Altered Leukocyte Responsiveness in Dairy Cows with Naturally Occurring Chronic *Staphylococcus aureus* Mastitis

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**ABSTRACT.** Changes in inflammatory parameters, leukocyte surface markers, functional responses and cytokine mRNA expression of leukocytes of dairy cows with naturally occurring chronic *Staphylococcus aureus* (S. aureus) mastitis and healthy cows were determined to elucidate the leukocyte responses to *S. aureus* infection of the mammary gland. Increased values in inflammatory parameters and matrix metalloproteinase activities in milk revealed the characteristics of cows with chronic mastitis. Expression of L-selectin and CD18 molecules on neutrophils and proportion of CD8 cells in milk from cows with *S. aureus* mastitis were significantly (P<0.05) increased compared with those found in healthy cows. The FeR-stimulated CL response of blood neutrophils was significantly (P<0.05) decreased in cows with *S. aureus* mastitis. Significant (P<0.05) decreased mitogenic responses of lymphocytes were found in cows with *S. aureus* mastitis; however, the values were not restored to those of healthy cows when stimulated with both mitogens and the cytokine IL-1\( \beta \). The mRNA expression of TNF-\( \alpha \), IL-1\( \beta \) and IL-8 on milk leukocytes from cows with *S. aureus* was found to be increased compared with that of healthy cows. The changes of immune responses found in cows with *S. aureus* mastitis appear to be influenced by the severity and duration of inflammation in infected quarters. The down-regulation of the leukocyte functions found in cows with *S. aureus* mastitis appears to be associated with the progress of the chronic stage of *S. aureus* mastitis.

**KEY WORDS:** inflammatory measures, leukocyte phenotypic marker, leukocyte response, *Staphylococcus aureus* mastitis.

Mastitis is the most costly disease affecting the dairy industry worldwide. Prevention of bovine mastitis and production of high quality milk are essential to favorable development of the dairy business and proper response to consumer demand. *Staphylococcus aureus* (S. aureus) is a major pathogen that causes a contagious type of bovine mastitis, and is widely prevalent in dairy herds [2, 7, 35, 36]. On dairy farms producing high-quality milk, *S. aureus* continues to be an important and seemingly ubiquitous mastitis pathogen [7, 36]. The persistence of *S. aureus* and the poor response of the pathogen to antibiotic therapy make *S. aureus* a common cause of culling [18, 36].

Several studies have been performed to investigate the phenotypic profiles of leukocytes in cows with experimental *S. aureus* mastitis [21, 22, 26, 27]; however, variations of immunophenotypes and their activities are observed even in experimental studies with the same challenge of pathogens used. As for the relationship between host immune functions and chronic *S. aureus* mammary infections of dairy cows, few studies have evaluated the immune responses in dairy cows with naturally occurring chronic *S. aureus* mastitis in the field. The chronic nature of *S. aureus* mastitis in dairy cows and the ability of the bacteria to withstand strong inflammatory responses may be associated with a decreased immune response [6]. The elucidation of leukocytic functions as well as their phenotypic changes on leukocytes in cows with *S. aureus* mastitis is necessary to understand the host defense capability against chronic *S. aureus* mastitis in dairy cows. In order to evaluate the status of inflammatory responses of the mammary gland in cows with *S. aureus* mastitis, major physical, chemical and immunological properties in milk were selected as inflammatory parameters.

The aim of this study was to evaluate the changes in the inflammatory parameters, expression of surface markers and functional responses of leukocytes in dairy cows with naturally occurring chronic *S. aureus* mastitis.

**MATERIALS AND METHODS**

**Cows:** Seven lactating Holstein dairy cows, 3.7 ± 1.6 (mean ± SD) years old, with naturally occurring *S. aureus* mastitis in one quarter and the remaining quarters free of infection and at the mid- to late lactation stages, were selected by Dairy Herd Improvement(DHI) monitoring (Hokkaido Dairy Milk and Testing Association, Sapporo, Hokkaido, Japan). Cows with *S. aureus* mastitis but free from any clinical signs of disease were selected based on the results of monthly somatic cell counts (SCC: >300 x 10\(^3\) cells/mL) in dairy herds, and the infected quarters were subsequently identified by bacteriological analysis of quarter milk. The cows having *S. aureus*-positive milk for at least more than one month were not treated with antibiotics.
before collection of milk and blood samples.

Six age- and lactation-stage-matched healthy lactating cows (3.2 ± 1.3 {mean ± SD} years old) with negative results of bacteriological analysis of quarters and SCC of less than 209 × 10^6 cells/ml were used as healthy cows. The animals were housed, fed, and milked twice daily at 3 local dairy farms according to the standard management procedures (Hokkaido DHI, Japan). The collection of quarter milk from dairy cows in this study was carried out in accordance with standard guidelines (Federation of Hokkaido Agricultural Mutual Relief Association, Hokkaido, Japan).

**Collection of milk samples from quarters:** Quarter foremilk samples were collected aseptically for bacteriological testing according to the procedure described previously [19]. Before sampling, the first 3 to 4 streams were discarded, and teat ends were disinfected with cotton swabs soaked in 70% alcohol. Two to 3 ml of quarter milk were collected into a 10-ml sterile culture tube (Eiken Co., Ltd., Tochigi, Japan) aseptically according to the procedure of the National Mastitis Council [19] for bacteriological analysis.

For leukocyte phenotypic analysis, 50 ml of quarter milk from the cows with *S. aureus* infection and 500 ml from normal cows were obtained individually from each cow by hand milking. Collected milk samples were filtered with nylon mesh and were centrifuged at 1,500 × g for 20 min at 4°C. The resulting cell pellets were washed twice with phosphate-buffered saline (PBS, pH 7.2) and used for staining of the cells.

**Parameters for quarter milk:** The electronic conductivity (EC) was measured using a portable conductivity meter (Oriental Instruments Ltd., Tokyo, Japan), and values were expressed as mS/cm. pH was measured using a pH meter (F-52, Horiba, Hitachi, Ltd., Tokyo, Japan). For determination of SCC, milk samples were analyzed with a Fossomatic cell counter (N90, A/S N, Foss Electric Ltd., Hillerød, Denmark). N-acetyl-β-D-glucosaminidase (NAGase) activity in milk was measured by a fluorometric procedure using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as a substrate, and values were expressed as the relative fluorescence intensity (RFI) of liberated 4-methylumbelliferone [14]. Concentrations of lactoferrin (LF) and immunoglobulin G (IgG) in milk were measured by use of single radial immunodiffusion assay kits (Ecos Co., Ltd., Miyagi, Japan).

**Bacteriological test:** Milk samples (10 µl) collected from quarter milk from lactating cows were swirl plated onto trypticase soy blood agar plates containing 5% sheep blood (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and incubated aerobically for 24 to 48 hr at 37°C. The identification of pathogens grown on the blood agar plates was carried out based on the procedure described previously [3]. *S. aureus* was identified as positive based on the hemolytic pattern on blood agar and positive coagulase reactions, as described previously [4]. To differentiate *S. aureus* from coagulase-positive *S. hyicus* and *S. intermedius*, the acetoin test (Voges-Proskauer test) was used [4]. Other mastitis pathogens were identified based on colony morphology and hemolytic patterns on blood agar, Gram’s staining, catalase and oxidase testing and additional biochemical tests [4]. Quarter milk was considered bacteriologically positive if growth of more than 250 colony forming units (CFU)/ml was detected in a sample [18].

**Gel electrophoresis:** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli [12], which was modified by utilizing a vertical slab gel (10% polyacrylamide) with stacking on the top (5% polyacrylamide). Defatted milk samples (100 µl) for SDS-PAGE were mixed with a loading buffer containing 12 mM Tris-HCl, 0.4% SDS, 5% glycerol and 0.02% bromphenol blue, pH 6.8, to a protein concentration of 50 µg/well and run for 1.5 hr at 100 V, followed by staining with Coomassie brilliant blue R-250. Bovine IgG (170 kDa), LF (80 kDa), albumin (Alb, 63 kDa), acid glycoprotein (AG, 42 kDa), haptoglobin (HP, 23, 35 kDa) and an unstained protein molecular weight marker (Fermentas, Cosmo-Biosciences, Tokyo, Japan) were used as markers.

**Matrix metalloproteinase (MMP)-2 and 9 activities:** The MMP-9 activity in milk evaluated by zymography that analyzed gelatinases was performed according to the methods described by Raulo et al. [25] with slight modification [9]. Samples were mixed with sample buffer (2:1, pH 6.8, containing 118 mmol/l Tris, 64 mmol/l H3PO4, 20% glycerol, 0.04% bromophenol blue and 6% SDS), incubated for 2 hr at room temperature and then loaded into 10% SDS-PAGE gels containing 1 mg of porcine skin gelatin/ml (Sigma Chemical Co., G2625, St. Louis, MO, U.S.A.) as a substrate. After the electrophoretic run, the gels were washed to remove SDS with 50 mmol/l Tris-HCl, 0.02%(W/W) Na2EDTA and 2.5% Tween 80 (pH 7.5) buffer containing 10 mmol/l EDTA and then with the same buffer supplemented with 100 mmol/l ZnCl2, 5 mmol/l CaCl2 and 10 mmol/l EDTA and incubated in the same buffer overnight at 37°C. Band densities of MMP-2 and MMP-9 on gels were evaluated.

**Isolation of mononuclear cells and neutrophils from blood:** Peripheral blood was collected from the jugular vein into a tube containing heparin (20 IU/ml). Mononuclear cells were isolated from heparinized blood by Ficoll-sodium iotamate gradients, as described previously [15, 16]. Isolated mononuclear cells were washed twice with PBS and used for staining of the cells. The purity of the mononuclear cells was 95 to 98% as assessed using Wright-Giemsa stain. Mononuclear cells were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 20% heat-inactivated bovine fetal serum (Cellect®, MP Biomedicals Inc, Eschwege, Germany) and 100 IU of penicillin/ml and 100 µg of streptomycin/ml for a lymphocyte stimulation assay.

Neutrophils were isolated from heparinized blood followed by hypotonic red blood cell lysis as described previously [15]. Isolated neutrophils were washed once with PBS, resuspended in HBSS (containing Ca²⁺ and Mg, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to a concentration of 5 × 10⁶ cells/ml. The cell populations comprised 90–95% neutrophils as determined by morphological evaluation.
More than 99% of both cell populations were viable when assessed by trypan blue dye exclusion.

**Leukocyte surface markers:** Immunofluorescent analysis of leukocytes was performed according to the procedure described previously [17]. Expression of the adhesion molecules, L-selectin and CD18 on leukocytes isolated from blood and quarter milk was measured by flow cytometric analysis using fluorescence-conjugated cross-reacting monoclonal antibodies [17]. Aliquots of leukocyte suspensions (3 × 10⁶ cells) were incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-L-selectin monoclonal antibody (CD62L, Dako A/S, Glostrup, Denmark) and FITC-conjugated mouse anti-CD18 monoclonal antibody (MHM23, Dako A/S, Glostrup, Denmark) according to the recommendations of the manufacturer. Mononuclear cells (3 × 10⁶ cells) were incubated with FITC-conjugated mouse anti-bovine CD4 and CD8 monoclonal antibodies (Bio-source Inc., CA, U.S.A.), respectively. FITC-conjugated rabbit anti-mouse IgG was used as a control. After incubation at 4°C for 30 min, cells were washed twice with PBS, fixed in 1% paraformaldehyde and analyzed on a flow cytometer (Coulter Epics Elite, Hialeah, FL, U.S.A.) using a logarithmic amplifier. Leukocytes expressing L-selectin, CD18, CD4 and CD8 were quantified by measurement of the fluorescent intensity of each population, and the percentage of gated cells staining positive for each marker was determined.

**Superoxide production of neutrophils:** Superoxide anion O₂⁻ production was determined by the cytochrome C (Cyt C) reduction assay as described previously [17]. The reaction mixture, containing 150 μl of 538 μM Cyt C (Sigma Chemical Co., St. Louis, MO, USA), 50 μl of neutrophils (1 × 10⁶ cells), 10 μl of opsonized zymosan (OPZ, 10 mg/ml; Sigma Chemical Co., St. Louis, MO, U.S.A.) and 40 μl of HBSS, was set up in triplicate and incubated for 30 min at 37°C. The reaction was stopped by immediate centrifugation at 4°C. In some experiments, 10 μl of recombinant bovine IL-1β (Microbiology Lab, RGU, Hokkaido, Japan) was added together with each stimulant to evaluate the reactivity of lymphocytes. The culture plates were centrifuged (∼ 150 g, 10 min) then the supernatants were removed, and 100 μl of dimethyl sulfoxide was added to each well to solubilize the cells. Absorbance was measured on a microplate reader (MTP 32, Corona Co., Ltd., Ibaraki, Japan) at a wavelength of 550 nm, and the results were expressed as optical density (OD).

**Cytokine mRNA expression:** Cytokine polymerase chain reaction (PCR) was performed using primers for bovine actin and tumor necrosis factor (TNF-α, IL-1β and IL-8 as previously described [26]. Total RNA was extracted from somatic cells using RNAesy Mini kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Polyadenylated RNA was primed with oligo (dt)(Roche Diagnostics, Mannheim, Germany) and reverse transcribed with AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany) for 1 hr at 42°C. Each PCR was performed in a total volume of 25 μl containing the following: 3 μl of cDNA, 11.3 to 12.8 μl of sterilized deionized H₂O, 2.5 μl of 10× PCR buffer, 1.5–3 μl of MgCl₂ (25 mM; Promega, Madison, WI, U.S.A.), 0.2 μl of dNTP mix (100 mM; Promega, WI, U.S.A.), 0.2 μl of Taq polymerase (5 U/μl, Promega, WI, U.S.A.), and 2.5 μl of each 10 μM sense/antisense primer (Hokkaido System Science Co., Ltd., Sapporo, Japan). PCR reactions were performed on a thermocycler (iCycler, Bio Rad, CA, U.S.A.) using the following program: one cycle of 3 min at 94°C, 29 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 52°C and 30 sec of extension at 72°C and then a final 3-min extension cycle at 72°C. Reaction products (10 μl) were visualized on ethidium bromide-stained 1.5% agarose gels.

**Statistics:** To determine the statistical significance of differences between the groups of S. aureus infected cows and the healthy cows, values of inflammatory measures in the 2 groups were evaluated using the unpaired Student’s t-test. One-way analysis of variance was used to analyze overall changes in the proportions of leukocytes. Differences in leukocyte responses were evaluated using Duncan’s multiple range test. Values for chemiluminescence response of neutrophils were logarithmically transformed for analysis. A P value of <0.05 was considered to be significant.
RESULTS

**Inflammatory parameters:** Values for the SCC, EC, pH, NAGase, LF and IgG concentrations in quarter milk from the cows with *S. aureus* mastitis and the healthy cows were determined (Table 1). The SCC, EC, SCC, NAGase, LF and IgG concentrations in milk from the cows with *S. aureus* mastitis were significantly (P<0.05) higher than those of the healthy cows. In the analysis of milk proteins by SDS-PAGE, LF, albumin and IgG were found to be increased in milk from the cows with *S. aureus* mastitis (Fig. 1).

Activities of MMP-2 and MMP-9 were clearly found in milk from quarters obtained from the cows with chronic *S. aureus* mastitis, Nos. 1, 2, 4, 5 and 6, compared with those found in healthy cows. These activities were found to be lowered in cows with chronic *S. aureus* mastitis, Nos. 3 and 7 (Fig. 2).

**Expression of adhesion molecules:** The percentages of L-selectin and CD18 molecules on blood and milk leukocytes from the cows with *S. aureus* mastitis and the healthy cows were compared (Fig. 3). The expression of L-selectin on neutrophils, lymphocytes and monocytes from blood of *S. aureus*-infected cows was similar to that of the healthy cows. L-selectin expression on neutrophils and macrophages of milk from the cows with *S. aureus* mastitis was significantly (P<0.05) decreased compared with that for blood. L-selectin expression on neutrophils and macrophages in milk from the cows with *S. aureus* mastitis was significantly (P<0.05) higher than in healthy cows.

Expression of CD18 on blood leukocytes from the cows

<table>
<thead>
<tr>
<th>Number of cows</th>
<th>Cows with chronic <em>S. aureus</em> mastitis</th>
<th>Healthy cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>9120.0 (330–28270)</td>
<td>80.3 (29–209)</td>
</tr>
<tr>
<td>SCC (×10³ cells/ml)</td>
<td>6.7 (4.6–9.3)</td>
<td>5.3 (4.3–6.4)</td>
</tr>
<tr>
<td>pH</td>
<td>6.79 (6.35–7.15)</td>
<td>6.63 (6.34–6.67)</td>
</tr>
<tr>
<td>NAGase (RFI)</td>
<td>41.2 (3.7–94)</td>
<td>4.5 (3.3–7.8)</td>
</tr>
<tr>
<td>LF (μg/ml)</td>
<td>651.7 (41.7–1104)</td>
<td>177.1 (20.8–458.3)</td>
</tr>
<tr>
<td>IgG (mg/ml)</td>
<td>0.47 (0.12–1.0)</td>
<td>0.26 (0.20–0.32)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean and range of the samples measured. SCC: somatic cell counts. EC: electronic conductivity. NAGase: N-acetyl-β-D-glucosaminidase. Values are expressed as relative fluorescence intensity (RFI). LF: lactoferrin.

a) Significantly different from healthy cows at P<0.05.
LEUKOCYTE RESPONSE IN S. AUREUS MASTITIS

with S. aureus mastitis was similar to that of healthy cows. CD18 expression on neutrophils and lymphocytes in milk from the cows with S. aureus mastitis was significantly ($P<0.05$) higher than for healthy cows.

**Lymphocyte subpopulation:** Lymphocyte markers, CD4 and CD8 cells, in blood and milk from the cows with S. aureus mastitis and healthy cows were compared (Fig. 4). CD4 and CD8 cells among blood lymphocytes from the cows with S. aureus mastitis were similar to those of healthy cows. CD8 milk lymphocytes from the cows with S. aureus mastitis were significantly ($P<0.05$) increased compared with blood lymphocytes.

**Neutrophil superoxide production and CL response:** The mean ($\pm$ SD) OD values for OPZ-stimulated superoxide production of blood neutrophils from 5 cows with S. aureus mastitis and 5 healthy cows were 0.45($\pm$ 0.03) and 0.43 ($\pm$ 0.05), respectively, and the values increased 5.7($\pm$ 3.3)$\%$ and 3.4($\pm$ 2.1)$\%$ when neutrophils were stimulated with OPZ and IL-1$\beta$.

As shown in Fig. 5, OPZ and OPZ together with IL-1$\beta$-stimulated CL responses of blood neutrophils from both S. aureus-infected and healthy cows were almost the same; however, the Agg-IgG, IgG-FcR stimulant-induced CL response of blood neutrophils from the cows with S. aureus mastitis was significantly ($P<0.05$) decreased compared with that of the healthy cows.

**Lymphocyte mitogenic response:** Con A- and PHA-induced mitogenic responses of blood lymphocytes from the cows with S. aureus mastitis were significantly ($P<0.05$) decreased compared with those of healthy cows (Fig. 6). The mitogenic responses of blood lymphocytes from the healthy cows were clearly increased when lymphocytes were stimulated with both mitogens and cytokine IL-1$\beta$ compared with those of cows with S. aureus mastitis.

**Cytokine-mRNA expression:** Expression of cytokines IL-1, IL-8 and TNF-$\alpha$ mRNA on leukocytes from the cows with S. aureus mastitis and the healthy cows was compared (Fig. 7). The mRNA expression of IL-1$\beta$, IL-8 and TNF-$\alpha$ on blood neutrophils from the cows with S. aureus mastitis was similar to that of the healthy cows (data not shown). The mRNA expression of IL-1$\beta$, IL-8 and TNF-$\alpha$ on milk leukocytes from the cows with S. aureus mastitis was found to be increased compared with the healthy cows (Fig. 7).

DISCUSSION

To characterize the inflammatory response of the bovine mammary gland during chronic staphylococcal infections, we evaluated the inflammatory parameters in quarter milk. Increased levels of SCC, EC, pH, NAGase, LF and IgG in quarter milk from cows with S. aureus indicated that inflammatory responses occurred in the mammary gland, and the presence of IgG, MMP-2 and MMP-9 in milk confirmed that inflammation in the mammary gland continued [24, 25]. Increased levels of MMPs in milk infected with S. aureus may be associated with the enhancement of shedding of L-selectin [23] and promotion of the migration of neutrophils into the inflamed quarters.
L-selectin and leukocyte integrin molecules play major roles in adhesion of leukocytes to the endothelial cells of blood vessels and complement 3b-mediated phagocytosis of neutrophils [13, 33]. The present study revealed changes in the leukocyte surface molecule expression and leukocyte responses in cows with naturally occurring *S. aureus* mastitis. The levels of leukocyte L-selectin and CD18 expression in peripheral blood from cows with *S. aureus* were found to be similar to those of healthy cows and to be a finding that was consistent with Riollet et al. [26]. However, this result contrasts with the report by Soltys & Quinn [32], who found that significant down-regulation of L-selectin and up-regulation of CD18 expression occurred in blood neutrophils from cows with *S. aureus* mastitis. The reason for the difference in the phenotypic features of leukocytes in cows with *S. aureus* mastitis is unclear, though it may be associated with the severity, extent and stage of mammary infection. However, in quarter milk, significantly higher L-selectin expression on neutrophils and macrophages, and CD18 expression on neutrophils and lymphocytes, in cows with *S. aureus* mastitis were found compared with those from healthy cows. The functional integrity of migrating leukocytes in inflamed quarters appears to be higher than in uninfected cows in which the cells migrate into the quarter milk without inflammation, i.e., spontaneous and random movements of leukocytes. This may indicate that a number of leukocytes, predominantly neutrophils, that are activated and have directional movement toward pathogens present in sites of inflammation could migrate into the inflamed mammary gland.

L-selectin on bovine neutrophils that migrate into tissues has been found to be diminished after activation [17] and diapedesis [31]. Diez-Fraille et al. [5] reported that in cows with experimental *Escherichia coli* (*E. coli*) mastitis, increases in L-selectin and CD18 on milk neutrophils were observed during the first 6 hr after inoculation of *E. coli*.

The level of CD4 cells in milk from healthy cows was decreased compared with that of blood. In contrast, CD4 cells in milk from cows with chronic *S. aureus* mastitis increased more than in blood. This result was similar to the report of Rivas et al. [27], who observed increases of CD4 cells in the mammary gland in an experimental study using...
cows with *S. aureus* infection. A significant increase in CD8 cells in milk from cows with *S. aureus* mastitis was found compared with blood in our study, and this finding was consistent with previous findings [26, 30] that CD8 cells were the main subpopulation of lymphocytes in quarters from cows inoculated with *S. aureus*. The extent of CD8 cells in milk was not consistent and varied dependent on the time after infection, and the cells in milk were increased at 9–14 days post-inoculation [27]. However, variations of immunophenotypes and their activities are commonly observed even in experimental studies with the same challenge of pathogens used [21, 28, 29]. In the present study, increases in respective CD4 and CD8 cells were found in milk from cows with naturally occurring chronic *S. aureus* mastitis, suggesting that inflammatory responses continue for a relatively long period in the mammary gland and cause the characteristic changes of lymphocyte profiles reflecting chronic infection by the *S. aureus* pathogen in dairy cows. It is likely that the humoral immunity mediated by antigen-response mechanisms is promoted, followed by enhancement of cell cytotoxicity of CD8 cells, which appear to play a role in chronic *S. aureus* infection in the bovine mammary gland. The significantly (*P*<0.05) decreased mitogenic responses of lymphocytes detected in cows with *S. aureus* mastitis were not restored to those of healthy cows when stimulated with both mitogens and cytokine IL-1β. This finding suggested that the reactivity of lymphocytes from cows with chronic *S. aureus* mastitis was down-modulated by *S. aureus* infection.

A significantly decreased FcR-mediated CL response of blood neutrophils was newly found in cows with *S. aureus* mastitis in our study, suggesting that FcR-mediated neutrophil functions were down-regulated, which resulted in decreased responsiveness of the host defense capability. It remains to be elucidated why expression of FcR on neutrophils was changed by *S. aureus* infection and FcR-mediated neutrophil functions were decreased. It is likely that several factors may depress the immune functions in cows with *S. aureus* mastitis and contribute to chronic infection by *S. aureus* in the mammary gland [2, 6]. Staphylococcal enterotoxins and inflammatory products produced by host-pathogen interaction mediated by inflammatory cytokines may modulate the functional responses in cows with chronic *S. aureus* mastitis, as suggested previously [6, 34]. The superoxide production of bovine neutrophils was increased when they were stimulated with both stimulants and IL-1β, as found in our study, and with LF, as previously reported [11]. LF [10], inflammatory cytokines and other immunological components such as IgG and complement fragments activate the leukocyte functions in the mammary gland [20]; however, leukocyte activity in cows with *S. aureus* mastitis appears to be far from sufficient to eliminate the *S. aureus* infection.
pathogen and subsequently results in the stage of chronic infection.

The mRNA expression of inflammatory cytokines was increased in leukocytes from milk obtained from cows with *S. aureus* mastitis, a finding that confirmed previous reports [1, 26]. Increased mRNA expression of IL-8 on milk leukocytes, mainly neutrophils, was observed in milk from cows with *S. aureus* mastitis, suggesting that IL-8 could promote the migration of neutrophils into the inflamed mammary gland.

In conclusion, characteristic findings of phenotypic changes of leukocyte markers, decreased FcR-mediated neutrophil and lymphocyte responses, and cytokine responses were detected in cows with naturally occurring chronic *S. aureus* mastitis. The down-modulation of the immune responses may at least be associated with the progress of the chronic stage of *S. aureus* mastitis.
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