Journal of Animal and Veterinary Advances 11 (14): 2588-2593, 2012
ISSN: 1680-5593

Inflammatory Response of Peripheral Blood Mononuclear Cells Post Intramammary Challenge with *Staphylococcus aureus* in Dairy Cows

Li Liu, Zhengrong Yuan, Cui Chen, Limin Zhang, Xiaojie Chen, Xidong Liu,
Xue Gao, Junya Li, Huijiang Gao, Lupei Zhang and Shangzhong Xu
Laboratory of Molecular Biology and Bovine Breeding,
Chinese Academy of Agricultural Sciences, Institute of Animal Science,
100193 Beijing, China

Abstract: To study the inflammatory response of Peripheral Blood Mononuclear Cells (PBMCs) to actue phases of *Staphylococcus aureus* (*S. aureus*) mastitis in dairy cows using gene expression data, the mRNA expression levels of six genes (Iinterleukin (IL)-8, Differentiation (CD) 14, Macrophage Colony Stimulating Factor Receptor (M-CSFR), Toll-Like Receptor 2 (TLR2), Chemokine Receptor 2 (CCR2) and CX_3C Chemokine Receptor 1 (CX_3CR1) in Peripheral Blood Mononuclear Cells (PBMCs) in infected cows (n = 3) with *S. aureus* relative to uninfected controls (n = 3) were measured at 0 day before inoculation and 1-3 days post inoculation (dpi) by the real-time quantitative PCR. Results indicated the expression of *IL-8*, *CD14* and *CCR2* gene were exhibited significantly ($p<0.05$) up-regulated within 2 dpi while there was no significant difference in the expression of *M-CSFR*, *TLR2* and *CX_3CR1* genes at 1-3 dpi. Additionally, concentrations of plasma lipopolysaccharide Binding Protein (LBP) and Somatic Cell Counts (SCCs) were evaluated. Together these data reveal that the upregulation of gene expression is probably related to the recruitment and activation of PBMCs.

Key words: Dairy cow, mastitis, *S. aureus*, gene expression, PBMCs, China

INTRODUCTION

Bovine mastitis, an inflammation of the udder caused by invading pathogens is one of the most important infectious diseases in dairy production (Blosser, 1979). One of the most economically important pathogenic bacteria causing bovine mastitis is *Staphylococcus aureus* (*S. aureus*) which results in increased Somatic Cell Count (SCCs) that diminish milk quality and decrease milk yield. Recent studies showed that *S. aureus* mastitis was implicated in 41% cases in part region of China and were more frequently associated with clinical mastitis than sub-clinical case which is difficult to control (Cheng *et al*., 2010). Evaluating the inflammatory response of Peripheral Blood Mononuclear Cells (PBMCs) may help in understanding the mechanism of the defense of the mammary glands against *S. aureus*.

PBMCs provide a mobile source of functionally competent cells of the innate immune system is a prevalent pathogen of relevance to both human and veterinary medicine, the consist mainly of lymphocytes (T-cells and B-cells and smaller amounts of NK cells) and monocytes (Ahmed *et al*., 2007). The change of cells population of PBMCs during bovine *S. aureus* mastitis have been evaluated (Gronlund *et al*., 2006; Kiku *et al*., 2010) however, very few studies focus on the gene expression of PBMCs.

Due to vascular permeability, some different inflammatory cytokines, chemokines and virulence factors will be released into blood circulation during *S. aureus* mastitis such as Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, Interferon-gamma (IFN-γ), fractalkine and virulence factors (Alluwaimi *et al*., 2003; Bannerman *et al*., 2004) which affect gene expression of PBMCs directly or indirectly. IL-8 is believed to be a major mediator of acute inflammation in response to infection (Standiford *et al*., 1994). The Cluster of Differentiation (CD) 14 and Toll-Like Receptor 2 (TLR2) are important player in host innate immunity in that they mediate host defense against Gram-positive bacterial infections (Schroder *et al*., 2003). Macrophage Colony Stimulating Factor Receptor (M-CSFR) is important for the development of the mononuclear phagocyte lineage (Geissmann *et al*., 2010). The Chemokine Receptor 2

Corresponding Author: Shangzhong Xu, Laboratory of Molecular Biology and Bovine Breeding, Chinese Academy of Agricultural Sciences, Institute of Animal Science, No. 2 Yuanmingyuan West Road, Haidian District, 100193 Beijing , P.R. China

(CCR2) and CX_3C Chemokine Receptor 1 (CX_3CR1) play an important role in inflammatory cell migration (Jerath *et al*., 2010).

Researchers hypothesized that PBMCs from *S. aureus* mastitis and healthy cattle may have distinctive gene expression and immune regulation. In this study, researchers use a *S. aureus*-induced bovine mastitis model to study the inflammatory response of PBMCs to actue phases of bovine *S. aureus* mastitis. Using Real Time Quantitative PCR, expression of six genes (*IL-8*, *CD14*, *M-CSFR*, *TLR2*, *CCR2* and *CX_3CR1*) were examined on the time course in PBMCs in infected cows (n = 3) with *S. aureus* relative to uninfected controls (n = 3) post intramammary inoculation. In addition, the levels of plasma LBP and SCC at different time points were also investigated.

MATERIALS AND METHODS

Animals: Six Holstein cows recruited from herds of a cattle farm in Tianjin, China were used for this study, all of which were in their first lactation (aged between 27-33 and 3-5 months post partum), daily milk yields were between 15 and 25 L. Animals were kept at the Dairy Development Center of Tianjin (China), none of the cows were pregnant at the time of the study and never had clinical or subclinical mastitis before. Milk from each quarter was bacteriologically negative and milk SCC<200,000 cells mL^{-1}. The experimental protocol was approved by the China Laboratory Animal Care and Use Committee.

Preparation of bacteria: *S. aureus* strain CVCC3056 (China) Veterinary Microorganism Preservation Center, Beijing which was originally isolated from a clinical case of mastitis was used to induce experimental intramammary infection. Before challenge exposure, 10 mL of brain heart infusion broth (Beijing Land Bridge Technology Co., Ltd.) were inoculated with the strain and incubated for 6 h at 37°C. Thereafter, 1 mL of the inoculum was transferred to cones bottle containing 99 mL of tryptic soy broth and incubated overnight at 37°C, the stock culture was stored at 4°C until used. On the day of inoculation, 1 mL of the stock culture was inoculated into 5 mL of tryptic soy broth and incubated under continual incubated for 4 h. Bacteria in the exponential phase of growth were harvested and washed once in pyrogenfree PBS. Total bacterial cell counts were determined using dry medium plates and the bacterial suspension was adjusted to achieve concentrations of 10^8 bacteria mL^{-1} by dilution in 5 mL of pyrogen-free PBS.

Intramammary challenge: The cows were randomly assigned to 2 groups, all udders were milked by hand, the cows in the controls (n = 3) were administered an intramammary inoculation with 5 mL of sterile pyrogen-free PBS whereas the treatment cows (n = 3) were inoculated with 10^8 bacteria mL^{-1}. Experimental infection was induced in the Right Front (RF) quarter of each animal so as to eliminate potential quarter-dependent effects that could arise if different quarters on the various cows were infected. Prior to intramammary challenge, the teat at the RF of the mammary gland was carefully washed and disinfected with ethanol. A blunt needle was introduced through the teat canal on the RF teat and the inoculum (5 mL) was infused into mammary gland.

Sample collection: Blood samples were collected via jugular venipuncture into 15 mL centrifuge tubes (Corning, USA) that contained 2 mL Acid Citrate Dextrose (ACD) as the anticoagulant at 0 day immediately before inoculation and 1-3 days post inoculation (dpi) with PBS or *S. aureus*. Following cleaning and disinfection of the teats, milk samples were collected into sterile tubes at the same time points.

PBMCs isolation: PBMCs were separated from ACD anticoagulated blood of cows (3 infected and 3 uninfected controls) by density gradient centrifugation using Ficoll separation solution (1.083 g mL^{-1} Sigma Chemical Co., St. Louis, Mo). From 10 mL of blood, researchers obtained approximately $2\text{-}5\times10^6$ PBMCs. The cell pellets were suspended in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and incubated for 10 min at room temperature to ensure cell lyses and preservation of the released RNA. This mixture was then frozen at -70°C until use for RNA isolation which was performed according to the TRIzol manufacture's instructions.

RT-PCR and quantitative RT-PCR: Total RNA was extracted from the PBMCs using the RNASimple Total RNA kit protocol (Tiangen Co., Ltd. China) and DNAse digested according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA using Quantscript RT kit (Tiangen Co., Ltd. China). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) used as control gene for animal samples to normalize expression data for target genes. The expression levels of four genes mRNA were detected using 7500 Real-Time PCR System (Applied Biosystems, USA) with SYBR Green PCR Master Mix Reagent (TaKaRa Inc., Japan). The specific gene primer pairs are shown in Table 1. The

Table 1: Information on the primers used in this study

Genes	Primers sequences (5'-3')	Accession number	Products (bp)
IL-8	F:AGAACTTCGATGCCAATGCAT	NM_173925	150
	R:GGGTTTAGGCAGACCTCGTTT		
CD14	F:GTA AATGACCTGACTCTGGACGG	NM_174008.1	195
	R:ATTCCTCTTCCCTCTCTTCCC		
M-CSFR	F:GGCACAGCGGTGACCTTGCGATG	NM_001075403.2	109
	R:TGGTGAGGATGCTTCTGGGGGCG		
TLR2	F:TGCGTTGGTTTGGATAGTGA	NM_174197	231
	R:AGACCAGAGGGGATGGAGTT		
CCR2	F:CACAATGTGCTTCCCACATC	NM_001194959.1	138
	R:GAGCTGTGCTTCGATTTGTC		
CX_3CR1	F:CCATCCTGACCACCTCAGTT	NM_001102558.1	107
	R:CATCTCCATCGCTGGTGTAA		
GAPDH	F:CTCCCAACGTGTCTGTTGTG	NM_001034034	222
	R:TGAGCTTGACAAAGTGGTCG		

real-time PCR program started with denaturing at 94°C for 2 min followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The last stage for the dissociation curve was as follows 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec.

ELISA for LBP and quantification of milk SCC: Anticoagulated blood centrifuged at 1500 g for 15 min and the clear plasma supernatant was collected, aliquoted and stored at -70°C. Plasma LBP levels were determined with a commercially available LBP ELISA kit that crossreacts with bovine LBP (Cell Sciences, Inc., Norwood, MA) as previously described (Bannerman *et al*., 2003). For the quantification of somatic cells, milk samples were heated to 60°C and subsequently maintained at 40°C until the cells were counted on an automated milk somatic cell counter (Fossomatic Model 90; Foss Food Technology, Hillerod, Denmark).

Data analysis: Data were analyzed with ABI 7500 SDS Software (ABI) with the baseline being set automatically by the software. The relative expression level (Fold change) was analyzed using the $2^{-\Delta\Delta Ct}$ Method (Livak and Schmittgen, 2001). Results were expressed as the mean fold-change in gene expression at each time point, from triplicate analyses using the sample at 0 day as the calibrator (assigned an expression level of 1). Briefly, the relative level of each mRNA normalized to the *GAPDH* gene was calculated using the following equation:

$$\text{Fold change} = \frac{2^{(\text{CT, Target - CT, GAPDH) Time x}}}{2^{(\text{CT, Target - CT, GAPDH) Time 0}}}$$

Mann-Whitney U test for comparison of two independent samples was used to evaluate differences between infected cows and uninfected controls. Treatments were considered statistically significant when at $p<0.05$ or $p<0.01$ using a Paired Two-Tailed test, statistical analyses were undertaken using SPSS 17.0 Software.

RESULTS AND DISCUSSION

Detection of differential gene expression in PBMCs: The mRNA expression of *IL-8*, *CD14*, *M-CSFR*, *TLR2*, *CCR2* and *CX_3CR1* genes at 0 day before inoculation and 1-3 dpi were examined in PBMCs in infected cows relative to uninfected controls, the expression of IL-8 exhibited significantly ($p<0.05$) up-regulated at 1 and 2 dpi with fold changes of 1.41±0.23 and 1.85±0.25, respectively (Fig. 1a). The *CD14* and *CCR2* gene significantly ($p<0.05$) enhanced expression only at 1 dpi and diminished on days 2 and 3, reaching a fold change value of 1.42±0.11 and 1.34±014 (Fig. 1b and e). There were no significant difference in M-CSFR, TLR2 and CX_3CR1 expression at 1-3 dpi (Fig. 1c-f).

Milk somatic cell analysis and LBP ELISA assays: As a local indicator of inflammation, milk Somatic Cell Counts (SCCs) were also enumerated prior to and after infection (Fig. 2). SCC of infected quarters increased significantly from 1-3 dpi and were significantly ($p<0.05$) higher than those of uninfected quarters. Average log 10 transformed SCC values were lower ($p<0.05$) in milk samples obtained from uninfected controls compared to the corresponding values in milk obtained from mastitic cows (5.0 versus 6.65). All inoculated udders produced marked clinical symptom (udder swelling or hardness accompanied by heat and pain) from 1-3 dpi and showed a gradual decrease in milk production, together with milk clots.

Intramammary infection with *S. aureus* did evoke the synthesis of the acute phase proteins, LBP. From 1-3 dpi, increased circulating blood concentrations of LBP were detected (Fig. 3). Plasma LBP levels of infected cows reached a maximum level of 254.55±5.9 μg mL^{-1} at 1 dpi and were approximately 10 fold higher ($p<0.01$) than uninfected controls (23.75±11.9 μg mL^{-1}).

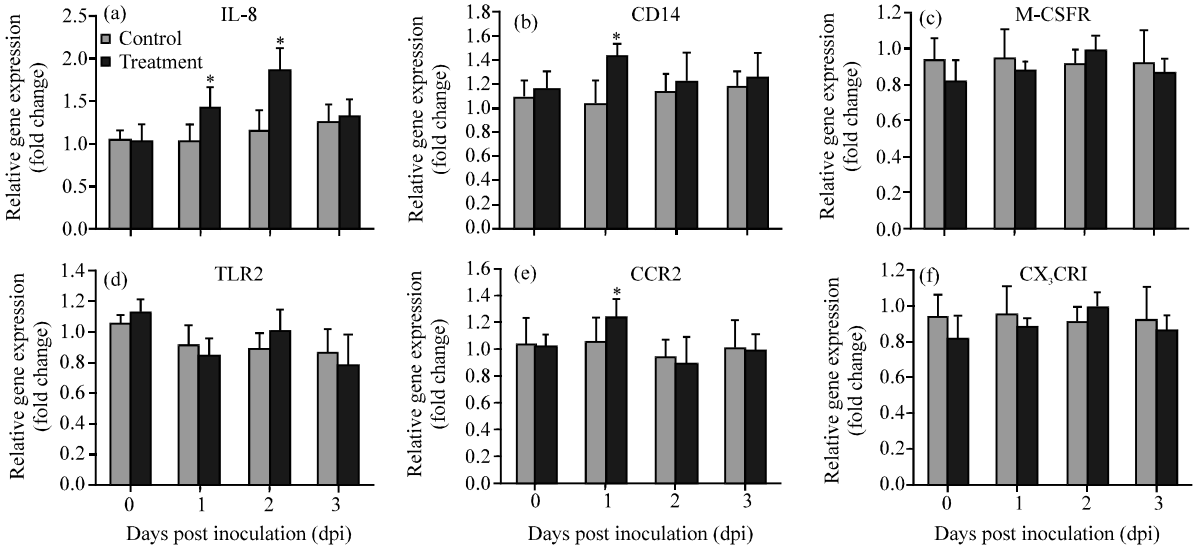

Fig. 1: Fold-changes in gene expression of; a) IL-8 ; b) CD14; c) M-CSFR; d) TLR2; e) CCR2 and f) CX_3CR1 in PBMC from infected cows (n = 3) with *S. aureus* compared to controls (n = 3) at 0 day before inoculation and 1-3 days post intramammary inoculation. Each bar represents the Mean (±SE) *($p<0.05$)

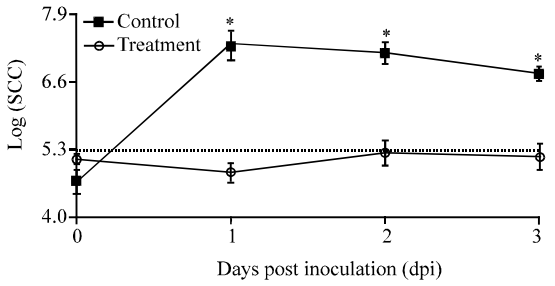

Fig. 2: Somatic Cell Counts (SCCs) in milk of udder quarters following intramammary *S. aureus* infection. Milk samples were collected immediately before (time 0 day) and at various time points post inoculation of *S. aureus*. The horizontal lines within the data sets represent 200,000 cells mL^{-1}. Mean (±SE) milk SCC are reported in millions of log10 cells *($p<0.05$)

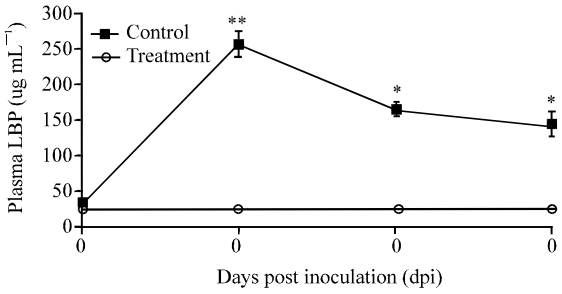

Fig. 3: Induction of acute phase protein synthesis following intramammary *S. aureus* infection. Blood samples were collected immediately before (time 0 day) and at various time points post inoculation of *S. aureus*. Plasma derived from the blood samples was assayed for Lipopolysaccharide binding Protein (LBP). Mean (±SE) concentrations of LBP are reportedin μg mL^{-1} (* $p<0.05$, **$p<0.01$)

The primary objective of the current study was to shed some light as to the inflammatory response of PBMCs to actue phases of bovine *S. aureus* mastitis using gene expression data. Although, Tao examined the demonstrated differential expression of innate immunity related genes, the gene expression of PBMCs to actue phases of *S. aureus* mastitis are still poorly defined in cows. In this study, six candidate genes (*IL-8*, *CD14*, *M-CSFR*, *TLR2*, *CCR2* and *CX_3CR1*), SCC and plasma LBP implicated in immune responses were examined. The up-regulation of IL-8 expression in PBMCs at 1-2 dpi, indicating IL-8 may play an important role in the inflammatory response of PBMCs. In human, IL-8 can each rapidly cause rolling monocytes to adhere firmly onto vascular endothelium (Gerszten *et al*., 1999), another study showed that IL-8 has the potential to decrease somatic cell counts of milk in *S. aureus*-infected Holstein cows (Takahashi *et al*., 2005). Bovine CD14 molecule in blood is mainly expressed by monocytes (Sohn *et al*., 2004) which play a central role in innate immunity during infection and also help initiate cell-mediated immunity. Study had shown the percentage of $CD14^+$ cells in milk

was significantly lower in *S. aureus* mastitis cows than in healthy cows (Kiku *et al*., 2010). CD14 expression was significantly increased at 1 dpi, this may be related to monocyte activation. However, the higher levels of M-CSFR expression which is associated with the generation of monocytes (Olweus *et al*., 1996) were not detected in infected cows indicating blood monocytes were not depleted in circulation during the actue phases of *S. aureus* mastitis. Although, IL-8, CD14 and LBP are crucial components of TLRs signal pathway (Schroder *et al*., 2003), the significant difference in TLR2 expression were not detected between infected cows and uninfected controls. This demonstrated that TLR2 signal pathway of PBMCs could not be pre-activated in circulation during tissue inflammation.

The recruitment of PBMCs has important implications for tissue inflammation, therefore two important chemokine receptor CCR2 and CX_3CR1 which play a role in inflammatory cell trafficking were examined in the study (Jerath *et al*., 2010). Previous studies in human and mice have show a pronounced accumulation of CCR2 and CX_3CR1-positive PBMCs occurs in inflammatory tissue (Mack *et al*., 2001; Sunderkotter *et al*., 2004). The results showed that only CCR2 expression in PBMCs was more significantly ($p<0.05$) up-regulated at 1 dpi in infected cows, it is suggested that the CCR2 signal pathway may play a larger role in PBMC trafficking in early of *S. aureus* mastitis. In addition to the evaluation of cytokine production, two markers of the systemic acute phase response, SCC and LBP were evaluated. The change of SCC and plasma LBP concentration from 1-3 dpi were consistent with a previous report (Bannerman *et al*., 2004).

CONCLUSION

Taken together, the present study documents that the subtle and complex nature of changes in gene expression of PBMCs during actue phases of bovine *S. aureus* mastitis, the upregulation of gene expression is probably related to the recruitment and activation of PBMCs.

ACKNOWLEDGEMENTS

New breed selection of quality beef and the key technologies research and demonstration (2011BAD28B04). The optimization scheme for genome-wide selection of Chinese beef groups is founded on high-density SNP (2010jc-2).

REFERENCES

Ahmed, M., Z. Shaban, D. Yamaji, Y. Okamatsu-Ogura and M. Soliman *et al*., 2007. Induction of proinflammatory cytokines and caspase-1 by leptin in monocyte/macrophages from holstein cows. J. Vet. Med. Sci., 69: 509-514.

Alluwaimi, A.M., P.V. Rossito, C.M. Leutenegger, T.B. Farver, W.L. Smith and J.S. Cullor, 2003. The cytokines marker in the *Staphylococcus aureus* mastitis of bovine mammary gland. J. Vet. Med., 50: 105-111.

Bannerman, D.D., M.J. Paape, J.W. Lee, X. Zhao, J.C. Hope and P. Rainard, 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol., 11: 463-472.

Bannerman, D.D., M.J. Paape, W.R. Hare and E.J. Sohn, 2003. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. J. Dairy Sci., 86: 3128-3137.

Blosser, T.H., 1979. Economic losses from and the national research program on mastitis in the United States. J. Dairy Sci., 62: 119-127.

Cheng, D.R., S.Y. Zhu, Y. Yin, W.W. Ding, Z.X. Mu, Z.R. Su and H.C. Sun, 2010. Prevalence of bacterial infection responsible for bovine Mastitis. Afr. J. Microbiol. Res., 4: 1110-1116.

Geissmann, F., M.G. Manz, S. Jung, M.H. Sieweke, M. Merad and K. Ley, 2010. Development of monocytes, macrophages and dendritic cells. Science, 327: 656-661.

Gerszten, R.E., E.A. Garcia-Zepeda, Y.C. Lim, M. Yoshida and H.A. Ding *et al*., 1999. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature, 398: 718-723.

Gronlund, U., A. Johannisson and K.P. Waller, 2006. Changes in blood and milk lymphocyte sub-populations during acute and chronic phases of *Staphylococcus aureus* induced bovine mastitis. Res. Vet. Sci., 80: 147-154.

Jerath, M.R., P. Liu, M. Struthers, J.A. DeMartino and R. Peng *et al*., 2010. Dual targeting of CCR2 and CX3CR1 in an arterial injury model of vascular inflammation. Thromb. J., Vol. 8. 10.1186/1477-9560-8-14.

Kiku, Y., T. Ozawa, S. Kushibiki, M. Sudo and K. Kitazaki *et al*., 2010. Decrease in bovine CD14 positive cells in colostrum is associated with the incidence of mastitis after calving. Vet. Res. Commun., 34: 197-203.

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using Real-time quantitative PCR and the 2 (delta delta C (T) method. Methods, 25: 402-408.

Mack, M., J. Cihak, C. Simonis, B. Luckow and A.E.I. Proudfoot *et al*., 2001. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J. Immunol., 166: 4697-4704.

Olweus, J., P.A. Thompson and F. Lund-Johansen, 1996. Granulocytic and monocytic differentiation of CD34hi cells is associated with distinct changes in the expression of the PU.1-regulated molecules, CD64 and macrophage colony-stimulating factor receptor. Blood, 88: 3741-3754.

Schroder, N.W., S. Morath, C. Alexander, L. Hamann and T. Hartung *et al*., 2003. Lipoteichoic Acid (LTA) of *Streptococcus pneumonia* and *Staphylococcus aureus* activates immune cells via Toll-Like Receptor (TLR)-2, Lipopolysaccharide-Binding Protein (LBP) and CD14, whereas TLR-4 and MD-2 are not involved. J. Biol. Chem., 278: 15587-15594.

Sohn, E.J., M.J. Paape, R.R. Peters, R.H. Fetterer, N.C. Talbot and D.D. Bannerman, 2004. The production and characterization of anti-bovine CD14 monoclonal antibodies. Vet. Res., 35: 597-608.

Standiford, T.J., D.A. Arenberg, J.M. Danforth, S.L. Kunkel, G.M. VanOtteren and R.M. Strieter, 1994. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: A cellular and molecular analysis. Infect. Immun., 62: 119-125.

Sunderkotter, C., T. Nikolic, M.J. Dillon, N. van Rooijen, M. Stehling, D.A. Drevets and P.J.M. Leenen, 2004. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J. Immunol., 172: 4410-4417.

Takahashi, H., T. Komatsu, K. Hodate, R. Horino and Y. Yokomizo, 2005. Effect of intramammary injection of rbil-8 on milk levels of somatic cell count, chemiluminescence activity and shedding patterns of total bacteria and S. aureus in holstein cows with naturally infected-subclinical mastitis. J. Vet. Med. Ser. B, 52: 32-37.