) was isolated from a milk sample taken from cow with subclinical mastitis and was repeatedly isolated from the same quarter during 6 months; was considered highly adapted to bovine mammary gland and designated as persistent-P. To confirm persistency, in a previous study by Pereyra et al. (2016), the genotypic profiles of *S. aureus* isolates obtained from the same quarters were compared using pulsed-field gel electrophoresis (PFGE). Strain B came from a dairy farm with low prevalence of *S. aureus* (1–3%), with a somatic cells count average ≤350.000 cells/mL at the time it was isolated, sensitive to penicillin by *in vitro* antimicrobial susceptibility testing, β-hemolysin producer, strong biofilm producer, low invasion ability in MAC-T cells (2.142 ± 0.46%), low *in vitro* bacterial growth, presence of *cap5* gene, absence of *blaZ* gene and overexpression of *fnbA* and *icaD* genes *in vitro*. Both strains belonged to *agr* group I. Strains were further characterized by a microarray-based *S. aureus* genotyping system (Alere GmbH, Jena, Germany). Genomic DNA was obtained as previously described (Monecke et al., 2008). Probe sequences used in this system were derived from published genome sequences of *S. aureus* strains and have been published previously (Monecke et al., 2008). The hybridization profiles allowed the assignment of isolates to clonal complexes (CC); strain A belonged to CC188, while strain B to CC97.

2.2. Experimental animals

All the procedures were carried out according to the Guide for the Care and Use of Laboratory Animals (ILAR, 2010) and approved by the Committee of Animal Ethics and Security of the Facultad de Ciencias Veterinarias, UNL. Female BALB/cJ lactating mice were used 10–15 days after birth of the offspring and at the beginning of

Table 1
Antibodies, source and commercially purchased reagents used.

Antibodies	Source	Company/catalogue number	pAB/mAB	Dilution
Primary antibodies				
Anti-TLR2	Rabbit	GenWay Biotech (18-732-292538)	pAB	1:200
Anti-NF-kB-p65 (clone C-20)	Mouse	Santa Cruz Biotechnology (sc-372)	pAB	1:500
Anti-CD14 (clone RPA-M1)[a]	Mouse	Zymed (18-0121)	mAB	1:150
Anti-CD4 (clone GK1.5)[b]	Rat	Santa Cruz Biotechnology (sc-13573)	mAB	1:75
Anti-CD8 (clone 53 − 6.7)[c]	Rat	Santa Cruz Biotechnology (sc-18913)	mAB	1:75
Anti-CD79 (clone HM57)[d]	Mouse	Abcam (ab62650)	mAB	1:200
Secondary antibodies				
Polyvalent Biotinylated link (anti-mouse/anti-rabbit igG2a)	Goat	Cell Marque (961D-22)	pAB	1:200
Biotinylated anti-rat IgG-B	Goat	Santa Cruz Biotechnology (sc-2041)	pAB	1:200

pAb, polyclonal antibody; mAb, monoclonal antibody.
[a] anti-CD14 (identify monocytes-macrophages).
[b] Anti-CD4 (identify T-helper cells).
[c] Anti-CD8 (identify T-cytotoxic cells).
[d] Anti-CD79 (identify B lymphocytes).

experiments these mice weighed 30–35 g. This mouse strain has been widely used for *S. aureus* mastitis experimental infections in previous works (Boulanger et al., 2007; Gogoi-Tiwari et al., 2016, 2017). The animals were provided by Centro de Medicina Comparada, Instituto de Ciencias Veterinarias del Litoral, (UNL-CONICET). During the experiments, the animals were kept with a controlled cycle of light-darkness (lights on between 8:00 AM and 8:00 PM), and at a temperature of 20–24 °C with free access to water and commercial balanced food.

2.3. Mice intramammary infection

Prior to challenge, A and B *S. aureus* strains were thawed from frozen stocks (−80 °C) and cultured in Columbia agar overnight at 37 °C. Then, plated on Columbia agar with 2.5% NaCl, incubated overnight at 37 °C for induction of capsule polysaccharide expression. The colonies were suspended in saline solution to produce a suspension equivalent to turbidity of a 0.5 McFarland standard to contain approximately 1×10^8 colony forming units (CFU/ml). Then, this suspension was diluted to reach the different inoculation doses. Intramammary inoculation was based on the method described by Brouillette and Malouin (2005). For both strains, three doses of *S. aureus* were evaluated (1×10^4, 1×10^5 and 1×10^6CFU/gland). Since only a dose of 1×10^6CFU/gland was able to establish an IMI lasting 11 days that allowed to studying adequately different immune components involved in the infectious process, this was selected for further experiments. Briefly, the litter was removed 1–2 h before inoculation of the mammary glands and a mixture of 87 mg/kg ketamine (Holliday-Scott S.A., Argentina)/13 mg/kg xylazine (Rompun, Bayer, Argentina), was used for anesthetizing the lactating mice. Three groups of 30 lactating mice in each group were inoculated. Teats ducts of both the L4 (left) and R4 (right) abdominal mammary glands were exposed under a binocular stereoscopic microscope and 100 µl of 1×10^6CFU/gland of *S. aureus* strain A (group I) and *S. aureus* strain B (group II) were delivered into mammary glands using a syringe with a 30 G blunt needle. Control mammary glands were infused with 100 µl of saline solution (group III). The litter was returned to their mothers 3 h later. Mice (3 at each time) were euthanized at 6, 12, 24, 48, 72, 96, 120, 168, 216 and 264 h post inoculation (pi) and the mammary glands were aseptically removed. One portion of the removed mammary gland tissue was homogenized in 2 ml of phosphate buffered saline (PBS, pH 7.2; 0.01 M) to further evaluate the level of bacterial infection. Another portion of the tissue sample was immediately fixed in 4% neutral buffered formalin for 12 h at 4 °C, washed in PBS and embedded in paraffin wax for histological studies. Another mammary tissue portion was immediately frozen at −80 °C in a freezing vial until further use in mRNA genes expression assays. Each group of mice were clinically examined daily until 264 h pi by recording changes in behavior, fur aspect and presence of inflammatory signs in the mammary gland.

2.4. Staphylococcus aureus infection profile

At the indicated time points after inoculation, a portion of both L4 and R4 abdominal mammary glands were aseptically collected, weighted and homogenized in 2 ml of PBS using a tissue grinder under sterile conditions. For each gland homogenate, six serial dilutions were performed in duplicate. Each series of dilutions was plated on Columbia agar plates, incubated overnight at 37 °C and *S. aureus* colonies were counted. A detection limit of 200 CFU/g of tissue was established. Raw bacterial CFU counts were transformed to based-10 logarithm values and data were expressed as CFU/g of mammary gland.

2.5. Histopathological studies

Tissue samples from mammary glands were placed in 4% buffered formalin and then washed in PBS. For light microscopy, tissues were dehydrated and embedded in paraffin wax. Histological blocks were cut on a rotary microtome manually. Serial sections of 4 µm were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO), dried for 24 h at 37 °C and assigned for staining with haematoxylin and eosin (HE) for mammary gland histopathological examination or for use in immunohistochemistry (IHC) procedures.

2.6. Immunohistochemistry

For IHC, the streptavidin–biotin immunoperoxidase method was performed as described by Baravalle et al. (2015). Briefly, after deparaffinization and hydration, endogen peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol for 20 min, and non-specific binding sites were blocked with 10% (vol/vol) normal goat serum for 15 min. Sections were incubated with primary antibodies (Table 1) for 18 h at 4 °C. Slides were washed with PBS and incubated at room temperature for 30 min with a biotinylated secondary antibody selected specifically against each one of the primary antibodies used (Table 1). The antigens were visualized by ExtrAvidin-Peroxidase, and 3.3-diaminobenzidine was used as chromogen. Subsequently, samples were rinsed in distilled water, counterstained with hematoxylin, dehydrated, and mounted. Negative control sections in which primary antibody was replaced by non-immune rabbit serum were included. Some sections were incubated only with DAB reagent to exclude the possibility that endogenous peroxidase activity had been unsuccessfully blocked. The homology between the target peptide of each primary antibody and the correspondent murine protein was tested with the

Basic Local Alignment Search Tool (BLAST software; http://www.ncbi.nlm.nih.gov/BLAST).

2.7. Image analysis and immunohistochemistry quantification

Image analysis was performed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA). Images were digitized via a color video camera (Motic 2.000, Motic China Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of 40×. 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 details were previously described (Baravalle et al., 2015). The immunohistochemical stained area (IHCSA) for antibody reaction was calculated as a percentage of the total area evaluated through color segmentation analysis. Briefly, the average density (% of positive area) of the TLR2 antibody reaction was calculated from at least 50 images of each area (parenchymal and stromal cells) in each section as a percentage of the total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain).

Cells positive for NF-κB-p65 were quantified in the epithelium (parenchyma) and stroma of mammary gland as described previously (Baravalle et al., 2015). Briefly, a minimum of 1000 cells in at least 20 microscopic fields at 40× magnification were counted in each slide. Epithelial and stromal cells showing an intense nuclear staining were quantified and results were expressed as a percentage of immunopositive cells. For measuring number of monocytes-macrophages labeled with anti-CD14 antibody, T-helper cells labeled with anti-CD4 antibody, T-cytotoxic cells labeled with anti-CD8 antibody and B lymphocytes labeled with anti-CD79 antibody, 30–40 random images from each slide were digitized at 40× and number of cells per mm^2 was obtained.

2.8. Real-time RT-PCR

Total RNA was isolated from mammary gland tissue after treatment with Trizol LS reagent (Invitrogen, Life technology, CA, USA) according to the manufacturer's instructions. Quantity and quality of RNA for all obtained samples from the different treatment groups were determined using a fluoroscopic method (Qubit, Invitrogen, CA, USA) and by visualization on a denaturing agarose gel. Two micrograms of RNA samples were treated with DNAse I (Invitrogen) in accordance with the manufacturer's instructions. RNA was reverse-transcribed into cDNA as described previously (Baravalle et al., 2015). Absence of genomic DNA was validated running for each sample and gene, non-transcriptional controls (without the reverse transcriptase and with RNA as sample). Subsequently, relative quantification of gene expression (qPCR) was performed using a StepOne Real Time PCR System (Applied Biosystems, Life Technology, CA, USA). Briefly, each PCR was performed in a total volume of 20 µl containing: 4 µl of cDNA (500 ng/ml), 4 µl of 5× Phire reaction buffer, 0.5 µl of each 10 µM forward/reverse primers, 0.2 mM dNTPs, 1 µl SYBR Green I (Invitrogen), 0.05 µl Phire Taq polymerase (Thermo Fisher Scientific Company, Finland) and 14 µl of sterilized DEPC treated water. Sequences of specific primer sets used to amplify target genes are listed in Table 2. Optimal conditions for PCR amplification of cDNA were established using routine methods. Each qPCR was performed using the comparative Ct method ($2-^{\Delta\Delta Ct}$) and β-actin as an internal control (Livak and Schmittgen, 2001; Baravalle et al., 2015). Specificity of the PCR products was verified by DNA sequencing using the Macrogen Sequencing Service (Macrogen, Korea). Oligonucleotide primers and amplification products were then tested using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Table 2
PCR primers and conditions.

Primer	Sequence (5′- 3′)	aT (°C)	Amplicon size (bp)	Reference sequence
TLR2	F-CAAGTACGAACTGGACTTCTCC R-CAGGTAGGTCTTGGTGTTCATT	60	148	NM_011905
IL-1α	F-CTCTGAGAACCTCTGAAACGTC R-GAAACTCAGCCGTCTCTTCTT	60	116	NM_010554
TNF-α	F-GTCGTAGCAAACCACCAAGT R-TTGAAGAGAACCTGGGAGTAGA	60	149	NM_013693
INF-γ	F-CAAGTGGCATAGATGTGGAAGA R-GACGCTTATGTTGTTGCTGATG	62	164	NM_010548
IL-10	F-ATACTGCTAACCGACTCCTTAAT R-TCAAATGCTCCTTGATTTCTGG	60	140	NM_008337
β-actin	F-AACTCCATCATGAAGTGTGA R-ACTCCTGCTTGCTGATCCAC	61	248	NM_007393

aT: annealing temperature.

2.9. Statistical analysis

A statistical software package (SPSS 11.0 for Windows; SPSS, Inc., Chicago, IL) was used to perform statistical analysis. The general linear model procedure was used for data analysis. The model tested for main effects of treatment, time and the treatment * time interaction. Individual means were compared by Duncan test or *t*-test. The level of significance was set at $P < 0.05$. Results were expressed as mean ± SEM (standard error of the mean).

3. Results

3.1. Bacterial growth in infected mammary glands

Fig. 1 shows number of CFU recovered from mice mammary glands inoculated with *S. aureus* strains A and B at different time points. Both *S. aureus* strains were present in all inoculated mammary glands throughout the study. A significant effect of sampling time ($P < 0.001$) and interaction between sampling time and treatment were observed ($P < 0.001$); however, the effect of infecting strain on the number of bacteria recovered from mammary glands homogenates was not significant ($P = 0.826$).

At 6 and 12 h pi CFU number of *S. aureus* strain A recovered was greater than those recovered from strain B (6 h, $P = 0.024$; 12 h, $P = 0.030$). At 24 h pi, CFU number recovered for strain A and B was similar ($P = 0.126$); however the bacterial burden in the mammary glands of mice infected with strain A reached the maximum observed

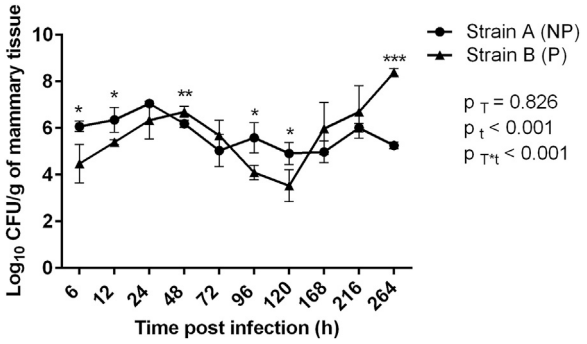

Fig. 1. Bacterial growth after intramammary infection with *S. aureus* strains A (non-persistent-NP) and B (persistent-P) in murine mammary gland at different time points. Values represent mean $\log_{10}$ CFU/g of mammary tissue ± standard error of the mean (SEM). The main effects of treatment (T) with *S. aureus* strains A and B, time of sampling (t) and treatment * time interaction (T*t) are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (levels of significance for individual time comparisons).

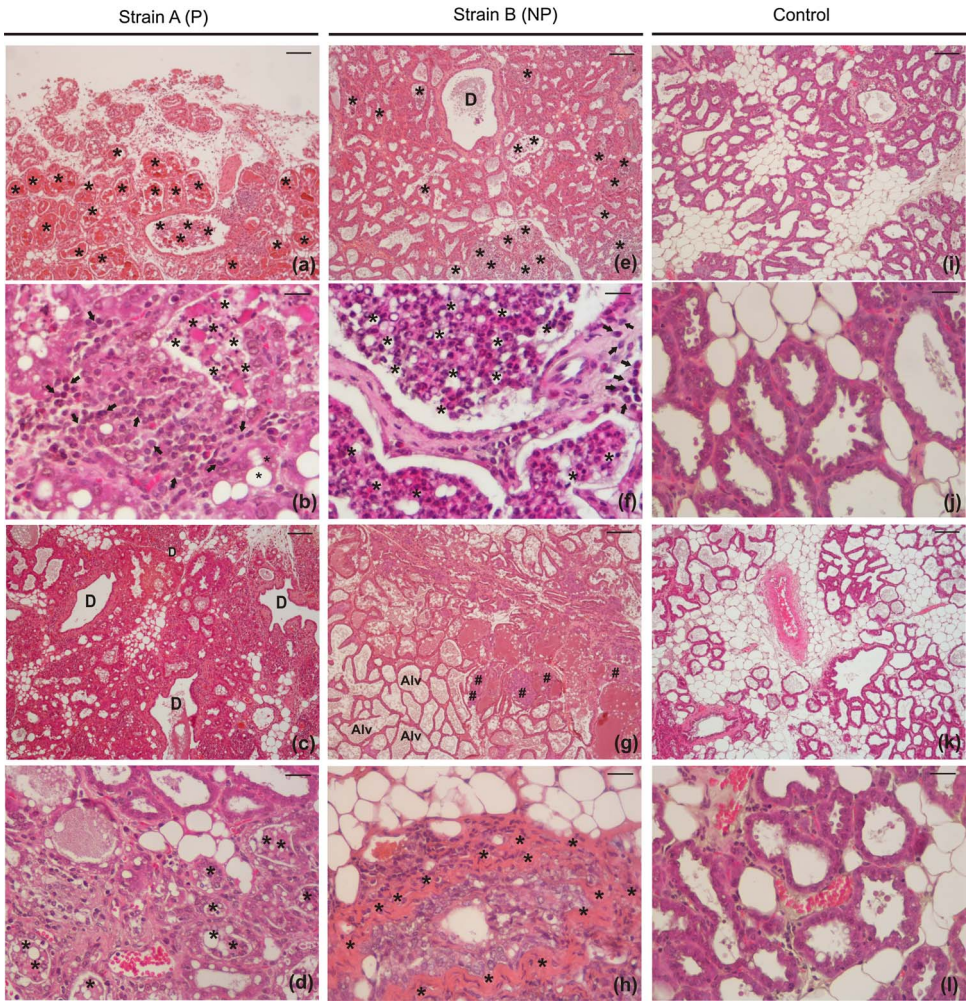

Fig. 2. Histopathogical changes in lactating mammary glands of mice inoculated with 1×10^6 CFU/gland of *S. aureus* strains A (non-persistent-NP) and B (persistent-P) and saline solution (controls) at different time points. (a-b) Mammary tissue of mice inoculated with *S. aureus* strain A (NP) at 48 h post inoculation (pi). Asterisks represent exudate of inflammatory cells and secretory products in the lumen of alveoli and ducts. Arrows indicate macrophages, neutrophils and lymphocytes infiltrating the interalveolar stroma. (c–d) Mammary tissue of mice inoculated with *S. aureus* strain A (NP) at 216 h pi. Extensive multifocal mammary necrosis surrounding the main mammary ducts (D). Asterisks indicate alveoli with loss of histological structure and replacement of mammary parenchyma with fibrous tissue. (e–f) Mammary tissue of mice inoculated with *S. aureus* strain B (P) at 48 h pi. Parenchymal zones with alveoli and ducts (D) with secretory products in the lumen and exudate of inflammatory cells (asterisks) with preserved alveolar and tubular structure. Infiltration of macrophages, neutrophils and lymphocytes in the interalveolar stroma (arrows). (g-h) Mammary tissue of mice inoculated with *S. aureus* strain B (P) at 216 h pi. Focalized areas of parenchyma with alveoli and ducts with conserved histological structure (Alv); areas with exudate of inflammatory cells and secretory products in the lumen of alveoli and ducts (#); areas with loss of tissue architecture with infiltration of lymphocytes and fibroblast and fibrous tissue circumscribing injuries (asterisks). (i–j) Mammary tissue of mice inoculated with saline solution (controls) at 48 h pi and 216 h pi (k,l). No histopathological alterations were observed in the mammary tissue of control mice. In figures a, c, e, g, i, k, bars represent 100 μm. In figures b, f, and j bars represent 20 μm. In figures d, h, and l bars represent 25 μm. Haematoxylin and eosin (HE) staining.

during the sampling period for this strain. At 48 h pi, although CFU number recovered in mice mammary glands infected with strain B continued to increase, CFU number obtained for strain A decreased significantly compared with strain B ($P = 0.010$). At 72 h pi, CFU number recovered decreased for both strains evaluated, and no differences between strains were observed ($P = 0.220$). At 96 and 120 h pi, CFU number recovered in mammary glands infected with strain B decreased compared with the number recovered for strain A (96 h, $P = 0.015$; 120 h, $P = 0.046$). At 168 and 216 h pi, CFU number recovered of mammary glands for both *S. aureus* strains increased compared with the previous period, reaching similar CFU numbers recovered for both strains (168 h, $P = 0.222$; 216 h, $P = 0.361$). At 264 h pi, a significant increase in CFU number recovered from mammary glands infected with strain B compared with CFU number recovered for strain A was observed ($P < 0.001$). Bacterial colonization in mammary glands of control mice was not found.

3.2. Histopathology

Changes in mammary tissue were evaluated by microscopy following HE staining (Fig. 2). At 6 h and 12 h pi, the histopathological changes in mammary glands inoculated with both *S. aureus* strains were similar, showing focalized areas of mammary tissue with neutrophils and macrophages exudate in the alveoli lumens and ducts with epithelial structure preservation and neutrophils and macrophages infiltration in the perivascular and perialveolar connective tissue. In the mammary glands inoculated with strain A, from 24 h to 96 h pi, large areas of damaged mammary tissue were observed with hyperemia and presence of abundant exudate of neutrophils, macrophages and epithelial cells in the lumens of alveoli and ducts. Moreover, multifocal necrotic areas were observed with structure loss and mild proliferation of connective tissue (Fig. 2a,b). In addition, a diffuse lymphoplasmacytic infiltrate was observed in the perialveolar connective tissue. In the mammary glands inoculated with strain B, from 24 h to 96 h pi, different degrees of mammary tissue injury were observed. In defined parenchymal zones, abundant neutrophils and macrophages exudate

within the lumens of alveoli and ducts were observed, with preservation of the epithelial structure. In other defined zones, exudates of neutrophils, epithelial cells and macrophages in the lumens of alveoli and ducts with loss of alveolar structure were observed (Fig. 2e,f). Hyperemia and macrophages, eosinophils, lymphocytes and plasmatic cells infiltration was observed in the interalveolar and interlobular connective tissue. Mice mammary glands inoculated with strain A, at 120 h pi, showed zones of mammary parenchyma with conserved histological structure and zones with abundant lymphoplasmacytic perialveolar infiltration, epithelial hyperplasia of ducts, high number of large macrophages, fibroblasts, collagen fibers, presence of foci with neutrophils and connective tissue proliferation. From 168 h to 264 h pi, large focal areas were evident with necrosis and loss of normal tissue architecture surrounding the main ducts, mild tubular epithelial hyperplasia, lymphoplasmacytic and macrophages infiltration and proliferation of dense connective tissue (Fig. 2c,d). Mice mammary glands inoculated with strain B, from 120 to 264 h pi, showed in the same histological section focalized zones of parenchyma with preserved structure of alveoli and ducts, focalized zones of functional alveoli with irregular lumens, focalized zones with necrosis and loss of normal architecture with infiltration of lymphocytes, fibroblasts and replacement of mammary parenchyma with fibrous tissue and focalized zones of alveoli and ducts with exudate of neutrophils in their lumens (Fig. 2g,h). No histopathological alterations in the mammary tissue of control mice were observed (Fig. 2i–l).

3.3. Immunohistochemistry for TLR2 and NF-κB-p65

Staining for TLR2 was present in all tissue sections at all experimental groups and was primarily associated with the apical cytoplasm of epithelial cells lining alveoli and ducts of the mammary parenchyma (Fig. 3). Furthermore, immune cells as macrophages and neutrophils showed intense staining for TLR2 in their cytoplasm. The percentages of IHCSA for TLR2 in mammary tissue (Fig. 3a) were influenced by the treatments ($P < 0.001$) and by time of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). The protein expression of TLR2 was higher in *S. aureus*-infected mammary glands than in control glands during all experimental period ($P < 0.05$). At 6, 48 and 72 h pi, significant differences in percentages of IHCSA for TLR2 among treatments were observed ($P < 0.05$), showing the mammary glands inoculated with strain A the highest percentages of immunostaining. From 96 to 264 h pi, percentages of IHCSA for TLR2 in mammary glands inoculated with *S. aureus* strains A and B were similar and higher than control group ($P < 0.05$).

Activated NF-κB was detected in stroma and parenchyma mammary cells by the nuclear localization of p65 subunit in all evaluated groups (Fig. 3). Fig. 3b and c show the percentages of immunopositive cells for NF-κB-p65 in parenchyma and stroma of mammary glands inoculated with *S. aureus* strains A and B and controls. Percentages of immunopositive parenchymal and stromal cells were influenced by the treatments ($P < 0.01$) and by the times of sampling ($P < 0.01$), showing interaction between these factors ($P < 0.01$). The percentages of immunopositive parenchymal and stromal cells were higher in mammary glands inoculated with *S. aureus* strains A and B compared with controls during all sampling times ($P < 0.05$). From 6 to 72 h pi a progressive increase in percentages of immunopositive parenchymal and stromal cells for NF-κB-p65 in mammary glands inoculated with *S. aureus* strains A and B compared with controls was observed

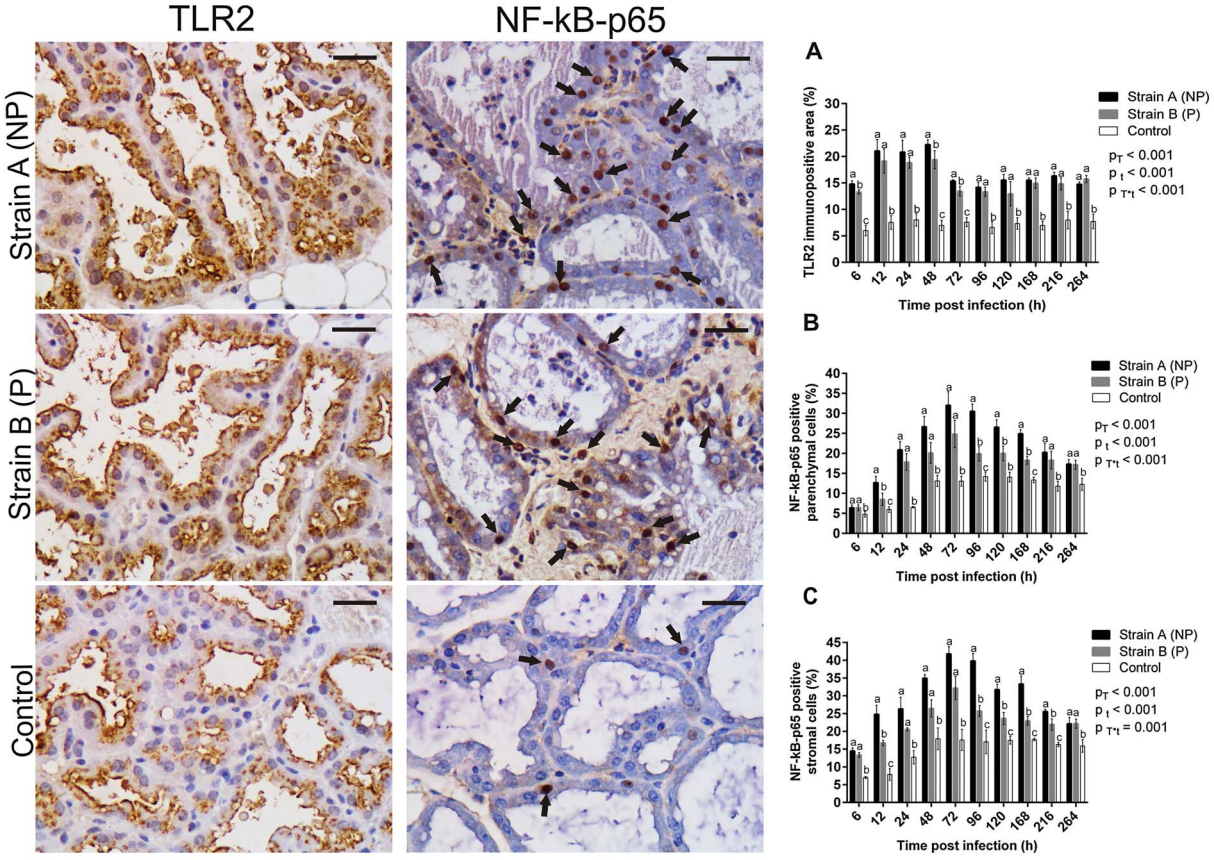

Fig. 3. Representative images of TLR2 and NF-kB-p65 immunostaining in murine mammary glands inoculated with *S. aureus* strains A (non-persistent-NP) and B (persistent-P) and saline solution (controls) at 48 h post inoculation are shown in the left panels. Nuclear immunoreactivity of mammary epithelial and stromal cells to antibody against NF-κB-p65 is indicated by black arrows. Magnification is the same for all panels and is indicated by 25 μm bars. A) Relative expression (measured as percentages of immunopositive area) of TLR2; B) Percentages of immunopositive cells for NF-κB-p65 in parenchyma (B) and stroma (C) in murine mammary gland inoculated with *S. aureus* strains A and B and saline solution (controls) at different sampling times. At each sampling time, different letters correspond to statistically significant differences ($P < 0.05$).

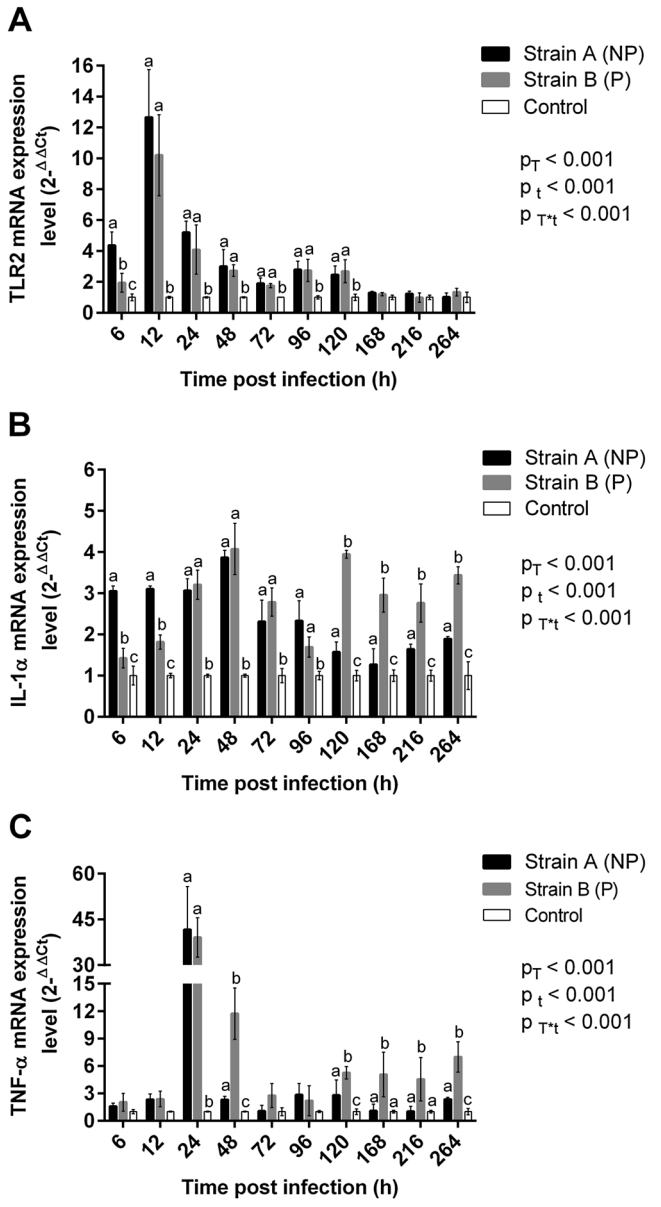

Fig. 4. Relative expression of TLR2 mRNA (A), IL-1α mRNA (B) and TNF-α mRNA (C) in mammary glands obtained from mice inoculated with 1×10^6CFU/g of mammary tissue of *S. aureus* strains A (non-persistent-NP) and B (persistent-P) and saline solution (controls) at different time points. Values are expressed as mean ± standard error of the mean (SEM). The main effects of treatment (T), time of sampling (t) and treatment * time interaction (T*t) are shown. At each sampling time, different letters correspond to statistically significant differences ($P < 0.05$).

($P < 0.05$). From 96 to 264 h pi a progressive decrease in percentages of immunopositive parenchymal and stromal cells for NF-κB-p65 was observed in *S. aureus*-infected glands (both strain A and B), however these percentages were higher than those observed in controls ($P < 0.05$). In parenchymal cells, the percentages of immunopositive cells were higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls at 12, 96, 120 and 168 h pi ($P < 0.05$). In stromal cells, the percentages of immunopositive cells were higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls at 12, 96, 120, 168 and 216 h pi ($P < 0.05$).

3.4. TLR2 and cytokines mRNA expression

The mRNA expression for TLR2 (Fig. 4a) was influenced by the treatments ($P < 0.001$) and by times of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). At 6 h pi, TLR2 gene expression was higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls ($P < 0.05$). At 12 h pi, TLR2 gene expression in mammary glands inoculated with both *S. aureus* strains was higher than in control mammary glands ($P < 0.05$), showing a peak in gene expression for both inoculated strains. From 24 h to 120 h pi a gradual decrease of TLR2 gene expression in mammary glands inoculated with both *S. aureus* strains was observed, showing the mammary glands inoculated with *S. aureus* strain A and B similar TLR2 gene expression that was higher than controls ($P < 0.05$). At 168, 216 and 264 h pi, no differences were observed in TLR2 gene expression between treatments.

We found transcripts for cDNA of IL1-α, TNF-α, INF-γ and IL-10 in mammary tissue from the three groups at every sampling period. However, mRNA relative levels for INF-γ and IL-10 could not be examined in mammary tissue since these samples fall outside of the dynamic linear range. The mRNA expression for IL-1α (Fig. 4b) was influenced by the treatments ($P < 0.001$) and by times of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). The mRNA expression for IL-1α was higher in mammary glands inoculated with *S. aureus* strains A and B than in controls at all times of sampling ($P < 0.05$). At 6 and 12 h pi, IL-1α gene expression was higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls ($P < 0.05$). At 24 and 48 h pi, IL-1α gene expression in mammary glands inoculated with strains A and B was higher than that observed in controls ($P < 0.05$), showing a peak at 48 h pi for both evaluated strains. At 72 and 96 h pi, a gradual decrease of mRNA expression for IL-1α in mammary glands inoculated with both *S. aureus* strains was observed, but remained at higher levels than those observed in controls ($P < 0$, 05). At 120 h pi, a marked increase of IL-1α gene expression in mammary glands inoculated with strain B was observed, showing higher relative gene expression levels compared with mammary glands inoculated with strain A and controls ($P < 0.05$). This elevated gene expression for IL-1α in mammary glands inoculated with strain B compared with mammary glands inoculated with strain A and controls was maintained from 120 h until 264 h pi.

The mRNA expression for TNF-α (Fig. 4c) was influenced by treatments ($P < 0.001$) and by times of sampling ($P < 0.001$), showing significant interaction between both factors ($P < 0.001$). At 24 h pi, TNF-α gene expression in mammary glands inoculated with strains A and B was higher than those observed in controls ($P < 0.05$), showing a peak in gene expression for both strains evaluated. At 48 h pi, TNF-α gene expression decreased sharply in mammary glands inoculated with both *S. aureus* strains compared with the previous period, showing a more pronounced decrease in mammary glands inoculated with strain A. In this period, TNF-α gene expression was higher in mammary glands inoculated with *S. aureus* strain B compared with mammary glands inoculated with strain A and controls ($P < 0.05$). From 120 at 216 h pi, an increase of TNF-α gene expression in mammary glands inoculated with strain B was observed, showing higher relative gene expression levels compared with mammary glands inoculated with strain A and controls ($P < 0.05$).

3.5. Identification and quantification of immune cells

Monocytes-macrophages labeled with anti-CD14 were found within lining epithelial cells and in the lumens of alveoli and ducts (Fig. 5). Immunopositive cells were observed infiltrating interalveolar and intralobular stromal tissue in the mammary glands from all groups. The number of monocytes-macrophages in mammary tissue (Fig. 6a) were influenced by treatments ($P < 0.001$) and by time of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). The number of monocytes-macrophages was higher in mammary glands inoculated with *S. aureus* strains A and B compared

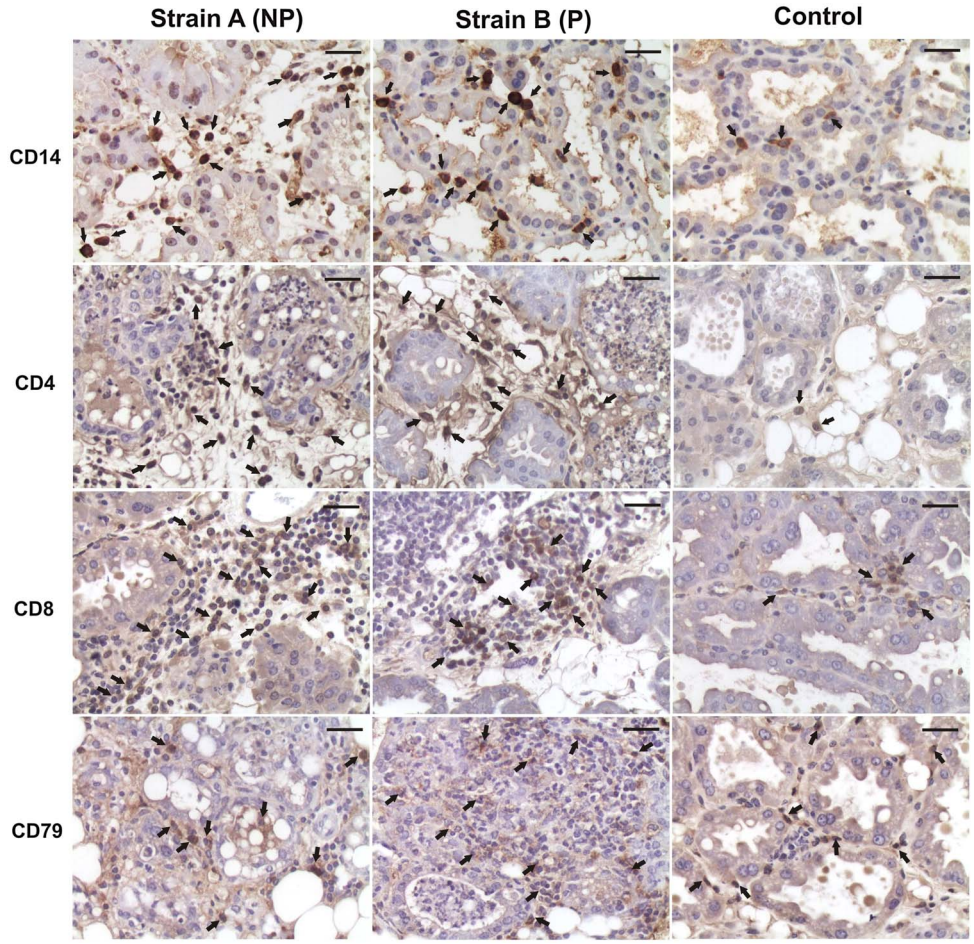

Fig. 5. Representative images of CD14, CD4, CD8 and CD79 immunopositive cells in murine mammary glands inoculated with *S. aureus* strains A (non-persistent-NP) and B (persistent-P) and saline solution (controls) at 96 h post inoculation. Immunoreactivity of monocytes-macrophages (labeled with anti-CD14), T-helper cells (labeled with anti-CD4), T-cytotoxic cells (labeled with anti-CD8) 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.

with controls at all sampling times ($P < 0.05$). At 6 h pi, the number of monocytes-macrophages was higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls ($P < 0.05$). From 12 to 48 h pi a gradual increase in the number of monocyte-macrophage in mammary glands inoculated with *S. aureus* strains A and B compared with controls was observed ($P < 0.05$). At 96 and 120 pi h a marked decrease was observed in the number of monocytes-macrophage in mammary glands inoculated with *S. aureus* strain B compared with the number in mammary glands inoculated with strain A ($P < 0.05$). At 168 and 216 h pi the number of monocytes-macrophages in mammary glands inoculated with strain B increased markedly compared with the previous period reaching similar numbers than those of strain A, differing significantly with respect to controls ($P < 0.05$). At 264 h pi, the number of monocytes-macrophages in mammary glands inoculated with *S. aureus* strain B was higher than the number quantified in mammary glands inoculated with strain A and controls ($P < 0.05$).

T lymphocytes labeled with anti-CD4 were found infiltrating the interalveolar and intralobular stromal tissue in mammary glands from all groups (Fig. 5). The number of CD4+ cells in mammary tissue (Fig. 6b) was influenced by the treatments ($P < 0.001$) and by the time of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). The number of CD4+ cells was higher in mammary glands inoculated with *S. aureus* strains A and B compared with controls at all sampling times ($P < 0.05$). At 12, 48 and 72 h pi a gradual increase in the number of CD4+ cells in the mammary glands inoculated with both *S. aureus* strains was observed; being this increase higher in mammary glands inoculated with *S. aureus* strain B compared with strain A and controls ($P < 0.05$). At 96 h pi a peak in the number of CD4+ cells in mammary glands inoculated with both *S. aureus* strains was observed, showing those glands inoculated with strain A higher number of cells than those inoculated with strain B and controls ($P < 0.05$). At 120 and 168 h pi a decrease in the number of CD4+ cells in mammary glands inoculated with both S. aureus strains was observed, showing those inoculated with strain A and B similar number of cells that were higher than those observed in controls (P 0.05). At 216 and 264 h pi the number of CD4+ cells increased significantly in mammary glands inoculated with S. aureus strain B showing differences with the number of quantified cells in mammary glands inoculated with strain A and controls (*P*0.05).

T lymphocytes labeled with anti-CD8 were found infiltrating the interalveolar and intralobular stromal tissue in mammary glands from all groups (Fig. 5). The number of CD8+ cells in mammary tissue (Fig. 6c) was influenced by the treatments ($P < 0.001$) and by the time of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). The number of CD8+ cells was higher in mammary glands inoculated with *S. aureus* strains A and B than in controls at all sampling times (*P*0.05). At 6 h pi, the number of CD8+ cells was higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls ($P < 0.05$). At 12, 24 and 48 h pi a gradual increase in the number of CD8+ cells in the mammary glands inoculated with both *S. aureus* strains was observed; being this increase higher in mammary glands inoculated with strain B than in those with strain A and controls ($P < 0.05$). At 96 h pi a peak in the number of

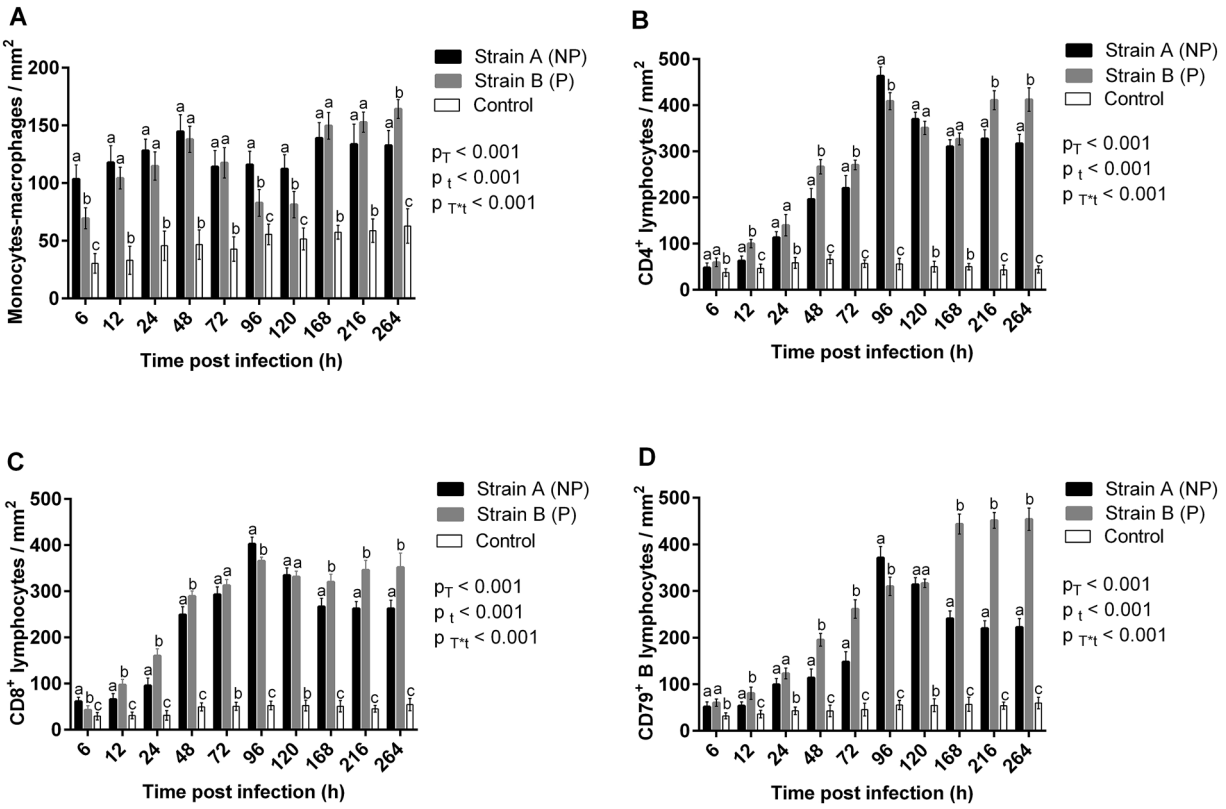

Fig. 6. Number of CD14+ monocytes-macrophages (a), CD4+ T cells (b), CD8+ T cells (c) and CD79+ B cells (d) in murine mammary glands inoculated with *S. aureus* strains A (non-persistent-NP) and B (persistent-P) and saline solution (controls) at different time points. Values represent mean numbers of immune cells/mm^2 ± standard error of the mean. The main effects of treatment (T) with *S. aureus* strains A and B, time of sampling (t) and treatment * time interaction (T*t) are shown. At each sampling time, different letters correspond to statistically significant differences ($P < 0.05$).

CD8+ cells in mammary glands inoculated with both *S. aureus* strains was observed, showing those inoculated with strain A higher number of cells than those inoculated with strain B and controls ($P < 0.05$). At 120 h pi a decrease in the number of CD8+ cells in mammary glands inoculated with both *S. aureus* strains was observed, showing those mammary glands inoculated with strain A and B similar number of cells and higher than controls ($P < 0.05$). At 168, 216 and 264 h pi the number of CD8+ cells increased significantly in mammary glands inoculated with *S. aureus* strain B showing differences with the number of quantified cells in mammary glands inoculated with strain A and controls ($P < 0.05$).

B lymphocytes labeled with anti-CD79 were found infiltrating the interalveolar and intralobular stromal tissue in the mammary glands from all groups (Fig. 5). The number of CD79+ cells in mammary tissue (Fig. 6d) were influenced by the treatments ($P < 0.001$) and by the time of sampling ($P < 0.001$), showing significant interaction between the two factors ($P < 0.001$). The number of CD79+ cells was higher in mammary glands inoculated with *S. aureus* strains A and B compared with controls at all sampling times ($P < 0.05$). At 12, 48 and 72 h pi a gradual increase in the number of CD79+ cells in the mammary glands inoculated with both *S. aureus* strains was observed; being this increase higher in mammary glands inoculated with strain B than those inoculated with strain A and controls ($P < 0.05$). At 96 h the number of CD79+ cells in mammary glands inoculated with *S. aureus* strain A was higher than the number quantified in mammary glands inoculated with strain B and controls ($P < 0.05$) reaching a peak. At 168, 216 and 264 h pi the number of CD79+ cells increased significantly in mammary glands inoculated with *S. aureus* strain B showing differences with the number of quantified cells in mammary glands inoculated with strain A and controls ($P < 0.05$).

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 (Nagahata et al., 2011; Wellnitz and Bruckmaier, 2012). Moreover, it was shown that different *S. aureus* strains have varying effects on the immune response (Kim et al., 2011). In the current study, a murine mastitis model was used to compare the hosts' immune response against two selected *S. aureus* isolates from NP and P bovine IMI. The *S. aureus* strains selection was based on results obtained in a previous study including clinical manifestation, phenotypic and genotypic profiles, multiplication capacity, biofilm production, expression of adhesion and biofilm-producing genes and mammary epithelial cell invasion capability (Pereyra et al., 2016). In addition, strains were further assigned to Clonal Complexes. Strain A belonged to CC 188 methicillin susceptible *S. aureus*, which is mostly a human lineage sporadically isolated from cattle (Monecke et al., 2011). Strain B belonged to CC97 which is frequently isolated from cattle (Monecke et al., 2008).

The mouse model of mastitis provides a valuable tool for the study of the pathogenesis of *S. aureus* mammary gland infection (Notebaert and Meyer, 2006). In the present study, both *S. aureus* strains multiplied rapidly in mice mammary tissue after inoculation, showing differences in bacterial clearance during infection progression. Differences between strains observed in the present study can be related to their *in vitro* behavior. In a previous study we evaluated the multiplication rate *in vitro* of *S. aureus* strain A and B and observed that both strains reached a maximal density after 5 h of culture; however strain A showed significantly lower generation time than strain B (Pereyra et al., 2016). These results are consistent with those observed in the present study at 6 and 12 h pi, where the strain A showed a higher rate of bacterial replication compared with strain B. Moreover, we observed

that strain A showed higher invasion ability in MAC-T cell and overexpression of adhesion and biofilm producing genes following internalization compared with strain B (Pereyra et al., 2016), which could have influence in bacterial replication and colonization *in vivo*. Collectively these findings demonstrated that, although both strains were capable of establishing an IMI, strain A showed a more aggressive behavior than strain B at early stages, while strain B multiplied initially at a lower rate but from 120 h pi to the end of the study increased its replication capacity probably associated to a better adaptation to the mammary gland environment. Although CC are considered to reflect adaptation to host species, both lineage and strain specific immune responses to bovine mammary epithelial cells *in vitro* have been detected (Budd et al., 2016). Since only two strains from two different CC were evaluated, potential associations between strain CC and *in vivo* behavior observed in this study cannot be established.

Consistent with histopathologic results obtained in early studies by Anderson and Chandler (1975) during *S. aureus* IMI in mice, in the present study *S. aureus* strain A triggered an acute pro-inflammatory response from initial observations until 120 h pi that was associated with a reduction in the number of bacteria recovered from mammary glands, but inducing extensive mammary tissue damage. Compared with strain A, in mammary glands inoculated with strain B, the histopathological lesions were less severe from initial observations until 120 h pi. In precursor histological studies during *S. aureus* mastitis in mice, necrotic mammary tissue areas were observed and attributed to the action of alpha and beta toxins (Haraldsson and Jonsson, 1984). Moreover, Bramley et al. (1989) observed severe lesions in murine mammary tissue after inoculation of *S. aureus* toxigenic strains, particularly those that expressed alpha-toxin. Although detecting toxin presence in mammary tissue was beyond the scope of this study, both strains A and B were able to produce alpha and beta toxins *in vitro* (Pereyra et al., 2016), suggesting that the greater injure observed in mammary glands inoculated with strain A at early stages of infection, could be due to alpha toxin production associated with its higher replication rate in mammary tissue. After 120 h pi, in mammary glands inoculated with strain B, we observed parenchymal zones with functional and preserved structure of alveoli and ducts, zones with necrosis, loss of normal architecture and replacement of mammary parenchyma with fibrous tissue. However, we also observed zones of alveoli and ducts with exudate of neutrophils in their lumen coexisting with zones infiltrated by macrophages, lymphocytes and plasma cells, which could indicate a chronic inflammatory response induced by the persistence of the bacteria in mammary tissue. These results showed that while strain B appeared to be partially controlled by the immune system until 120 h pi compared with strain A, as indicated by a lower bacterial burden, the immune response was insufficient to inhibit bacterial replication during this sampling period. Although both *S. aureus* strains appeared to up-regulate recruitment of immune cells, *S. aureus* strain A triggered a stronger initial inflammatory response compared with strain B which influenced the outcome of the host-bacterium interaction.

Toll-like receptor 2 is a crucial recognizable receptor activated by *S. aureus* infection as demonstrated both in mammary epithelial cells and in rat mammary tissue (Yang et al., 2008; Wang et al., 2015). In the present study, protein expression of TLR2 was higher in mammary glands inoculated with *S. aureus* strains A and B compared with controls during all sampling times. Studies characterizing immunoexpression of TLR2 in *S. aureus* mouse mastitis model have not yet been carried out; however, our findings are consistent with previous reports in cows where TLR2 was detected by IHQ in the apical domain of ductal and alveolar epithelium and in the cytoplasm of immune cells like monocytes-macrophages, lymphocytes, neutrophils and plasma cells (Baravalle et al., 2015). At 6 h pi protein and gene expression of TLR2 was higher in mammary glands inoculated with strain A compared with strain B and controls, which could be related to a higher initial recognition and enhanced immune response triggered by strain A. Between 12 and 48 h pi the highest percentages of immunoexpression for both *S. aureus* strains were observed, which were directly related with the high TLR2 mRNA levels observed at 12 h pi. At 48 and 72 h pi, significant differences among the three treatments were observed, showing the mammary glands inoculated with strain A the highest percentages of immunostaining for TLR2. These results could indicate that *S. aureus* strain A was able to induce a higher TLR2 gene expression at onset of infection (6 h pi) resulting in a higher protein expression until 72 h pi. Although initial recognition by TLR2 was higher for strain A compared with strain B, bacterial clearance from mammary glands was significantly lower than the one observed for strain B between 96 and 120 h pi and this might be a contributing factor to the molecular mechanisms underpinning the immune evasion strategies of this pathogen from mammary defenses (Netea et al., 2004).

The activation of TLRs by pathogens is known to result in the activation of NF-κB (Hatada et al., 2000). Once activated, the NF-κB subunit p65 dissociates from its inhibitory protein IκB-α and translocates from the cytoplasm to the nucleus, where it triggers transcription of pro-inflammatory cytokines (Li and Verma, 2002). In an early study, Notebaert et al. (2008) using IHC detected activated NF-κB by the nuclear localization of p65 in mammary epithelial cells of mice challenged with *E. coli*. In the present study, using immunohistochemical staining, we demonstrated that for both *S. aureus* strains the subunit p65 of the transcription factor NF-κB was translocated to the nucleus of the murine mammary epithelial and stromal cells. These results agree with a previous *in vivo* study using NF-κB reporter mice that detected the NF-κB-p65 in the nucleus of mammary epithelial cells and immune cells at 12 and 24 h after intramammary infection with *S. aureus* (Breyne et al., 2014). In the present study, at 12 h pi and from 96 to 168 h pi both percentages of parenchymal and stromal NF-κB-p65 positive cells were higher in mammary glands inoculated with *S. aureus* strain A compared with strain B and controls. These results are consistent with the increased ability of strain A compared with strain B to induce a higher gene and protein expression of TLR2, higher capacity of replication *in vitro* (Pereyra et al., 2016) and *in vivo* in murine mammary gland from 6 to 24 h pi.

The activation of TLR-induced signaling pathways leads to the production of the major pro-inflammatory cytokines TNF-α and IL-1β, which are locally produced by several cell types and trigger an inflammatory cascade (Zbinden et al., 2014). It has previously been reported that *S. aureus* bovine IMI results in the upregulation of IL-1β, IL-6 and IL-8 but not TNF-α (Wellnitz and Bruckmaier, 2012). Breyne et al. (2014) compared the kinetics of the induced cytokine profiles following mice mammary gland challenge with *E. coli* and *S. aureus* showing similar increase of IL-1α and TNF-α for both pathogens after 12 and 24 h pi, but about 3-fold higher for *E. coli* compared with *S. aureus*. In the present study, mRNA levels for IL-1α were significantly higher in mammary glands inoculated with both *S. aureus* strains compared with controls at all times of sampling. At 6 h pi a different outcome for *S. aureus* strains A and B were observed, showing mammary glands inoculated with strain A higher IL-1α gene expression compared with strain B. These results could be associated with the higher initial bacterial recognition by TLR2, higher rate of bacterial replication and with the increased monocytes-macrophages recruitment in mammary glands inoculated with strain A compared with strain B at this sampling time; being indicative of greater immune response stimulation from strain A with respect to strain B in the initial stage of the IMI. In mammary glands inoculated with both *S. aureus* strains we observed a peak in IL-1α mRNA levels at 48 h pi, which could be associated with the gradual increase of monocytes-macrophages in mammary tissue at this sampling time. However, from 120 h and until 264 h pi a different outcome for *S. aureus* strains A and B was observed; showing a higher IL-1α gene expression mammary glands inoculated with strain B compared with strain A. Concomitantly, during this interval a higher CFU number was recovered from mammary glands inoculated with strain B compared with strain A and controls, which could be associated with the pathogen reactivation, and re-stimulation

of immune response consistent with a f chronic inflammatory process. The study of mechanisms that determine infection reactivation were beyond the scope of this study, but this characteristic may allow *S. aureus* to colonize other areas of the gland promoting bacterial persistence *in vivo*.

In accordance with results of Breyne et al. (2014) in *S. aureus* infected murine mammary glands, mRNA levels for TNF-α showed a peak at 24 h pi for both strains evaluated compared with controls. These authors also detected a strong induction of IL-8 like chemokine (KC and MIP-2) after 24 h pi coincident with an influx of neutrophils to mammary gland. In our study, although chemokines were not evaluated, histopathological studies corroborate the infiltration of neutrophils at this time for both *S. aureus* strains. At 48 h pi, TNF-α gene expression decreased sharply in mammary glands inoculated with both *S. aureus* strains compared with the previous period, showing higher TNF-α gene expression mammary glands inoculated with strain B compared with strain A and controls. Similarly to IL-1α, from 120 to 216 h pi, an increase of TNF-α gene expression in mammary glands inoculated with strain B was observed, showing higher relative gene expression levels compared with mammary glands inoculated with strain A and controls. A recent study in lactating mice infected with large doses of strong versus weak biofilm-forming *S. aureus* to induce acute mastitis suggested potential association of mammary tissue damage with the strength of biofilm formation and production of high levels of TNF-α (Gogoi-Tiwari et al., 2017). Based on results obtained previously (Pereyra et al., 2016) strain A was characterized as a moderate biofilm producer while strain B as a strong biofilm producer, which may be related to the increase in TNF-α gene expression and chronic inflammatory reaction induced by strain B as infection progressed. Collectively, these results suggest a strong and sustained inflammatory reaction in mammary glands inoculated with strain B compared with strain A as the IMI progressed, demonstrated by the increased number of monocytes-macrophages and exudates of neutrophils in the lumen of alveoli and ducts. Although previous *in vitro* studies have shown that TNF-α is a potent leukocyte activator that enhances the phagocytosis and killing of mastitis pathogens by neutrophils (Kabbur et al., 1995), recruitment of inflammatory cells induced by strain B was not sufficient to control bacterial replication.

Interferon-γ is an important mediator for activation of recruited neutrophils and macrophages that enhances microbicidal activity via receptor-mediated phagocytosis (Riollet et al., 2001; Bannerman et al., 2004). Studies characterizing gene expression of IFN-γ in a *S. aureus* mouse mastitis model until day 11 pi have not yet been carried out. Zhao et al. (2015) observed a slight but detectable increase in the mRNA levels of INF-γ up to 7 d pi in mammary glands from mice challenged with 4×10^6 CFU/gland of *S. aureus* isolated from a goat IMI. In this study, although the number of monocytes-macrophages was higher in mammary glands infected with both *S. aureus* strains compared with controls during all sampling times, the transcript levels for IFN-γ were too low to be quantified in all experimental groups. Discrepancies between studies may rely in the *S. aureus* strain origin, the concentration of inoculum used, which was lower in the present study, and the mouse strain.

IL-10 is often associated with suppression or resolution of inflammatory responses (Redpath et al., 2001). Although cDNA transcripts for IL-10 in mammary tissue from the three inoculated groups were found at all sampling times, levels were too low to be quantified. In contrast, Zhao et al. (2015) found that IL-10 mRNA expression was significantly increased on day 1 and 7 pi in mammary glands from mice challenged with *S. aureus*. Discrepancies between studies are coincident with those observed for the expression of IFN-γ.

Macrophages are the main immune cell type that invading pathogens encounter when entering the mammary gland. Upon bacterial recognition, macrophages activate the immune system through release of cytokines and other pro-inflammatory mediators and orchestrate the innate immune response, including neutrophil migration and bactericidal functions (Aitken et al., 2011). Our results showed that the number of monocyte-macrophages quantified increased significantly from 6 h until 48 h pi in mammary glands inoculated with both *S. aureus* strains with respect to control glands, except at 6 h pi where the number of cells was significantly higher in mammary glands inoculated with strain A compared with B and control. Interestingly, at 96 and 120 h pi the number of monocytes-macrophages in mammary glands inoculated with strain B decreased significantly compared with strain A, coinciding with the fewest number of CFU recovered from mammary glands inoculated with strain B compared with strain A. This result suggests that mammary glands inoculated with *S. aureus* strain B, between 96 at 120 h pi, were able to mount a partially effective immune defense and possibly other immune cells like neutrophils contributed to bacterial clearance as observed by histopathology. Instead, during the same sampling periods, mammary glands inoculated with strain A presented higher number of monocytes-macrophages, but lower bacterial clearance and mRNA levels of pro-inflammatory cytokines compared with mammary glands inoculated with strain B, indicating a less effective immune response. After 168 h pi the number of monocytes-macrophages in mammary glands inoculated with both *S. aureus* strains increased with respect to the previous period, reaching mammary glands inoculated with strain B significantly higher numbers than strain A at 264 h pi. The higher number of phagocytic cells in mammary glands inoculated with strain B was related to the significantly higher number of CFU recovered from mammary tissue at this time, indicating a re-stimulation of monocytes-macrophages and pro-inflammatory cytokines expression. In mammary glands inoculated with strain A, although the number of phagocytic cells remained high at this time, the bacterial burden significantly decreased indicating a greater infection control.

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 (Nagahata et al., 2011). In the murine mastitis model, the number of T helper and T killer lymphocytes, as well as, the number of B lymphocytes was higher in mammary glands inoculated with both *S. aureus* strains compared with controls all along the sampling time. Moreover, a sharp increase in lymphocytes number was observed at 96 h pi in mammary glands inoculated with both strains tested. This increment could be a result of the inflammatory environment triggered by the innate immune response, which attracts to the site of infection naive lymphocytes that in physiological conditions circulate throughout the mammary gland (Yamaguchi et al., 1999).

Changes of lymphocytes subsets in mammary tissue of mice during pathogenesis of *S. aureus* IMI have rarely been reported (Cao et al., 2012; Zhao et al., 2015). In a murine *S. aureus* mastitis model, Zhao et al. (2015) observed increased percentages of CD4+ and CD8+ cells at 7 and 5 days post-challenge, respectively. In cows, Grönlund et al. (2006) demonstrated changes in proportions of CD4+ and CD8+ cells obtained from milk of quarters experimentally infected with *S. aureus*, showing an increment in CD4+:CD8+ cell ratio in the clinical phase of IMI and predominance of CD8+ cells in the chronic sub-clinical phase of IMI (from day 22 to day 33 pi). In the present study, inversion of CD4+:CD8+ cell ratio during the infectious process was not observed and the number of CD4+ cells was always higher than CD8+ cells in mammary glands inoculated with both *S. aureus* strains. The lymphocytes found in mammary tissue differ from milk and possibly play different roles in the immune response against pathogens which, besides the differences between animal species, could explain the discrepancies observed with the studies of Grönlund et al. (2006) from bovine milk.

Studies characterizing the dynamics of B cells in mouse model of *S. aureus* IMI have not yet been carried out. In cows, Grönlund et al. (2006) observed increased proportion and expression of B-lymphocytes in blood and milk during chronic sub-clinical mastitis. In the present study, increased number of B lymphocytes was evident in mammary

glands inoculated with *S. aureus* strain B compared with strain A and controls during advanced stages of infection (from 168 h to 264 h pi), suggesting development of a humoral response. As *S. aureus* can invade and survive intracellularly, a preferential stimulation of B cells and development of humoral response in mammary glands inoculated with strain B during advanced stages of infection, may not be sufficient to eliminate intracellular bacteria, which could explain the high number of CFU recovered from mammary tissue. The higher number of T and B lymphocytes in mammary glands inoculated with strain B from 168 to 264 h pi could be a result of the chronic inflammatory environment triggered by the presence of viable bacteria in mammary tissue. Collectively, these results indicate that both *S. aureus* strains were able to stimulate an adaptive immune response in the host after IMI coinciding with others studies in murine mammary gland (Cao et al., 2012; Zhao et al., 2015).

5. Conclusions

The present study revealed that both *S. aureus* strains isolated from bovine IMI with different clinical manifestation (clinical-NP and subclinical-P), phenotype and genotypic profile were able to induce immune responses in murine mammary glands in a strain-dependent manner. The host immune response was different for each *S. aureus* strain throughout the course of infection, showing in general a greater initial response to strain A compared with strain B and then a further immune response, mainly stimulated by strain B and consistent with a chronic inflammatory process. Strain B (P), compared with strain A (NP), achieved a greater adaptation to the mammary gland, inducing a higher immune response in the advance stages of IMI but with lower bacterial clearance from tissue suggesting the development of bacterial strategies for overcoming host immune response. In this study, strain-specific pathogenicity observed across the IMI, implies that pathogen factors rather than host factors and that *S. aureus* strains could influence the host immune response to achieve the persistence in mammary gland in this model. Our results collectively enhance the available information on host-pathogen interaction and point out for the need of further research to expand the knowledge about these interactions for developing new strategies to control this mastitis-causing pathogen.

Conflict of interest statement

None of the authors have any conflict of interest.

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