The cell wall component lipoteichoic acid of *Staphylococcus aureus* induces chemokine gene expression in bovine mammary epithelial cells

Yoshio KIKU1), Yuya NAGASAWA1), Fuyuko TANABE1), Kazue SUGAWARA1), Atsushi WATANABE1), Eiji HATA1), Tomomi OZAWA2), Kei-ichi NAKAJIMA3), Toshiro ARAI4) and Tomohito HAYASHI1)*

1)Hokkaido Research Station, National Institute of Animal Health, NARO, Sapporo, Hokkaido 062–0045, Japan
2)National Institute of Animal Health, NARO, Tsukuba, Ibaraki 305–0856, Japan
3)Hokkaido Agricultural Research Center, NARO, Sapporo, Hokkaido 062–8555, Japan
4)School of Veterinary Medicine, Nippon Veterinary and Life Science University, Musashino, Tokyo 180–8602, Japan

(Received 16 December 2015/Accepted 6 May 2016/Published online in J-STAGE 20 May 2016)

**ABSTRACT.** *Staphylococcus aureus* (SA) is a major cause of bovine mastitis, but its pathogenic mechanism remains poorly understood. To evaluate the role of lipoteichoic acid (LTA) in the immune or inflammatory response of SA mastitis, we investigated the gene expression profile in bovine mammary epithelial cells stimulated with LTA alone or with formalin-killed SA (FKSA) using cap analysis of gene expression. Seven common differentially expressed genes related to immune or inflammatory mediators were up-regulated under both LTA and FKSA stimulations. Three of these genes encode chemokines (IL-8, CXCL6 and CCL2) functioning as chemoattractant molecules for neutrophils and macrophages. These results suggest that the initial inflammatory response of SA infection in mammary gland may be related with LTA induced chemokine genes.

**KEYWORDS:** bovine mammary epithelial cell, cap analysis of gene expression, chemokine, lipoteichoic acid, *Staphylococcus aureus*

doi: 10.1292/jvms.15-0706; *J. Vet. Med. Sci.* 78(9): 1505–1510, 2016

Bovine mastitis involves inflammation of the mammary gland and is commonly caused by bacterial infection [27]. Despite extensive management practices, it continues to be an economically important disease of dairy ruminants worldwide, owing to reduced milk yield, loss of milk that must be discarded after treatment, and the high cost of veterinary services. The gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*), is the primary cause of typical bovine mastitis ranging between the clinical and sub-clinical stages, through infection of the mammary tissue [26]. *S. aureus* infection often causes chronic inflammation in bovine mammary glands for the entire life of dairy cattle, sometimes without visible signs of disease. Upon bacterial infection of the mammary tissues, the host must be able to immediately initiate elimination processes [18]. At the early stages of infection, the predominant defense strategy that is rapidly induced is the innate immune response. In response to damage or the presence of invading pathogens, a variety of host cells, such as monocytes, macrophages and epithelial cells, produce and secrete potent immune and inflammatory mediators. Most of the pathogenic studies conducted to date have focused on bovine mastitis caused by *Escherichia coli* infection and the role of its major virulent factor, lipopolysaccharide (LPS); however, there is a lack of information on the pathogenic mechanism of *S. aureus* in bovine mastitis, despite its recognized importance in the dairy industry. Among the virulence factors of *S. aureus*, the cell wall component lipoteichoic acid (LTA) has been shown to play a pathogenic role in infectious diseases [3] through its involvement in biofilm formation, which promotes bacterial adherence to the host [4, 7]. Recent studies have investigated the transcriptional response to killed or inactive gram-positive pathogens and evaluated the contribution of gram-positive cell wall constituents, such as LTA, to the triggering of specific host defense responses [4, 16]. Although *S. aureus* has been considered a major pathogenic bacterium of bovine mastitis, it has thus far been difficult to identify the exact role of the individual bacterial cell components, including LTA, in relation to the onset of mastitis.

Studies evaluating the responses to infections at various epithelial sites strongly suggest that epithelial cells are capable of responding to bacterial intrusion, suggesting that they play a major role in the initiation of inflammation [1, 16]. Indeed, in responding to microbial infection, intestinal [12] and respiratory [1, 16] epithelial cells are well known to initiate the recruitment of neutrophils through their inflammatory and immune responses. Kang et al. suggested that *S. aureus* stimulates the human intestinal epithelial cells to induce the chemokine interleukin (IL)-8 production through its LTA, potentially contributing to the development of intestinal inflammation [12]. Based on this background, we considered whether bovine mammary epithelial cells (BMECs) could act as sentinels for signal invading mastitis-causing pathogens. Therefore, we investigated the role of LTA on the early immune responses of BMECs against *S. aureus* infection.

To reveal whether LTA of *S. aureus* could induce immune or inflammatory response mediators in mastitis, we deter-
mained the gene expression profile of BMECs stimulated with purified LTA alone or formalin-killed *S. aureus* (FKSA) constituents containing all bacterial components, including LTA, using cap analysis of gene expression (CAGE) [13]. This technique has great advantages compared to classical microarray-based expression detection techniques [11, 13]. Specifically, CAGE can be identified the DNA regulatory elements that are specific for biological phenomenon by looking at the sequences that are in the promoters of the RNA isoforms being expressed in the analyzed samples. This technique also enables identification of transcriptional start sites (TSSs) of differentially expressed genes (DEGs) that are related to the stimulus. The expression level of each DEG was quantified, including annotated genes, in the target cells under the induction conditions to mimic bovine mastitis. The use of the CAGE technology is considered to be useful for the development of new diagnostics and therapy of bovine mastitis.

Purified LTA derived from *S. aureus* was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.), which was adjusted to 1 µg/ml in fetal bovine serum PBS-free fresh Dulbecco’s modified Eagle medium (DMEM) for LTA stimulation. The *S. aureus* strain BM1006, which was originally isolated from a cow at a Japanese dairy farm, was used for preparation of FKSA. Multilocus sequence typing analysis revealed that strain BM1006 was sequence type 352 (ST352), which is one of the most common isolates from *S. aureus* strains causing bovine mastitis [9, 22]. The washed *S. aureus* BM1006 was suspended in phosphate-buffered saline containing 0.5% formaldehyde, incubated overnight at room temperature for inactivation and then washed 3 times by PBS. Finally, the killed *S. aureus* was suspended in DMEM and adjusted to 2.5 × 10⁶ cells/ml for FKSA stimulation.

The procedure for isolation of BMEC clones was performed as described by Nakajima et al. [17]. The cloned BMECs were maintained in DMEM supplemented with 10% (vol/vol) FBS, 5 µg/ml of insulin, 50 U/ml of penicillin and 50 µg/ml of streptomycin in 25-cm² culture flasks. When the BMECs reached 80% confluence, fresh FBS-free DMEM was added to the flasks with or without LTA or FKSA. After incubation of the BMECs with these stimulators for 6 hr, the cells were harvested by directly adding 520 µl of lysis buffer to each flask according to the manufacturer’s protocol of Quick Gene RNA cultured cell Kit S (FUJIFILM Corp., Tokyo, Japan). After completion of BMEC lysis, total RNA was extracted from the BMECs using the Quick Gene RNA cultured cell Kit S and nucleic acid isolation system QG-810 (FUJIFILM Corp.).

The CAGE library was prepared following the protocol described by Kodzius et al. [13], which was modified by using adaptors suitable for direct sequencing on an Illumina GAII platform; the adaptors were prepared and obtained from DNAFORM (Yokohama, Japan). In brief, LTA- and FKSA-stimulated BMEC complementary DNA (cDNA) was synthesized from total RNA using a mixture of random and oligo-dT primers using PrimerScript RT Master Mix (TAKARA BIO INC., Tokyo, Japan) according to the manufacturer’s protocol. The 5’ end of cDNA was selected using the cap-trapper method, and cDNA was ligated to a linker containing a recognition site for EcoP151. After the second strand was synthesized, EcoP151 cleaved the cDNAs at a site 27 nucleotides away from the 5’ end to produce the CAGE tags. Next, a linker was attached to the 3’ end of the tag sequence for amplification. Sequencing of the CAGE library was performed on a Genome Analyzer II platform (Illumina, San Diego, CA, U.S.A.). Mapping of CAGE-tag sequences to the bovine genome (NCBI Btau 4.0) was performed at Genomatix (Genomatix Software, München, Germany). We used cluster analysis for genome-wide identification of DEGs, by determining the local enrichments of CAGE tags representing TSSs obtained from Genomatix. All TSS clusters were correlated with transcripts annotated in the EIDorado database (Genomatix, NCBI Btau 4.0 Version 07-2009).

First, we analyzed DEGs in the profile of BMECs stimulated with LTA alone according to the CAGE results. A total of 59,441 TSSs of DEGs were induced by the LTA stimulation, including 41 up-regulated and 141 down-regulated genes. Furthermore, 29 of the 41 up-regulated DEGs and 79 of the 141 down-regulated DEGs could be annotated to known genes. After filtering DEGs showing log FC > 0.61 and an adjusted P-value < 0.05, we found 5 immune response-related DEGs (IL-1α, CXCL6, RSAD2, CCL2 and IL-1α) and 5 inflammatory response-related DEGs (IL-8, CXCL6, CCL2, IL-1α and NFKBIZ) among the 41 up-regulated genes. Table 1 shows the top 20 up-regulated DEGs with the highest expression levels. The remaining up-regulated DEGs were not annotated to genes related to either immune or inflammatory responses in the BMEC genome. The CAGE results were confirmed using real-time RT-PCR of IL-8 and CXCL6 genes. Their expression levels after the stimulation of LTA were 17.1-fold and 22.2-fold of a control, respectively (data not shown). On the other hand, of the 141 down-regulated DEGs, after filtering with the criteria of DEGs showing a log FC > −1.42 and adjusted P-value < 0.05, no immune response- or inflammatory response-related DEGs were identified (data not shown).

Next, we analyzed the DEG profile in the BMECs stimulated with FKSA according to the CAGE results. A total of 57,417 TSSs of DEGs were induced by the FKSA stimulation, including 54 up-regulated genes and 15 down-regulated genes. Furthermore, 29 of the 54 up-regulated and 4 of the 15 down-regulated genes could be annotated with known DEGs. Table 2 shows the top 20 up-regulated genes with the highest expression levels. After filtering the up-regulated DEGs with the criteria of log FC > 0.64 and adjusted P-value < 0.05, we found 6 immune response-related DEGs (IL-8, CXCL6, Erap2, CCL2, IL-1α and Bcl3) and 6 inflammatory response-related DEGs (IL-8, Spp1, CXCL6, CCL2, IL-1α and NFKBIZ). The remaining genes were not annotated to immune or inflammatory response-related genes in the BMEC genome. The CAGE results were confirmed using real-time RT-PCR of IL-8 and CXCL6 genes. Their expression levels after the stimulation of FKSA were 10.4-fold and 8.3-fold of a control, respectively (data not shown). Similar to the results for LTA stimulation alone,
of the 15 down-regulated DEGs, after filtering for a log FC $>-1.42$ and adjusted \( P \)-value$<0.05$, no immune or inflammatory response-related DEGs were found (data not shown). Although the immune and inflammatory response mediators involved in the pathogenesis of \textit{S. aureus} in vivo have not been elucidated, the results of this CAGE study identified 7 commonly up-regulated DEGs, \textit{IL-8}, \textit{CXCL6}, \textit{MX2}, \textit{CCL2}, \textit{IL-1\( \alpha \)}, \textit{NFKBIZ} and \textit{PTGS2}, in both the FKSA and LTA stimulations. Moreover, three of these encode chemokines (\textit{IL-8}, \textit{CXCL6} and \textit{CCL2}), and one encodes a cytokine (\textit{IL-1\( \alpha \)}).

Interleukin (IL-\( \alpha \)), also known as chemokine (C-X-C motif) ligand 8 (CXCL8), is a small cytokine belonging to the CXC chemokine family. IL-\( \alpha \) is a chemokine produced by macrophages and other cell types, such as epithelial cells, airway smooth muscle cells and endothelial cells [10, 15]. IL-\( \alpha \), also known as neutrophil chemotactic factor, has primary functions of inducing chemotaxis to target cells, not only primarily neutrophils but also other granulocytes, to promote their migration toward the site of infection in bovine mastitis [20, 24]. IL-\( \alpha \) also induces phagocytosis once arriving at the infection site. In addition, an increased level of IL-\( \alpha \) is often found in acute diseases, such as mastitis, caused by \textit{E. coli}, as well as in the chronic inflammatory phase of sub-clinical dry-period mastitis [25].

### Table 1. Top 20 up-regulated genes\(^{a)}\) in bovine mammary epithelial cells stimulated with lipoteichoic acid

| Gene symbol | Gene name | Gene accession | Immune response | Inflammatory response | Log FC | \( P \)-value\(^{b)}\) | Common with FKSA\(^{a)}\) stimulation |
|-------------|-----------|----------------|----------------|----------------------|-------|----------------|----------------------------------|
| IL8         | interleukin 8, chemokine (C-X-C motif) ligand 8 (CXCL8) | NM_173925.2 | Yes | Yes | 6.1 | 3.20E-25 | Yes |
| CXCL6       | chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) | NM_174300.2 | Yes | Yes | 4.14 | 6.10E-78 | Yes |
| MCM7        | minichromosome maintenance complex component 7 | NM_001025345.2 | 3.66 | 0.03 |
| MX2         | myxovirus (influenza virus) resistance 2 (mouse) | NM_173941.2 | 2.73 | 0.0089 | Yes |
| NFKBIA      | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | NM_001045868.1 | 1.53 | 0.0065 |
| RSAD2       | radical S-adenosyl methionine domain containing 2 | NM_001045941.1 | 1.43 | 0.0089 |
| CCL2        | chemokine (C-C motif) ligand 2 | NM_174006.2 | Yes | Yes | 1.37 | 3.90E-13 | Yes |
| IL1A        | interleukin 1, alpha | NM_174092.1 | Yes | Yes | 1.2 | 0.0019 | Yes |
| MOV10       | Mov10 RISC complex RNA helicase | NM_001075839.1 | 1.19 | 0.017 |
| PRKCSH      | protein kinase C substrate 80K-H | NM_176662.1 | 0.96 | 0.028 |
| NFKBIZ      | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta | NM_174726.1 | Yes | 0.92 | 8.80E-07 | Yes |
| PTGS2       | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | NM_174445.2 | 0.88 | 0.0021 | Yes |
| RPS2        | ribosomal protein S2 | NM_001033613.1 | 0.86 | 0.033 |
| ZNF706      | zinc finger protein 706 | NM_001199073.1 | 0.8 | 0.022 |
| LOC504599   | histone H3.2 | NM_001166569.1 | 0.77 | 0.022 |
| KDELRE12    | KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 | NM_001079779.1 | 0.74 | 0.023 |
| ADM         | adrenomedullin | NM_173888.3 | 0.72 | 0.0065 |
| LOC505183   | histone H2B type 1-like | XM_581429.4 | 0.66 | 0.024 |
| HIST1H2AC   | histone cluster 1, H2ac | XM_603142.2 | 0.61 | 0.033 |
| CEBPD       | CCAT/enhancer binding protein (C/EBP), delta | NM_174267.2 | 0.61 | 0.0008 |

\( a \) Up-regulated genes showing a value greater than 1 log FC, defined as the logarithm of the gene expression level in a sample relative to that of the non-stimulated control. \( b \) Significance of the difference in gene expression levels between the sample and control. \( c \) Formalin-killed \textit{Staphylococcus aureus}. 
of ligand/receptor reactions and serve as important mediators of the immune reaction in the innate immune system response. A previous report also shows that intramammary infusion of LTA induced the secretion of IL-8 in milk [19]. Therefore, our findings suggest that the up-regulated IL-8 and CXCL6 in response to stimulation by whole S. aureus, including LTA, might function as immune and inflammatory mediators in S. aureus-induced mastitis.

CCL2 is a member of the C-C motif chemokine family and has been reported to recruit monocytes, memory T cells and dendritic cells to the sites of inflammation produced by either tissue injury or infection. CCL2 is secreted upon stimulation by monocytes and other innate cells [2]. In response to the CCR2-CCL2 interaction, monocytes are trafficked to the sites of microbial infection [21]. Monocytes differentiate into macrophages or dendritic cells to curtail the infection by directly phagocytizing and killing the pathogens. Thus, monocytes, along with neutrophils, form an integral part of the innate immune system and play a key role in the early containment of infections, such as mastitis.

IL-1α is mainly produced by activated macrophages, as well as neutrophils, epithelial cells and endothelial cells. It shows metabolic, physiological and hematopoietic activities and plays a central role in regulation of the immune response. IL-1α binds to the IL-1 receptor and is involved in the pathway that activates tumor necrosis factor-alpha (TNF-α). A previous study showed that BMECs stimulated with heat-inactivated preparations of E. coli displayed coordinated gene regulation governed by the activation of IL-1α and TNF-α signaling; however, this appears to be an E. coli-specific immune response feature, because stimulation of BMECs with S. aureus did not significantly alter IL-1α [8]. Similarly, other studies indicated that stimulation of BMECs with LTA did not significantly alter the expression of Toll-

### Table 2. Top 20 up-regulated genes\(^a\) in bovine mammary epithelial cells following stimulation with formalin-killed Staphylococcus aureus

| Gene symbol | Gene name | Gene accession | Immune response | Inflammatory response | Log FC | P-value\(^b\) | Common with LTA\(^c\) stimulation |
|-------------|-----------|----------------|-----------------|-----------------------|--------|------------|-------------------------------|
| IL8         | interleukin 8, chemokine (C-X-C motif) ligand 8 (CXCL8) | NM_173925.2 | Yes | Yes | 4.92 | 1.90E-10 | Yes |
| SPP1        | secreted phosphoprotein 1 | NM_174187.2 | Yes | Yes | 3.51 | 5.90E-39 | Yes |
| ANKRD37     | ankyrin repeat domain 37 | NM_001075392.1 | 3.07 | 0.0089 |
| CXCL6       | chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) | NM_174300.2 | Yes | Yes | 2.74 | 1.60E-24 | Yes |
| C29H11orf86 | chromosome 29 open reading frame, human C1orf86 | NM_001077090.1 | 2.7 | 0.035 |
| GN1         | guanine nucleotide binding protein (G protein), beta polypeptide 1 | NM_175777.3 | 2.66 | 0.0035 |
| MX2         | myxovirus (influenza virus) resistance 2 (mouse) | NM_173941.2 | 2.57 | 0.013 | Yes |
| ATP6V1C1    | ATPase, H+ transporting, lysosomal 42 kDa, V1 subunit C1 | NM_176676.1 | 2.07 | 0.045 |
| ERAP2       | endoplasmic reticulum aminopeptidase 2 | NM_001075628.2 | Yes | 2.07 | 0.015 |
| CYP1B1      | cytochrome P450, family 1, subfamily B, polypeptide 1 | NM_001192294.1 | 1.65 | 0.0043 |
| PTGS2       | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | NM_174445.2 | 1.49 | 2.20E-12 | Yes |
| CEBPD       | CCAAT/enhancer binding protein (C/EBP), delta | NM_174267.2 | 1.11 | 4.90E-11 |
| CCL2        | chemokine (C-C motif) ligand 2 | NM_174006.2 | Yes | Yes | 1.07 | 0.013 | Yes |
| IL1A        | interleukin 1, alpha | NM_174092.1 | Yes | Yes | 1.02 | 0.032 | Yes |
| C27H8orf4   | chromosome 27 open reading frame, human C8orf4 | NM_001035490.2 | 1.01 | 5.00E-09 |
| NFKBIZ      | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta | NM_174726.1 | 0.83 | 0.00019 | Yes |
| DUSP6       | dual specificity phosphatase 6 | NM_001046195.1 | 0.78 | 8.40E-05 |
| MASTL       | microtubule associated serine/threonine kinase-like | NM_00113765.1 | 0.71 | 0.011 |
| SIK1        | salt-inducible kinase 1 | XM_003581774.1 | 0.67 | 0.0024 |
| BCL3        | B-cell CLL/lymphoma 3 | NM_001205993.1 | Yes | 0.64 | 0.023 |

\(^a\) Up-regulated genes showing a value greater than 1 log FC, defined as the logarithm of the gene expression level in a sample relative to that of the non-stimulated control.

\(^b\) Significance of the difference in gene expression levels between the sample and control.

\(^c\) Lipoteichoic acid.
like receptor pathway genes, including IL-8 and CXCL6, or interferon-inducible genes, including CCL2 [6, 23]. However, the results of the present CAGE study demonstrated that 4 of the up-regulated DEGs (IL-8, CXCL6, CCL2 and IL-1α) were common to both the FKSA and LTA stimulations of BMECs. Thus, using CAGE, we were able to clarify the indeterminate expression of chemokine genes that have not been previously identified under induction conditions mimicking bovine mastitis. In addition, 5 DEGs expressed under both LTA and FKSA stimulations were associated with the inflammatory response, including IL-8, CXCL6, CCL2, IL-1α, and NFκBIZ. In particular, IL-8 and CXCL6 are predicted to play an important role in this response given their roles as chemoattractant for neutrophil granulocytes, as described above. Therefore, our findings imply that the up-regulated DEGs observed in response to both LTA and FKSA were mainly induced by LTA stimulation. These results suggest that the initial inflammatory response of S. aureus infection in mammary gland may be related with LTA induced chemokine genes. Further consideration will be needed to yield any findings about the differences between the bacterial species causing mastitis.

The simultaneous detection of temporal expression patterns of pathogens and host cells, especially during S. aureus mastitis, will help to define the pathogenesis of mastitis and provide further insight into the molecular cross-talk between pathogens and host cells. The CAGE results described herein have revealed novel mechanisms of chemokine induction by S. aureus LTA.

ACKNOWLEDGMENTS. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Research Program on Innovative Technologies for Vaccine Development, and Development of Dairy Management System using the Milking Robot) and JSPS KAKENHI Grant Number 25850215, 15K07731.

REFERENCES

1. Bals, R. 2000. Epithelial antimicrobial peptides in host defense against infection. Respir. Res. 1: 141–150. [Medline] [CrossRef]
2. Colotta, F., Borré, A., Wang, J. M., Tattanelli, M., Maddalena, F., Polentarutti, N., Peri, G. and Mantovani, A. 1992. Expression of a monocyte chemotactic cytokine by human mononuclear phagocytes. J. Immunol. 148: 760–765. [Medline]
3. De Kimpe, S. J., Kengatharan, M., Thiemermann, C. and Vane, J. R. 1995. The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure. Proc. Natl. Acad. Sci. U.S.A. 92: 10359–10363. [Medline] [CrossRef]
4. Fabretti, F., Theilacker, C., Baldassarri, L., Kaczynski, Z., Kropec, A., Holst, O. and Hubein, J. 2006. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect. Immun. 74: 4164–4171. [Medline] [CrossRef]
5. Fezer, R. J., Oberholzer, C., Baker, H. V., Novick, D., Rubinstein, M., Moldawer, L. L., Priddle, J., Souza, S., Dinarello, C. A., Ertel, W. and Oberholzer, A. 2003. Molecular characterization of the acute inflammatory response to infections with gram-negative versus gram-positive bacteria. Infect. Immun. 71: 5803–5813. [Medline] [CrossRef]
6. Gilbert, F. B., Cunha, P., Jensen, K., Glass, E. J., Foucras, G., Robert-Granité, C., Rupp, R. and Rainard, P. 2013. Differential response of bovine mammary epithelial cells to Staphylococcus aureus or Escherichia coli agonists of the innate immune system. Vet. Res. (Faisalabad) 44: 40. [Medline] [CrossRef]
7. Ginsburg, I. 2002. Role of lipoteichoic acid in infection and inflammation. Lancet Infect. Dis. 2: 171–179. [Medline] [CrossRef]
8. Günther, J., Esch, K., Poschadel, N., Petzl, W., Zerbe, H., Mitterhammer, S., Blum, H. and Seyfert, H. M. 2011. Comparative kinetics of Escherichia coli- and Staphylococcus aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. aureus elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1α or tumor necrosis factor alpha. Infect. Immun. 79: 695–707. [Medline] [CrossRef]
9. Hata, E., Katsuda, K., Kobayashi, U., Uchida, I., Tanaka, K. and Eguchi, M. 2010. Genetic variation among Staphylococcus aureus strains from bovine milk and their relevance to methicillin-resistant isolates from humans. J. Clin. Microbiol. 48: 2130–2139. [Medline] [CrossRef]
10. Hedges, J. C., Singer, C. A. and Gerthoffer, W. T. 2000. Mitogen-activated protein kinases regulate cytokine gene expression in human airway myocytes. Am. J. Respir. Cell Mol. Biol. 23: 86–94. [Medline] [CrossRef]
11. Im, J., Lee, T., Jeon, J. H., Baik, J. E., Kim, K. W., Kang, S. S., Yun, C. H., Kim, H. and Han, S. H. 2014. Gene expression profiling of bovine mammary gland epithelial cells stimulated with lipoteichoic acid plus peptidoglycan from Staphylococcus aureus. Int. Immunopharmacol. 21: 231–240. [Medline] [CrossRef]
12. Kang, S. S., Noh, S. Y., Park, O. J., Yun, C. H. and Han, S. H. 2015. Staphylococcus aureus induces IL-8 expression through its lipoproteins in the human intestinal epithelial cell, Caco-2. Cytokine 75: 174–180. [Medline] [CrossRef]
13. Kodzius, R., Kojima, M., Nishiyori, H., Nakamura, M., Fukuda, S., Tagami, M., Sasaki, D., Imamura, K., Kai, C., Harbers, M., Hayashizaki, Y. and Carninci, P. 2006. CAGE: cap analysis of gene expression. Nat. Methods 3: 211–222. [Medline] [CrossRef]
14. Lahouassa, H., Moussay, E., Rainard, P. and Rirollet, C. 2007. Differential cytokine and chemokine responses of bovine mammary epithelial cells to Staphylococcus aureus and Escherichia coli. Cytokine 38: 12–21. [Medline] [CrossRef]
15. Message, S. D. and Johnston, S. L. 2004. Host defense function of the airway epithelium in health and disease: clinical background. J. Leukoc. Biol. 75: 5–17. [Medline] [CrossRef]
16. Moreilhon, C., Gras, D., Hologne, C., Bajolet, O., Cottrez, F., Magnone, V., Merten, M., Groux, H., Puchelle, E. and Barbry, P. 2005. Live Staphylococcus aureus and bacterial soluble factors induce different transcriptional responses in human airway cells. Physiol. Genomics 20: 244–255. [Medline] [CrossRef]
17. Nakajima, K., Itoh, F., Nakamura, M., Kawamura, A., Yamazaki, T., Kozakai, T., Takusari, N. and Ishisaki, A. 2015. Short communication: opposing effects of lactoferrin on the proliferation of fibroblasts and epithelial cells from bovine mammary gland. J. Dairy Sci. 98: 1069–1077. [Medline] [CrossRef]
18. Oviedo-Boysó, J., Valdez-Alarcón, J. J., Cajarero-Julier, M., Ochoa-Zarzosa, A., López-Meza, J. E., Bravo-Patiño, A. and Baizabal-Aguirre, V. M. 2007. In innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. J. Infect. 54: 399–409. [Medline] [CrossRef]
19. Rainard, P., Fromageau, A., Cunha, P. and Gilbert, F. B. 2008.
Staphylococcus aureus lipoteichoic acid triggers inflammation in the lactating bovine mammary gland. *Vet. Res.* **39**: 52. [Medline] [CrossRef]

20. Raman, D., Sobolik-Delmaire, T. and Richmond, A. 2011. Chemokines in health and disease. *Exp. Cell Res.* **317**: 575–589. [Medline] [CrossRef]

21. Serbina, N. V., Jia, T., Hohl, T. M. and Pamer, E. G. 2008. Monocyte-mediated defense against microbial pathogens. *Annu. Rev. Immunol.* **26**: 421–452. [Medline] [CrossRef]

22. Smith, E. M., Green, L. E., Medley, G. F., Bird, H. E., Fox, L. K., Schukken, Y. H., Kruze, J. V., Bradley, A. J., Zadoks, R. N. and Dowson, C. G. 2005. Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* **43**: 4737–4743. [Medline] [CrossRef]

23. Strandberg, Y., Gray, C., Vuocolo, T., Donaldson, L., Broadway, M. and Tellam, R. 2005. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine* **31**: 72–86. [Medline] [CrossRef]

24. Swanson, K. M., Stelwagen, K., Dobson, J., Henderson, H. V., Davis, S. R., Farr, V. C. and Singh, K. 2009. Transcriptome profiling of Streptococcus uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. *J. Dairy Sci.* **92**: 117–129. [Medline] [CrossRef]

25. Watanabe, A., Hirota, J., Shimizu, S., Inumaru, S. and Kimura, K. 2012. Single intramammary infusion of recombinant bovine interleukin-8 at dry-off induces the prolonged secretion of leukocyte elastase, inflammatory lactoferrin-derived peptides, and interleukin-8 in dairy cows. *Vet. Med. Int.* **2012**: 1–8. [Medline] [CrossRef]

26. Wesson, C. A., Deringer, J., Liou, L. E., Bayles, K. W., Bohach, G. A. and Trumble, W. R. 2000. Apoptosis induced by *Staphylococcus aureus* in epithelial cells utilizes a mechanism involving caspases 8 and 3. *Infect. Immun.* **68**: 2998–3001. [Medline] [CrossRef]

27. Zhao, X. and Lacasse, P. 2008. Mammary tissue damage during bovine mastitis: causes and control. *J. Anim. Sci.* **86** Suppl: 57–65. [Medline] [CrossRef]