

Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR

Jai-Wei LEE^{a,b}, Douglas D. BANNERMAN^c, Max J. PAAPE^c,
Ming-Kuei HUANG^a, Xin ZHAO^{a*}

^a Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada

^b Department of Tropical Agriculture and International Cooperation,

National Pingtung University of Science and Technology, Neipu, Pingtung 91201, Taiwan

^c Bovine Functional Genomics Laboratory, USDA-Agriculture Research Service, Beltsville, MD 20705, USA

(Received 29 May 2005; accepted 9 September 2005)

Abstract – The expression of inflammatory cytokines, including interleukin (IL)-6, IL-8, IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)- α , and interferon (IFN)- γ , by milk somatic cells was characterized by real-time polymerase chain reaction (PCR) in dairy cows experimentally challenged with either *E. coli* ($n = 8$) or *S. aureus* ($n = 8$). The mRNA abundance of a target gene was calibrated with that of a reference gene (β -actin) and expressed as fold of induction over the control quarter at each time point. At no single time point did all eight quarters challenged with the same type of bacteria demonstrated increased expression of a target gene and there was large variation among animals at each given time. As a consequence, most tested comparisons were not statistically significant except the peak time points of IL-8 expression (75- and 29- fold in glands challenged with *E. coli* and *S. aureus*, respectively). However, the average fold induction of all targeted cytokines was increased in response to both bacterial challenges with the exception of IFN- γ . The expression of IFN- γ was only increased in milk somatic cells isolated from *E. coli*, but not *S. aureus*, challenged mammary glands. Moreover, upregulated expression of cytokine genes had higher magnitudes and/or faster responses in glands challenged with *E. coli* in comparison with those challenged with *S. aureus*. We propose that the compromised upregulation of inflammatory cytokines in *S. aureus* infected glands may, at least partially, contribute to the chronic course of infection caused by this pathogen. Further research on identifying factors responsible for the differentially expressed cytokine profiles may be fundamental to developing strategies that mitigate the outcome of bovine mastitis.

mastitis / cytokine / *Escherichia coli* / *Staphylococcus aureus* / SCC

1. INTRODUCTION

Escherichia coli and *Staphylococcus aureus* are the most prevalent pathogens to

induce bovine mastitis, one of the most costly diseases to the dairy industry [4]. The pathogenesis and clinical symptoms of *E. coli* mastitis are considerably different

* Corresponding author: xin.zhao@mcgill.ca

from those of *S. aureus* mastitis, which makes the nature of these two types of intramammary infection distinct. Mastitis induced by *E. coli* is usually acute and often resolves spontaneously within a short period. In contrast, *S. aureus* mastitis often becomes chronic and may persist for the life of infected animals. This may be attributed, at least partially, to the ability of *S. aureus* to survive intracellularly in mammary cells. Moreover, differential activation of the innate immune system by various virulence factors may also play a role in the course of infection. It has been well documented that a cell wall component of Gram-negative bacteria, lipopolysaccharide (LPS), is the key virulence factor eliciting the clinical symptoms associated with *E. coli* infections [2, 11]. The recognition of LPS is mediated by membrane CD14, LPS-binding protein (LBP), and Toll-like receptors (TLR) (primarily TLR4), which initiates the activation of leukocytes and subsequent cytokine production [6, 13]. In addition, soluble CD14 binds to LPS and forms a complex capable of stimulating epithelial cells to produce chemoattractants, such as interleukin (IL)-8 [26].

In contrast, the virulence factor(s) of Gram-positive bacteria that are recognized by the innate immune system and elicit its activation are less well-characterized. Multiple virulence factors, including peptidoglycan (PGN), lipoteichoic acid (LTA), and toxins, are involved in the pathogenesis of *S. aureus* mastitis [24]. Both PGN and LTA have been shown to activate immune cells, including monocytes and macrophages, to produce inflammatory cytokines [8, 14, 23]. Activation of immune cells by these cell wall components of Gram-positive bacteria seems to be TLR2, but not TLR4, dependent [28].

The induction of TLR-associated signal transduction pathways is best characterized by the activation of NF- κ B, which leads to production of a spectrum of inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF- α [13]. TLR2 and TLR4 are required for macrophage NF- κ B activation in response to PGN and LPS, respectively [25]. It has

also been indicated that human macrophages exposed to Gram-negative bacteria differentially expressed genes relative to those activated by Gram-positive bacteria [16], implying alternative activation pathways are initiated by Gram-negative bacteria. Moreover, the magnitude of most differentially expressed genes was higher in macrophages exposed to Gram-negative bacteria.

The inflammatory responses are sophisticatedly regulated by the cytokine network during an infection. It has been demonstrated that intramammary challenge with *E. coli* or *S. aureus* elicits differential innate immune responses in terms of clinical symptoms and milk cytokine profiles at the protein level [3, 19]. The difference in cytokine profiles might attribute to the natural properties of these two types of mastitis. Although cytokines can be produced by somatic cells and mammary epithelial cells, the somatic cells are probably the main sources of cytokines in milk. Therefore, the objective of this study is to investigate whether these two types of bacteria induce distinguishable cytokine profiles at the level of transcription in milk somatic cells. RNA was isolated from the milk somatic cells of challenged animals and the expression of selected cytokine genes, including IL-6, IL-8, IL-12, TNF- α , IFN- γ , and GM-CSF, was semi-quantified by real-time PCR at various times points during experimentally induced *E. coli* or *S. aureus* mastitis.

2. MATERIALS AND METHODS

2.1. Animals

The experiment was conducted in parallel to our previous study [3], using the same animals. Briefly, sixteen clinically healthy Holstein cows in mid lactation (214 ± 8.67 days in milk) were selected based on milk somatic cell counts (SCC) ($< 500\,000$ cells/mL) and the absence of bacteria from three daily, consecutive,

aseptically collected milk samples. Use of animals for this study was approved by the Animal Care and Use Committee of Beltsville Agriculture Research Center.

2.2. Preparation of bacteria

The organisms used were serum-resistant *E. coli* strain P4 and *S. aureus* strain 305, which were originally isolated from clinical cases of bovine mastitis and have been shown to induce experimental mastitis [1, 11]. Before challenge exposure, 10 mL of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Spark, MD, USA) were inoculated with either strain and incubated for 6 h at 37 °C. Thereafter, 1 mL of the inoculum was transferred to aerating flasks containing 99 mL of tryptic soy broth (TSB, Difco, Detroit, MI, USA) and incubated overnight at 37 °C. After incubation, the flasks were placed in an ice water bath and mixed by swirling. One millilitre from each flask was serially diluted in PBS and 1 mL of the resulting dilution was mixed with 9 mL of pre-melted trypticase soy agar in petri dishes. The plates were allowed to solidify at room temperature and then transferred to a 37 °C incubator overnight. The aerating flasks containing the stock inoculum were maintained at 4 °C overnight. Once the concentration of the stock had been determined based on the prepared pour plates, the stock was diluted in PBS to a final concentration of 40 CFU/mL.

2.3. Intramammary challenge

One front or rear quarter of each cow was infused with 2 mL (40 CFU/mL) inoculum of either *E. coli* ($n = 8$) or *S. aureus* ($n = 8$) immediately after the morning milking. The contralateral quarter of each challenged quarter was infused with 2 mL of sterile PBS. The actual number of bacteria infused, determined by the pour-plating, was confirmed to be 72 and 74 CFU/quarter for *E. coli* and *S. aureus*, respectively. Milk sample collection and rectal temperature measurement were carried out at 0, 8, 16,

24, 32, 40, 48, and 72 h relative to the challenge.

2.4. Bacteriology and determination of SCC

Aseptically collected milk samples, with or without serial dilutions, were plated onto blood agar plates and the number of CFU enumerated after 16 h of incubation at 37 °C. Confirmatory identification was performed by the Maryland Department of Agriculture Animal Health Section (College Park, MD, USA). For the determination of milk SCC, a 2-mL aliquot of milk was heated for 15 min at 60 °C and maintained at 40 °C until counted in duplicate on an automated cell counter (Fossomatic 90, Foss Electronic, Helleroed, Denmark).

2.5. Isolation of milk somatic cells

Fifty millilitre of aseptically collected milk samples were diluted with an equal volume of sterile PBS and centrifuged at $700 \times g$ for 20 min at 20 °C. After the fat layer and the supernatant were discarded, the cell pellet was washed twice and suspended in sterile PBS. A small portion of the cell suspension was properly diluted and cytospin-centrifuged for differential counting. The remaining portion was enumerated and spun down for total RNA extraction.

2.6. RNA extraction and reverse transcription

Total RNA extraction was performed using TRIZOL (GIBCO/BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. The pellet of 10^6 milk somatic cells was lysed by 1 mL of TRIZOL reagent, and centrifuged after adding 0.2 mL of chloroform (phase separation). The RNA, retained in the aqueous phase, was precipitated by mixing with an equal volume of isopropanol and washed twice with 75% ethanol. Afterward, an appropriate amount of diethylpyrocarbonate (DEPC)-treated water was added to dissolve the RNA, and

Table I. Sequences of primers for bovine cytokines and β -actin in real time PCR.

Gene	Primer	Sequence (5'-3')	Length	Accession
IL-6	IL-6.f209	TCATTAAGCGCATGGTCGACAAA	105	NM173923
	IL-6.r313	TCAGCTTATTTTCTGCCAGTGTCT		
IL-8	IL-8.f251	CACTGTGAAAATTTCAGAAATCATTGTTA	105	NM173925
	IL-8.r355	CTTCACAAATACCTGCACAACCTTC		
IL-12	IL-12.f623	TTATTGAGGTCGTGGTAGAAGCTG	115	U11815
	IL-12.r737	GGTCTCAGTTGCAGGTTCTTGG		
GM-CSF	GM-CSF.f170	AGTAATGACACAGAAGTCGTCTCTG	87	U22385
	GM-CSF.r256	GCCGTTCTTGTACAGCTTCAGG		
IFN- γ	IFN- γ .f296	TCATTAAGCGCATGGTCGACAAA	185	M29867
	IFN- γ .r480	TCAGCTTATTTTCTGCCAGTGTCT		
TNF- α	TNF- α .f2377	TCTTCTCAAGCCTCAAGTAACAAGC	418	AF011926
	TNF- α .r2794	CCATGAGGGCATTGGCATAAC		
β -actin	β -actin.f38	CCTTTTACAACGAGCTGCGTGTG	391	AH00130
	β -actin.r428	ACGTAGCAGAGCTTCTCCTTGATG		

the concentration was determined by the optical density value at 260 nm. The reverse transcription (RT) reaction was started by adding 2 μ g of total RNA and 0.5 μ g of oligo (dT₁₂₋₁₈) to 12 μ L of sterile, distilled water and heated at 70 °C for 10 min. After cooled on ice, 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 5 \times first strand buffer and 200 U Superscript II RNase H⁻ Reverse Transcriptase (Gibco/BRL) were added. The mixture was stabilized at 25 °C for 10 min and subsequently incubated at 42 °C, 50 min, for the RT reaction. Thereafter, the temperature was raised to 70 °C for 15 min to inactivate the reverse transcriptase. Synthesized cDNA was kept at -20 °C until being used.

2.7. Lightcycler real-time PCR

The Lightcycler real-time PCR was carried out as described [17] with modifications. Briefly, primers for specific bovine genes, as listed in Table I, were synthesized (Invitrogen, Burlington, Ontario, Canada) to have an equal annealing temperature of 60 °C. The reaction condition for each individual gene was optimized using a Quanti-

Tect SYBR Green PCR kit (Qiagen, Mississauga, Ontario, Canada) in a LightCycler system (Roche, Mississauga, Ontario, Canada) and applied to the following protocol. The cDNA was analyzed in 20 μ L PCR mixture containing a final concentration of 0.5 μ M primer, 1 μ L of cDNA, and 2 \times QuantiTect SYBR green PCR mastermix. The PCR master mix contains HotStartaq DNA polymerase, SYBR green PCR buffer, dNTP mix including dUTP, SYBR green I, ROX (passive reference dye) and MgCl₂ (3 mM for GM-CSF and TNF- α ; 2.5 mM for the others). The PCR mixture was added into a cold PCR capillary, centrifuged, and placed in the LightCycler system. The LightCycler was programmed in 4 steps: (1) denaturation at 95 °C for 15 min; (2) amplification for 50 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C (depending on the product length, 5 s per 100 bp); (3) melting curve by 95 °C for 5 s, 65 °C for 15 s, and 95 °C for 0 s; (4) cooling at 40 °C. In each reaction, the cycle number at which the fluorescence rises appreciably above the background fluorescence is determined as crossing point (CP).

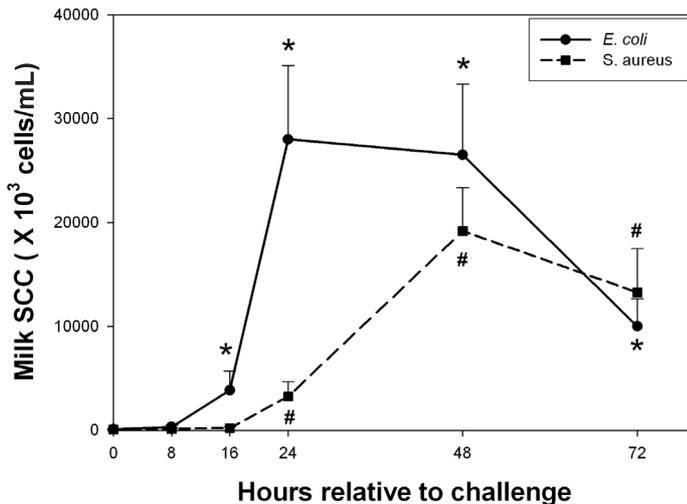


Figure 1. Effect of intramammary challenge with *E. coli* or *S. aureus* on milk somatic cell counts (SCC). Milk SCC were quantified in milk samples collected from challenged quarters at indicated time points. Data are presented as the mean \pm S.E.M. *, #: significantly increased in *E. coli*- or *S. aureus*-challenged quarters, respectively, relative to time 0 ($P < 0.05$).

2.8. Relative quantification

The expression of each gene was analyzed using the relative quantification method described by Pfaffl [17]. In brief, a slope was determined from the exponential phase, under the optimized real-time PCR amplification condition, of each target cytokine gene or the reference gene (bovine β -actin). The amplification efficiency (E) was calculated based on the slope, where $E = 10^{[-1/\text{slope}]}$. The expression of selected cytokine genes was calibrated by that of the reference gene, bovine β -actin, at each time point and converted to the relative expression ratio (fold of induction), where

$$\text{Fold of induction} = \frac{(E_{\text{target}})^{(\text{control gland CP}_{\text{target}} - \text{challenge gland CP}_{\text{target}})}}{(E_{\text{ref}})^{(\text{control gland CP}_{\text{ref}} - \text{challenge gland CP}_{\text{ref}})}}$$

2.9. Statistical methods

The mean of milk SCC and the fold of induction at each time point were compared

to the mean observed prior to intramammary challenge using the MIXED procedure of SAS [22]. Data on milk SCC were transformed to \log_{10} value before being analyzed. A P -value of < 0.05 was considered significant.

3. RESULTS

The clinical symptoms, bacterial growth, and other parameters associated with the infection were described in our previous publication [3]. The milk SCC data from challenged quarters at the time points which RNA samples were obtained are shown (Fig. 1). Milk SCC were significantly ($P < 0.05$) increased at 16 h and 24 h postinfection in *E. coli* and *S. aureus* challenged quarters, respectively. The mRNA expression of selected cytokine genes during the infection, represented as folds induction relative to the control gland, showed large variations among the animals in terms of magnitudes and kinetics. At no single time point did all eight quarters challenged with

the same type of bacteria demonstrate increased expression of a target gene. As a consequence, most of the tested comparisons were not statistically significant ($P > 0.05$), except the peak time points of IL-8 expression. The expression of IL-8 in milk SCC was significantly ($P < 0.05$) increased 75 and 29 folds at 16 h and 24 h postinfection, respectively, in comparison to expression prior to the challenge (Fig. 2A).

The temporal profiles of IL-12 and GM-CSF expression following infection with either bacteria were similar. The expression of IL-12 steadily increased after the challenge, peaked at 24 h, and decreased gradually afterward (Fig. 2B). Transient expression of GM-CSF mRNA was observed within the first 24 h of infection (Fig. 2C), however, a later induction also appeared in one *S. aureus* and four *E. coli* challenged glands. The average folds of induction for these two cytokines were always higher in glands challenged with *E. coli* at all time points.

A striking difference was observed in the expression of IFN- γ mRNA between the two types of infections. The folds of induction of IFN- γ were not changed in milk SCC isolated from glands challenged with *S. aureus* (Fig. 3A). On the other hand, the expression was gradually upregulated in glands challenged with *E. coli*, and reached its peak at 48 h postinfection. The profiles of TNF- α and IL-6 demonstrated different patterns not only between the two types of infections, but also among the animals. In *E. coli* challenged glands, the expression of TNF- α mRNA was elevated at 8 h postinfection and returned to basal levels afterward (Fig. 3B). Glands challenged with *S. aureus* showed different responses in TNF- α mRNA expression. Four out of the 8 glands peaked at 24 h, and 2 each peaked at 8 h and 72 h postinfection, respectively. A similar trend of variation was also observed on the expression of IL-6 in *E. coli* challenged quarters. The expression of IL-6 did not change at all in two quarters, and peaked at various time points in the others, result-

ing in a profile with 2 peaks. In contrast, glands infused with *S. aureus* had a sharp up-regulation of IL-6 only at 24 h postinfection (Fig. 3C).

4. DISCUSSION

E. coli and *S. aureus* are the common pathogens recovered from bovine mastitis isolates. Being an environmental pathogen, the transmission pathway of *E. coli* differs from that of *S. aureus*, a contagious pathogen that spreads from cow to cow. Moreover, distinct virulence factors associated with these two types of bacteria (i.e. Gram-positive versus Gram-negative) elicit different clinical symptoms and courses of infections. In a previous study, we reported on the clinical symptoms, bacterial dissemination, and induction of inflammatory cytokines at the protein level in milk from cows challenged with either *E. coli* or *S. aureus* [3]. In the present study, total RNA extracted from milk somatic cells collected at various time points was analyzed for selected cytokine genes using real-time PCR. Therefore, the present study evaluates the expression of inflammatory cytokines at the transcriptional level in milk somatic cells after intramammary challenge with *E. coli* or *S. aureus*.

The advantages of using real-time PCR over conventional PCR have been thoughtfully described [12]. However, we encountered large variations in the folds of induction of a target gene calculated based on a relative quantification method [17]. The variation may be attributed to several factors and the high sensitivity of real-time PCR probably further augmented the variation among animals. It has been reported that there is a high variation among the kinetics and magnitude of the inflammatory responses elicited in cows in response to intramammary infection [5]. In the animals challenged with *E. coli*, there was no single time point at which bacteria were recovered from all eight quarters. Since live bacteria were used in the challenge study, the kinetics of bacterial dissemination further increased

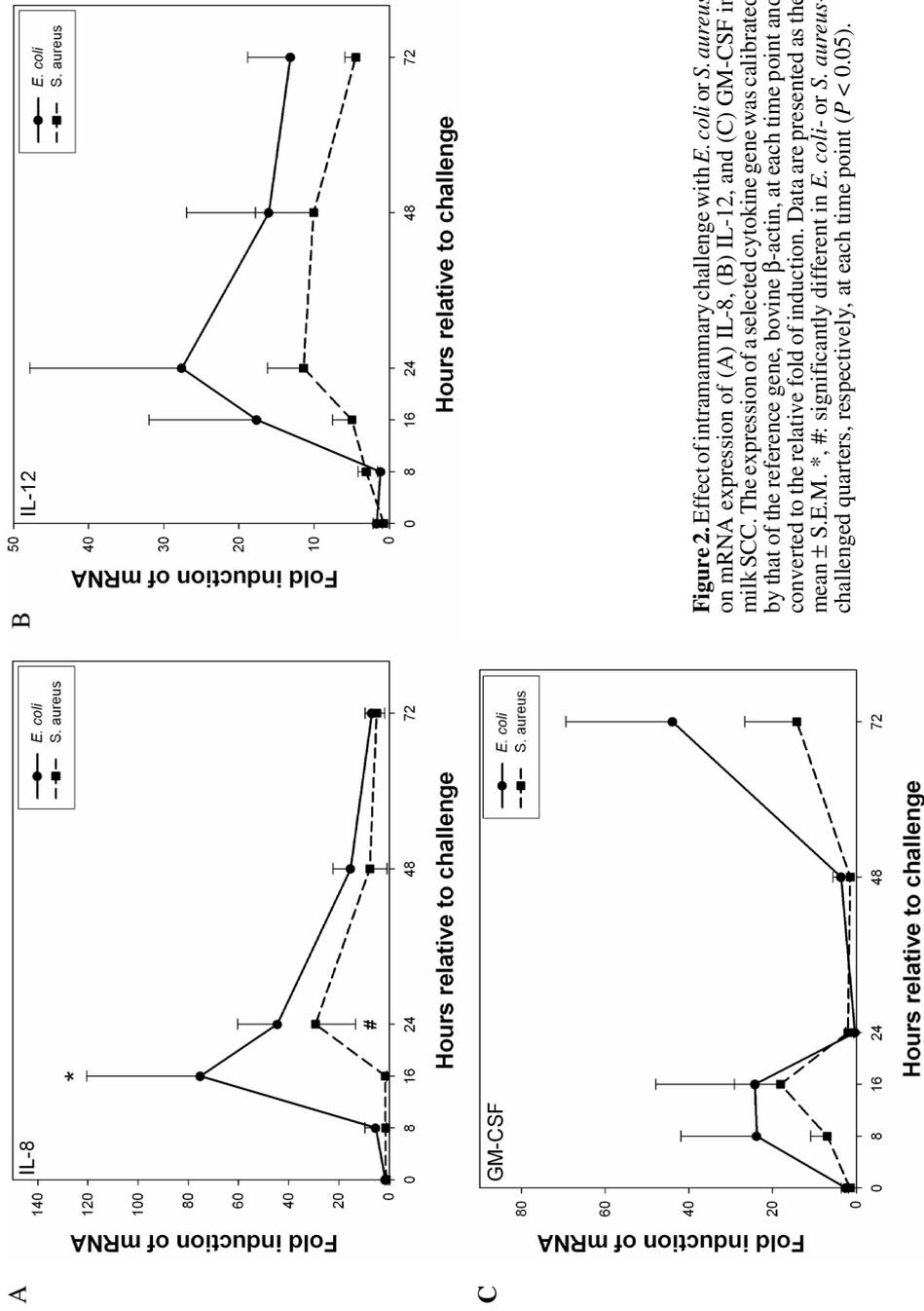


Figure 2. Effect of intramammary challenge with *E. coli* or *S. aureus* on mRNA expression of (A) IL-8, (B) IL-12, and (C) GM-CSF in milk SCC. The expression of a selected cytokine gene was calibrated by that of the reference gene, bovine β -actin, at each time point and converted to the relative fold of induction. Data are presented as the mean \pm S.E.M. *, #: significantly different in *E. coli*- or *S. aureus*-challenged quarters, respectively, at each time point ($P < 0.05$).

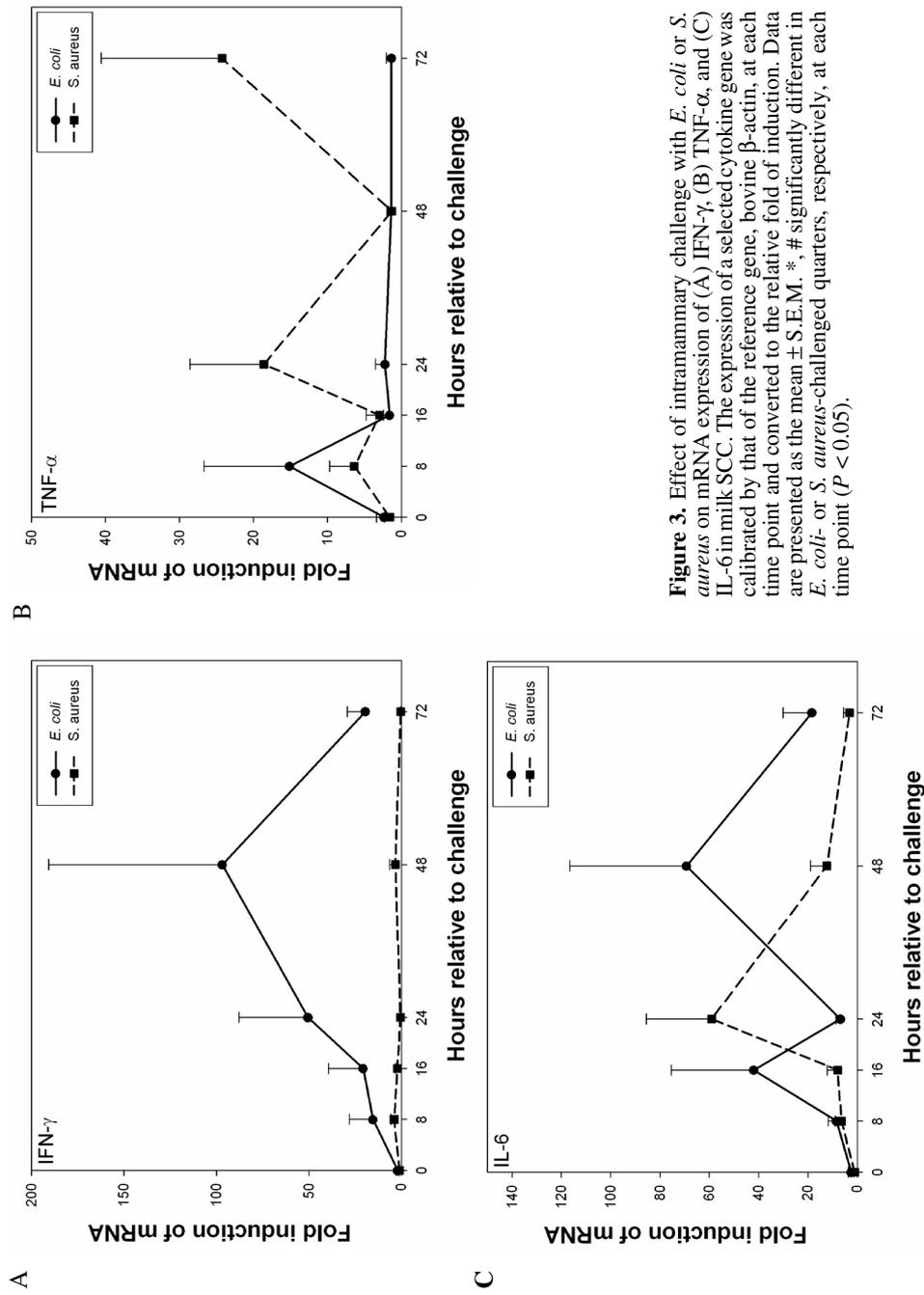


Figure 3. Effect of intramammary challenge with *E. coli* or *S. aureus* on mRNA expression of (A) IFN- γ , (B) TNF- α , and (C) IL-6 in milk SCC. The expression of a selected cytokine gene was calibrated by that of the reference gene, bovine β -actin, at each time point and converted to the relative fold of induction. Data are presented as the mean \pm S.E.M. *, # significantly different in *E. coli*- or *S. aureus*-challenged quarters, respectively, at each time point ($P < 0.05$).

the complexity of our observation. Different severities in each individual animal triggered a different level of defensive reactions to overcome the infection. Therefore, for some cytokines, including TNF- α , IL-6, and GM-CSF, variations were not only due to the magnitude, but also the time point showing a noticeable upregulation of the target gene. Second, the percentage of each subset of milk somatic cells varies from one animal to another in infection-free glands, confirmed by leukocyte differential counting (data not shown). Under normal conditions, the predominate cell type is the macrophage (54–83%), followed by the lymphocyte (10–27%), and the neutrophil (0–11%). However, the neutrophil presents more than 95% of milk somatic cells during mastitis [9]. Under this circumstance, the timing of neutrophil influx, which was different in each animal, dramatically affects the cell population of milk somatic cells at some time points. Therefore, the upregulation of the target genes, except IL-8, was not statistically significant due to the large variation. Nevertheless, the profiles still provide informative trends of gene expression in response to intramammary challenge.

The mRNA of all six cytokines, as well as β -actin, could be detected in milk somatic cells before the challenge, consistent with a previous study [1]. After the challenge, mRNA expressions of the six cytokine genes were upregulated. Generally speaking, our results demonstrate that the expression of all six cytokine genes had higher magnitudes and/or faster responses in glands challenged with *E. coli*. This is in agreement with a previous study using microarrays to examine the gene expression of human macrophages in response to various pathogens [16]. Most of the differentially regulated genes were more highly expressed in human macrophages exposed to Gram-negative pathogens. It is not clear why the expression of inflammatory cytokines was delayed and less pronounced in infections induced by Gram-positive bacteria, such as *S. aureus*. Since these two types of bacteria elicit inflammatory responses through dif-

ferent receptors, different pathways or levels of signal transduction might be responsible for this phenomenon. Nevertheless, the weaker and delayed upregulation of cytokine genes elicited by *S. aureus* bacteria may, at least partially, explain the chronic nature of *S. aureus* mastitis. Taking IL-8 as an example, in comparison with glands challenged with *S. aureus*, the induction of expression of IL-8 mRNA in glands challenged with *E. coli* was significantly upregulated earlier (16 versus 24 h) and stronger (75 versus 29 folds). The profile of SCC showed a similar tendency implying IL-8 is one of the chemoattractants for neutrophils in infected bovine mammary glands [10].

Interestingly, the upregulated expression of IL-8, as well as TNF- α , mRNA detected in glands challenged with *S. aureus* is in contrast to our previous results at the protein level [3]. By using ELISA, neither IL-8 nor TNF- α was detected in the milk after challenge with *S. aureus*. The discrepancy between mRNA level and protein level on these inflammatory cytokines was also reported in cows injected with α -toxin [20]. The authors proposed that lower sensitivity (> 30 pg/mL) of the ELISA kit can not be excluded. In addition, *S. aureus* might be able to mount defensive mechanisms that interfere with the translation of mRNA of these two critical cytokines. It has been also well established that increased expression of a given mRNA transcript does not necessarily correspond with upregulated expression of a protein, and vice versa. A significant amount of IFN- γ was detected in milk using ELISA after the challenge [3]. However, the expression of IFN- γ mRNA was not provoked in most glands challenged with *S. aureus*, which is in agreement with results after intramammary infusion of α -toxin [20]. As a matter of fact, the expression of mRNA was found to be downregulated after intramammary challenge with *S. aureus* [1]. Comparing stimulation by different pathogens, 26 out of 43 genes strongly induced by human macrophages exposed to Gram-negative organisms and LPS are IFN- γ dependent [15]. It seems that lacking IFN- γ

expression, at least at the transcriptional level, is common in *S. aureus* infections. TH1 type immune responses, including delayed-type hypersensitivity (DTH), are more effective in eliminating intracellular pathogens, and IFN- γ is an essential cytokine for TH1 responses. Considering the ability to survive intracellularly, *S. aureus* could teleologically deploy mechanisms to suppress the IFN- γ expression in milk somatic cells. If this is the case, IFN- γ detected by ELISA in milk could come from cells other than milk somatic cells in the mammary gland. Human oral epithelial cells have been shown to have the ability of producing IFN- γ [21]. Whether bovine mammary epithelial cells are able to produce IFN- γ in response to bacterial infections needs further investigation.

Accumulated lines of evidence reveal that the innate immune system recognizes Gram-positive and Gram-negative bacteria mainly through TLR2 and TLR4, respectively [25, 27, 28]. However, a recent study reported mastitis, induced by either Gram-positive or Gram-negative bacteria, increases mammary mRNA abundance of both TLR2 and TLR4 [7]. Although both TLR lead to similar signalling pathways, including the activation of NF- κ B, striking differences in cytokine transcription were observed in human dendritic cells stimulated with TLR2 or TLR4 agonists [18]. It is unclear whether these two recognition patterns mediated the differentially expressed cytokine profiles, at the transcriptional level, in response to different types of pathogens in bovine mammary glands. However, differentially expressed cytokine profiles may, at least in part, contribute to the different clinical symptoms that appear in *E. coli* or *S. aureus* mastitis. Delayed and less pronounced upregulation of inflammatory cytokine transcription could be associated with the chronic nature of *S. aureus* mastitis. Specific preventive and therapeutic strategies are required for each type of mastitis. Understanding the inflammatory responses elicited by these pathogens is fundamental to developing such strategies.

ACKNOWLEDGEMENTS

This study was sponsored by the Dairy Cattle Genetics Research and Development Council of Canadian Dairy Network, Valorisation Recherche Québec and Natural Sciences and Engineering Research Council of Canada.

REFERENCES

- [1] Alluwaimi A.M., Leutenegger C.M., Farver T.B., Rossitto P.V., Smith W.L., Cullor J.S., The cytokine markers in *Staphylococcus aureus* mastitis of bovine mammary gland, *J. Vet. Med. B Infect. Dis. Vet. Public Health* 50 (2003) 105–111.
- [2] Bannerman D.D., Paape M.J., Hare W.R., Sohn E.J., Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge, *J. Dairy Sci.* 86 (2003) 3128–3137.
- [3] Bannerman D.D., Paape M.J., Lee J.-W., Zhao X., Hope J.C., Rainard P., *Escherichia coli* and *Staphylococcus aureus* elicit different innate immune responses following intramammary infection, *Clin. Diagn. Lab. Immunol.* 11 (2004) 463–472.
- [4] Barkema H.W., Schukken Y.H., Lam T.J., Beiboer M.L., Wilink H., Benedictus G., Brand A., Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts, *J. Dairy Sci.* 81 (1998) 411–419.
- [5] Burvenich C., Van Merris V., Mehrzad J., Diez-Fraile A., Duchateau L., Severity of *E. coli* mastitis is mainly determined by cow factors, *Vet. Res.* 34 (2003) 521–564.
- [6] Chow J.C., Young D.W., Golenbock D.T., Christ W.J., Gusovsky F., Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction, *J. Biol. Chem.* 274 (1999) 10689–10692.
- [7] Goldammer T., Zerbe H., Molenaar A., Schuberth H.-J., Brunner R.M., Kata S.R., Seyfert H.-M., Mastitis increases mammary mRNA abundance of β -defensin 5, Toll-like receptor 2 (TLR2), and TLR4 but not TLR9 in cattle, *Clin. Diagn. Lab. Immunol.* 11 (2004) 174–185.
- [8] Gupta D., Kirkland T.N., Viriyakosol S., Dziarski R., CD14 is a cell-activating receptor for bacterial peptidoglycan, *J. Biol. Chem.* 271 (1996) 23310–23316.

- [9] Lee C.-S., Wooding F.B.P., Kemp P., Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretion, colostrums, and milk from normal cows, *J. Dairy Res.* 47 (1980) 39–50.
- [10] Lee J., Zhao X., Recombinant human interleukin-8, but not human interleukin-1 β , induces bovine neutrophil migration in an in vitro co-culture system, *Cell Biol. Int.* 24 (2000) 889–895.
- [11] Lee J.-W., Paape M.J., Elsasser T.H., Zhao X., Recombinant soluble CD14 reduces severity of intramammary infection by *Escherichia coli*, *Infect. Immun.* 71 (2003) 4034–4039.
- [12] Leutenegger C.M., Alluwaimi A.M., Smith W.L., Perani L., Cullor J.S., Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan[®] polymerase chain reaction, *Vet. Immunol. Immunopathol.* 77 (2000) 275–287.
- [13] Martin T.R., Recognition of bacterial endotoxin in the lungs, *Am. J. Respir. Cell Mol. Biol.* 23 (2000) 128–132.
- [14] Morath S., Stadelmaier A., Geyer A., Schmidt R.R., Hartung T., Synthetic lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus of cytokine release, *J. Exp. Med.* 195 (2002) 1635–1640.
- [15] Nau G.J., Richmond J.F.L., Schlesinger A., Jennings E.G., Lander E.S., Young R.A., Human macrophage activation programs induced by bacterial pathogens, *Proc. Natl. Acad. Sci. USA* 99 (2002) 1503–1508.
- [16] Nau G.J., Schlesinger A., Richmond J.F.L., Young R.A., Cumulative toll-like receptor activation in human macrophages treated with whole bacteria, *J. Immunol.* 170 (2003) 5203–5209.
- [17] Pfaffl M.W., A new mathematic model for relative quantification in real-time RT-PCR, *Nucleic Acids Res.* 29 (2001) 2002–2007.
- [18] Re F., Strominger J.L., Toll-like receptors 2 (TLR2) and TLR4 differentially activate human dendritic cells, *J. Biol. Chem.* 276 (2001) 37692–37699.
- [19] Riollet C., Rainard P., Poultré B., Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*, *Clin. Diagn. Lab. Immunol.* 7 (2000) 161–167.
- [20] Riollet C., Rainard P., Poultré B., Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with *Staphylococcus aureus* α -toxin, *Inflamm. Res.* 49 (2000) 486–496.
- [21] Rouabhia M., Ross G., Pagé N., Chakir J., Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation, *Infect. Immun.* 70 (2002) 7073–7080.
- [22] SAS/STAT User's Guide, Version 8, SAS Institution Inc., Cary, NC, 2000.
- [23] Schwandner R., Dziarski R., Wesche H., Rothe M., Kirschning C.J., Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2, *J. Biol. Chem.* 274 (1999) 17406–17409.
- [24] Sutra L., Poultré B., Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*, *J. Med. Microbiol.* 40 (1994) 79–89.
- [25] Takeuchi O., Hoshino K., Kawai T., Sanjo H., Takada H., Ogawa T., Takeda K., Akira S., Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components, *Immunity* 11 (1999) 443–451.
- [26] Wang Y., Zarlenga D.S., Paape M.J., Dahl G.E., Recombinant bovine soluble CD14 sensitizes the mammary gland to lipopolysaccharide, *Vet. Immunol. Immunopathol.* 86 (2002) 115–124.
- [27] Werling D., Jungi T.W., Toll-like receptors linking innate and adaptive immune response, *Vet. Immunol. Immunopathol.* 91 (2003) 1–12.
- [28] Yoshimura A., Lien E., Ingalls R.R., Tuomanen E., Dziarski R., Golenbock D., Cutting edge: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2, *J. Immunol.* 163 (1999) 1–5.