Veterinary Immunology and Immunopathology 132 (2009) 237–242

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

Effect of a biological response modifier on expression of CD14 receptor and tumor necrosis factor-alpha in *Staphylococcus aureus*-infected mammary glands at drying off

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 9 April 2009
Accepted 11 May 2009

Keywords:
Biological response modifier
Bovine mammary gland
TNF-α
CD14 receptor
Staphylococcus aureus

ABSTRACT

Agents that increase natural protective mechanisms have been proposed for prevention and treatment of intramammary infections. The objectives of this study were to get an insight of innate immune mechanisms that occur during bovine mammary involution in both uninfected and chronically *Staphylococcus aureus*-infected glands and to describe the effects on those mechanisms of a single intramammary infusion of a LPS-based biological response modifier (BRM) at the end of lactation. Three groups of 12 cows, each one including 6 *S. aureus*-infected and 6 uninfected, were infused in two mammary quarters with BRM or placebo and sacrificed at 7, 14 and 21 d of involution. In uninfected and *S. aureus*-infected quarters treated with a BRM, the number of monocytes/macrophages detected with CD14 antibody was significantly higher ($P < 0.05$) than in placebo-treated quarters at every sampling evaluation period. In uninfected quarters, the TNF-α staining area was not affected by BRM treatment. However, in infected quarters, the immunostained area for TNF-α was significantly higher than in uninfected quarters and BRM treatment was associated with increased staining at 21 d of involution.

1. Introduction

Susceptibility of the bovine mammary gland to new intramammary infections (IMIs) is markedly increased during early involution and the periparturient period (Oliver and Sordillo, 1988). Conversely, fully involuted bovine mammary glands are markedly resistant to IMI (Oliver and Sordillo, 1988). These observations led to the hypothesis that hastening mammary gland involution while increasing natural protective mechanisms during the early nonlactating period could favor prevention of IMI during the involution process (Oliver and Smith, 1982a).

An improved understanding of components implicated in the immunological response of the mammary gland has led researchers to address alternative approaches to classic mastitis control measures based on hygiene and antibiotic therapy, such as manipulation of local immune responses to mastitis pathogens. Among these, biological response modifiers (BRMs) or immunomodulators are compounds capable of interacting with the immune system to regulate specific aspects of the host response (Tzianabos, 2000). Biological response modifiers have been used in an attempt to enhance innate immune mechanisms against bovine mastitis pathogens (Zecconi, 2000; Takahashi et al., 2004; Kimura et al., 2008). However, the exact mechanisms of

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doi:10.1016/j.vetimm.2009.05.004

action and effect of these compounds on the bovine mammary tissue are not fully understood. Studies performed in cows showed that intramammary inoculation of *Escherichia coli* lipopolysaccharide (LPS) at drying off resulted in acceleration of mammary gland involution (Oliver and Smith, 1982a). Intramammary LPS-based BRM in *Staphylococcus aureus*-infected mammary glands induced an increase in pro-apoptotic proteins (Bax), apoptogenic proteases and apoptosis, without inhibiting mammary cell proliferation (Dallard et al., 2008). In addition, inoculation of LPS (Oliver and Smith, 1982b) and LPS-based BRM (Dallard et al., 2007) at drying off was shown to transiently reduce the isolation of mastitis pathogens from mammary glands during the nonlactating period.

LPS is a complex glycolipid with different biological effects that include nonspecific activation of the immune system, activation of the complement cascade and induction of endotoxin-shock syndrome (Van Miert, 1991). LPS binds to a 60-kDa serum acute-phase protein, LPS binding protein (LBP) (Erridge et al., 2002) to conform a LPS-LPB complex which binds to CD14, a glycosylphosphatidylinositol-linked receptor that lacks a transmembrane domain, present on monocytes, macrophages and, to a lesser degree, on neutrophils (Landmann et al., 1991). This binding initiates signal transduction through the toll-like receptor-4 (TLR-4) resulting in the release of pro-inflammatory cytokines by macrophages, such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL)-1β, -6, -8 (Van Miert, 1991; Guha and Mackman, 2001), inducing an innate defence response against Gram-negative bacteria (Van Miert, 1991). Furthermore, little is known about the role of the cell membrane receptor CD14 in pathogenesis of mastitis caused by Gram-positive bacteria. Sladek and Rysanek (2006) suggested the possibility of involvement of CD14 surface receptor in recognition of apoptotic neutrophils by macrophages during *S. aureus* and *Streptococcus uberis* experimentally induced mastitis.

TNF-α is a potent leukocyte activator that enhances the phagocytosis and killing of mastitis pathogens by bovine neutrophils (Kabbur and Jain, 1995). Although TNF-α has been detected in normal and infected bovine mammary glands (Hagiwara et al., 2000; Riollet et al., 2001; Alluwaimi et al., 2003), its exact role in mammary gland regulatory processes that take place during physiological or pathological states is not fully understood (Watanabe et al., 2000).

There is only scarce information about innate immune mechanisms that take place during involution of uninfected or infected bovine mammary glands (Rainard and Riollet, 2006). The objectives of this study were to get an insight of innate immune mechanisms that occur during bovine mammary involution in both uninfected and chronically *S. aureus*-infected glands and to describe the effects on those mechanisms of a single intramammary infusion of a LPS-based BRM at the end of lactation.

2. Materials and methods

2.1. Biological response modifier

The product contained LPS of *E. coli* strain (LN02) at 0.35 μmol concentration and 4.5 mg of membranous and ribosomal fractions of the same strain incorporated into liposomes contained in 10 mL of aqueous-based vehicle (Laboratorio Neomar, Buenos Aires, Argentina). Liposomes (The Liposystem Complex®, I.R.A., Milan, Italy) composition was 40% phospholipids and 60% hydrophilic medium and active principle. Concentration of LPS was determined by quantification of 2-keto-3-deoxyoctonate by a colorimetric method (Osborn, 1963).

2.2. Animals

Holstein nonpregnant cows in late lactation from the Rafaela Experiment Station of INTA herd producing approximately 8 kg of milk per day before experimentation were used. Based on previous bacteriological studies, animals were identified as either uninfected or infected with *S. aureus*. Infections were naturally acquired either in the dry period or during the first 2 months of the lactation preceding initiation of the study.

2.3. Experimental design

Infectious status of mammary quarters was determined within 6 months before initiation of the experiment and confirmed 20 and 3 d before inoculation. Infected quarters were randomly selected from cows showing at least two quarters infected with *S. aureus*. From these cows, only two infected quarters were infused either with BRM or placebo (vehicle alone). Uninfected quarters were selected from cows free of infection at the time of sampling. Only two quarters from each cow were infused either with BRM or placebo. Cows whose mammary quarters became infected during the experimental period were excluded from the study. Uninfected ($n = 6$) and *S. aureus*-infected ($n = 6$) mammary quarters were included in each group (7, 14 and 21 d). In all cases milking was interrupted after intramammary infusion of either BRM or placebo. Mammary secretion samples were aseptically collected for bacteriological analysis using standard procedures (Hogan et al., 1999) 3 d before BRM or placebo administration, immediately before inoculation and every 48 h after infusion. Samples for somatic cell counts (SCCs) were collected immediately after taking samples for bacteriological analysis before BRM or placebo administration and every 48 h after infusion. Animals included in the three groups were slaughtered at 7, 14 and 21 d after inoculation at a local abattoir and samples for histological analysis were taken. According to the eligibility criterion used, 36 out of 44 cows that initiated the experiment were included in the study.

2.4. Bacteriological examination

Ten microlitres of mammary secretion samples 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 and 48 h. Isolated colonies were identified according to standard procedures (Hogan et al., 1999). Presence of one colony of *S. aureus* on blood agar was considered as a positive identification; therefore detection limit was 100 colony

forming units/mL. Intramammary infection was defined as isolation of the same organism from two consecutive samples.

2.5. Somatic cell counts

The SCC determinations were performed by a commercial laboratory (Laboratorio Regional de Servicios Analíticos, Esperanza) using an automated counter Somacount 300 (Bentley Instruments, MN, USA). Since most significant changes in SCC occur during the first week following lactation interruption (McDonald and Anderson, 1981), for data analysis only a subset of samples from preinoculation to day 8 post-inoculation (PI) belonging to the group that was sacrificed 21 d PI were considered.

2.6. Tissue samples preparation

Immediately after cows were sacrificed, tissue samples were taken from the dorso-lateral portion of the gland (deep parenchyma) at a depth of 4 cm following previous descriptions (Nickerson et al., 1992). Tissue samples of approximately 1 cm^3 were fixed in 10% neutral buffered formalin, washed in phosphate buffered saline (PBS, pH 7.4) and processed as previously described (Dallard et al., 2005).

2.7. Immunohistochemistry

A streptavidin-biotin immunoperoxidase method was performed as previously described (Dallard et al., 2008). Briefly, sections were deparaffinized, hydrated and microwave pre-treated (antigen retrieval). The endogen peroxidase activity was inhibited with 1% H_2O_2 and nonspecific binding was blocked with 10% normal goat serum. Sections were incubated overnight at 25 °C with rabbit polyclonal antiserum against ovine TNF-α (Chemicon, San Francisco, CA) and CD14 monoclonal antibody (clone RPA-M1; Zymed, San Francisco, CA). Slides were incubated for 30 min at room temperature with rat-preabsorbed biotinylated secondary antibodies selected specifically against one of each of the two types of primary antibodies used (monoclonal or polyclonal). The visualization of antigens was achieved by the streptavidin-peroxidase method (BioGenex, San Ramon, CA) and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit – Zymed, San Francisco, CA) was used as chromogen.

2.8. Image analysis

Image analysis was performed using Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA). Images were digitized by a CCD color video camera (Sony, Montvale, NJ) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co., Japan) using an objective magnification of 40×. The details of image analysis as a valid method for quantifying expression levels and the methodological details were previously described (Dallard et al., 2005, 2007). Briefly, the immunohistochemical stained area (IHCSA) for 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 colors 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. TNF-α expression was evaluated by positive IHCSA. For measuring number of monocytes/macrophages stained with anti-CD14, 40 random images from each slide were digitized at 40× and number of cells per mm^2 was calculated.

2.9. Statistical analysis

A three way factorial model with fixed factors for treatment (BRM and placebo), infection status (*S. aureus*-infected and uninfected) and days of involution (7, 14 and 21 d) was applied. For SCC a similar model was applied using time intervals of 48 h instead of days of involution. A Proc GLM was used and means were compared by Duncan test (SAS, 1999).

3. Results and discussion

3.1. Analysis of somatic cell counts

At cessation of milking, the mammary gland tissue undergoes intense physiological changes. In the early dry period, cell concentrations augment for the first 7 d to reach about 2–5 × 10^6 mL^{-1}, then decrease to stabilise during most of the period at 1–3 × 10^6 mL^{-1} (McDonald and Anderson, 1981). In the present study, the pattern of SCC in uninfected placebo-treated quarters was similar to that previously described in cows after interruption of lactation (Oliver and Smith, 1982a); showing a steady increase along the sampling period and a peak 8 d PI (Fig. 1). A slow increase in SCC was observed in uninfected and *S. aureus*-infected quarters treated with placebo, reaching a peak at 6 d PI of 6.877 and 6.974 for uninfected and infected mammary glands, respectively (Fig. 1). The

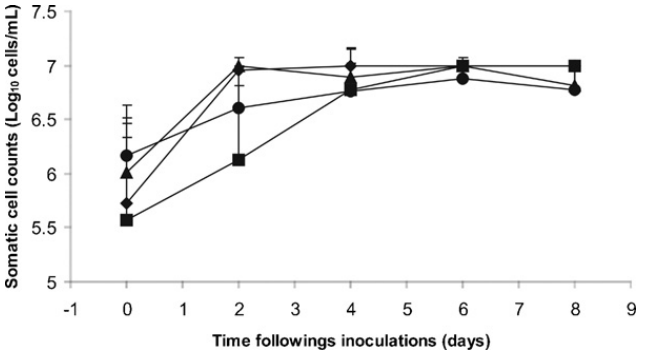

Fig. 1. Kinetics of somatic cell counts (SCC) after intramammary infusion of a biological response modifier (BRM) or placebo in *S. aureus*-infected and uninfected quarters. The values represent means of SCC at 48 h intervals for an 8-d period during involution. Samples were collected immediately before BRM administration and at every 48 h post-infusion. (◆): Uninfected mammary quarters treated with BRM; (■): Uninfected mammary quarters treated with placebo (▲): *S. aureus*-infected quarters treated with BRM; (●) *S. aureus*-infected quarters treated with placebo.

response profile for SCC in mammary secretions from infected placebo-treated quarters was in accord with previous observations for subclinical mastitis (Sordillo et al., 1987).

A pronounced increase in SCC at 48 h post-BRM inoculation was observed in both *S. aureus*-infected and uninfected mammary quarters, followed by a slow decrease through the sampling period. The peak log SCC at 48 h PI in mammary secretions from infected and uninfected quarters was 7.002 and 7.013, respectively (Fig. 1). These results were in accord with previous observations of Oliver and Smith (1982a) following a single intramammary administration of 100 μg of *E. coli* LPS or 100 μg of LPS plus 20 mg of colchicine. The different nature of the preparations used in both studies do not allow for direct comparisons; however, massive influx of cells, which is considered to contribute to bacterial clearance, was achieved at the same time PI and followed a similar pattern of decline along the experimental period. In addition, Kauf et al. (2007) observed a SCC peak 48 h after inoculation of 100 μg of *E. coli* LPS in mammary quarters that were experimentally infected with *S. aureus* 24 h before.

Interactions between sampling day and infectious status, as well as between sampling day and treatment group were detected. Significant differences in SCC were detected between BRM- and placebo-treated quarters only at 48 h post-inoculation, irrespectively of infectious status ($P < 0.05$).

3.2. Monocytes/macrophages quantification

Capability of the immune system to respond to a number of bacterial pathogens is mediated by its ability to recognize pathogen-associated molecular patterns (PAMPs). PAMPs include bacterial cell wall components of both Gram-negative and -positive bacteria. Recognition of PAMPs is mediated by toll-like receptors (TLR) and accessory molecules, like CD14 and LPS binding protein (LBP). In TLR-2- and TLR-4-mediated activation, CD14 serves as a key receptor for LPS, peptidoglycan and lipoteichoic acid (Wright et al., 1990). The role of the cell membrane receptor CD14 in activation of cells from bovine mammary glands was studied mainly in LPS-mediated mastitis. It was observed that the inflammatory reaction of mammary glands to LPS was accompanied with expression of the CD14 surface receptor on neutrophils and macrophages (Sladek et al., 2002). LPS binds to LPB, an acute-phase protein that facilitates the transfer of LPS to membrane-associated CD14 (Wright et al., 1990). This binding initiates signal transduction through the TLR-4 resulting in the release of pro-inflammatory cytokines by macrophages, such as TNF-α and IL-1β, IL-6, IL-8 (Guha and Mackman, 2001), inducing an innate defence response against Gram-negative bacteria (Van Miert, 1991).

Previous investigations by several authors described that CD14 is found on the membranes of monocytes/macrophages, and in a 10-fold-lower amount on polymorphonuclear granulocytes (Landmann et al., 1991). This observation might explain the different pattern showed in either neutrophils or monocytes/macrophages immunoreactivity for CD14 in the present study, since immunostaining was minimally observed in neutrophils and widely expressed in monocytes/macrophages. The CD14+ cells 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 the staining occurred strongly on their surface.

Sladek and Rysanek (2006) evaluated the role of CD14 during resolution of experimentally induced *S. aureus* and *S. uberis* mastitis. Infections induced a local immune response characterized by an increase in the total counts of CD14+ neutrophils and macrophages in milk In the present study, regarding CD14+ monocytes/macrophages number, no interactions among the three factors were detected (Table 1). Interactions between infectious status and week of involution were detected ($P < 0.05$). The number of monocytes/macrophages/mm^2 staining with CD14 antibody in mammary tissue was significantly higher in placebo-treated chronically *S. aureus*-infected than in uninfected quarters during every observation period ($P < 0.05$), indicating the involvement of cells bearing this receptor in the local immune reaction of mammary gland against this organism. In uninfected placebo-treated quarters, a significant increment of monocytes/macrophages was observed at 7 d of involution, compared with 14 and 21 d ($P < 0.05$).

Interactions between treatment and infectious status were detected ($P < 0.05$). In *S. aureus*-infected and uninfected quarters, treated with LPS-based BRM, we observed a significant increase in the number of CD14+ monocytes/macrophages/mm^2 at every sampling evaluation period ($P < 0.05$), suggesting that BRM stimulus maintained recruitment of this cellular type along the period irrespectively of the infectious status at inoculation time. Although recruitment of this cell type and a parallel

Table 1
Effect of treatment with a biological response modifier on CD14+ monocytes/macrophages number in uninfected and *S. aureus*-infected mammary quarters during involution.

Involution	Quarters infected with *S. aureus*		Uninfected quarters	
	BRM[a]	PLACEBO	BRM	PLACEBO
7 d	110.11 (2.25)a	103.14 (1.44)b,c	103.12 (3.16)b,c	82.13 (1.17)e
14 d	107.25 (1.48)a,b	91.29 (2.11)d	100.92 (1.30)c	75.67 (0.71)f
21 d	100.57 (0.83)c	81.53 (1.26)e	102.94 (0.79)b,c	74.98 (1.43)f

Values represent means of number monocytes/macrophages/mm^2 ± standard error of the mean. Means for each treatment and days of involution without a common letters (a–f) differ ($P < 0.05$).

[a] Biological response modifier.

increment of SCC are associated with resolution of inflammation (Sladek and Rysanek, 2006) and clearance of infection (Kauf et al., 2007) we observed in a previous study that BRM treatment only contributed to transient clearance of *S. aureus* IMI at 7 d of involution (Dallard et al., 2007).

Interactions between treatment and week of involution were detected ($P < 0.05$). In *S. aureus*-infected quarters treated with BRM, the number of monocytes/macrophages at 7 and 14 d was significantly greater than at 21 d of involution. In infected, placebo-treated quarters, the number of immunopositive cells was higher at 7 d of drying off ($P < 0.05$) diminishing with the progress of involution.

3.3. TNF-α immunostaining

The physiological or pathological roles of TNF-α in the regulation of mammary functions *in vivo* have not been well defined (Watanabe et al., 2000). In the present study, TNF-α immunostaining was associated with mammary parenchyma and stroma structures both in infected and uninfected quarters at every sampling period. Intense immunostaining of alveolar epithelial cell cytoplasms and ducts was observed. Intraepithelial macrophages, neutrophils, lymphocytes and fibroblast, as well as endothelium and vascular smooth muscle cells, showed intense immunostaining. Regarding TNF-α expression, no interactions among the three factors were detected (Table 2).

Since TNF-α can stimulate apoptosis in various cell types (Laster et al., 1988), it may also be involved in mammary gland remodelling during involution. In the present study, interactions between infectious status and week of involution were detected ($P < 0.05$). In uninfected placebo-treated quarters, the percentages of IHCSA for TNF-α showed a significant increase at 7 and 21 d of drying off compared with 14 d of involution. In addition, infected placebo-treated quarters showed increased stained area for TNF-α compared with uninfected placebo-treated quarters, which paralleled an increase in stromal cells apoptosis as detected in a previous study (Dallard et al., 2008), suggesting a role for this cytokine in apoptosis induction during chronic infections. However, although several apoptosis markers were found to be increased during the involution period, a possible role of TNF-α in the induction of apoptosis during bovine mammary involution in uninfected quarters could not be ascribed (Dallard et al., 2008). Further studies are required to determine potential changes in the TNF-α and TNFR mRNA protein levels during mammary involution process.

Interactions between treatment and infectious status were detected ($P < 0.05$). A significant increase of immunostained area for TNF-α was observed in *S. aureus*-infected quarters treated with BRM compared with uninfected BRM-treated quarters at every sampling evaluation period ($P < 0.05$).

It should be remarked that the trigger for the release of TNF-α and its exact source in the mammary gland remains largely unknown to date. Certain interstitial cells, including mast cells, fibroblast, macrophages, lymphocytes, neutrophils and endothelial cells found within the complex architectural network that makes up the mammary gland, have been shown to produce TNF-α (Ip et al., 1992) and could serve as a source for this factor. In the present study, in uninfected quarters, BRM infusion caused a significant increase of CD14+ monocytes/macrophages number at every observation period. However, BRM treatment did not modify the percentages of staining area for TNF-α compared with placebo-treated controls during every evaluated period. TNF-α is significantly increased in milk and serum in both coliform and LPS-induced mastitis (Alluwaimi, 2004) and has been associated with severe clinical cases (Sordillo and Peel, 1992). Albeit LPS dose used in the present study elicited a CD14+ monocyte/macrophage response, it appeared that stimulus was insufficient to achieve high TNF-α level in mammary tissue. In addition, lack of association of CD14+ monocytes/macrophages number and TNF-α expression could have been due to the source of this factor, since other cell types not quantified in this study also produce TNF-α (Ip et al., 1992).

Percentages of IHQSA for TNF-α in *S. aureus*-infected quarters, were significantly greater than in uninfected quarters, at every evaluation period irrespective of treatment, suggesting that infection by this organism, rather than BRM infusion, appeared to be the main factor affecting this cytokine expression during early involution. A recent study demonstrated that experimental *S. aureus* IMI did not elicit production of TNF-α and IL-8 in milk during a 7-d observation period following bacteria inoculation, which has been considered to favor establishment of chronic infections (Bannerman et al., 2004). Although establishing a time course of appearance of TNF-α in mammary tissue during development of a chronic *S. aureus* infection was beyond the scope of the present study; mRNA of TNF-α and other pro-inflammatory cytokines in milk cells during chronic IMI caused by this organism has been detected in a previous study (Riollet

Table 2
Effect of treatment with a biological response modifier on percent of immunohistochemical stained area for TNF-α in uninfected and *S. aureus*-infected mammary quarters during involution.

Involution	Quarters infected with *S. aureus*		Uninfected quarters	
	BRM[a]	PLACEBO	BRM	PLACEBO
7 d	11.88 (0.31)b	11.11 (0.36)b,c	7.22 (0.73)e,f	8.55 (0.45)d,e
14 d	9.75 (0.74)c,d	8.71 (0.57)d,e	4.90 (0.79)g	5.84 (0.46)f,g
21 d	17.20 (0.50)a	12.28 (0.61)b	8.20 (0.42)d,e	8.81 (0.44)d,e

Values represent means of percent immunohistochemical stained area ± standard error of the mean. Means for each treatment and days of involution without a common letters (a–g) differ ($P < 0.05$).

[a] Biological response modifier.

et al., 2001), suggesting a role for this cytokine during chronic IMI.

Interactions between treatment and week of involution were detected ($P < 0.05$). In uninfected quarters, BRM treatment did not alter TNF-α expression during involution. While, inoculation of LPS-based BRM in chronically *S. aureus*-infected mammary glands, produced a significant increase of TNF-α immunostaining at 21 d of involution, compared with placebo-treated quarters. The nature of this response is difficult to explain and does not seem to be related to an immediate effect of BRM infusion. In addition, increased TNF-α immunostaining, coincided with a significant rise of CD14+ monocytes/macrophages in infected BRM-treated quarters at 14 and 21 d of drying off compared with placebo-treated quarters. In conclusion, these findings showed that administration of BRM to mammary glands increases SCC and recruitment of CD14+ monocytes/macrophages in both infected and non-infected glands and increases TNF-α expression in *S. aureus*-infected glands. The results suggest that BRM can act to promote innate immune defence against bacterial infections and also point towards a role for TNF-α in chronic *S. aureus* IMI during involution.

Acknowledgements

The authors express their appreciation to O. Warnke, M. Marín for field technical assistance and to O. Quaino and M. Signorini for assistance with statistical analysis. This work was supported by CAI + D (Universidad Nacional del Litoral), Argentine National Agency for the Promotion of Science and Technology (PICT 604) and Fundación ArgenINTA.

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