Comparative Immunology, Microbiology and Infectious Diseases 54 (2017) 51–60

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

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

Full Length Article

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

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ARTICLE INFO

Keywords:
Immune response
Staphylococcus aureus
Mastitis
Involution

ABSTRACT

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

1. Introduction

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

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

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http://dx.doi.org/10.1016/j.cimid.2017.08.005
Received 19 February 2017; Received in revised form 28 August 2017; Accepted 29 August 2017

subsequent lactation [7].

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

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

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

2. Materials and methods

2.1. Animals and experimental design

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

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

2.2. Bacteriological examination

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

2.3. Tissue sample preparation

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

2.4. Antigen/antibody specificity

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