Characterization of the bovine innate immune response in milk somatic cells following intramammary infection with *Streptococcus dysgalactiae* subspecies *dysgalactiae*

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ABSTRACT

The innate immune response of milk somatic cells in cows to *Streptococcus dysgalactiae* ssp. *dysgalactiae* was investigated by deliberate intramammary challenge. Cows were challenged with 2,500 colony-forming units of *Strep. dysgalactiae* DPC 5435, previously isolated from a clinical mastitis case. Eight of the 9 cows treated showed clinical signs of mastitis (swollen udders, increased somatic cell score, and clotted milk) within 1 wk of challenge. Messenger RNA levels of *IL-1β* and toll-like receptor 4 (*TLR4*) in milk somatic cells increased approximately 40 fold within 48 h of infusion, whereas tumor necrosis factor α increased 16 fold within the same time frame. Interestingly, cows homozygous for the G allele of the C-X-C chemokine receptor type 1 (*CXCR1*)-777 polymorphism had higher *IL-8* and *CXCR1* transcript abundance at 24 h postinfusion compared with cows homozygous for the C allele. The difference in expression of these genes at this critical time point may influence the severity of disease within different genotypes.

Key words: mastitis, *Streptococcus dysgalactiae* ssp. *dysgalactiae*, immune response, *CXCR1*

INTRODUCTION

Streptococcus dysgalactiae, a gram-positive bacterium, accounts for a notable proportion of both clinical and subclinical bovine mastitis infections (Hogan et al., 1989; Todhunter et al., 1995). It exhibits characteristics of both a contagious and an environmental pathogen (Bramley and Dodd, 1984). Due to the adaptability of this pathogen, traditional mastitis control programs have had minimal effect on reducing the incidence of *Strep. dysgalactiae* (Bolton et al., 2004). Different

strains of *Strep. dysgalactiae* isolated from bovine IMI exhibit variations in infectivity (Higgs et al., 1980) and express a range of surface receptors that bind to bovine proteins such as IgG, α_2 -macroglobulin, albumin, fibronectin, fibrinogen, collagen, vitronectin, and plasminogen (Calvinho et al., 1998). In addition, the bacterium has also been shown to produce extracellular enzymes, such as fibrinolysin, hyaluronidase (Sting et al., 1990), and streptokinases, which promote establishment and persistence of IMI (McCoy et al., 1991). In vitro, *Strep. dysgalactiae* has demonstrated the ability to adhere to (Calvinho and Oliver, 1998a) and internalize (Almeida and Oliver, 1995; Calvinho and Oliver, 1998b) within mammary epithelial cells. Once internalized, the bacterium is capable of survival within the viable host cell (Almeida and Oliver, 1995; Calvinho and Oliver, 1998b). This ability to adhere and internalize no doubt confers protection from therapeutics and the immune surveillance system.

The innate immune response to pathogen assault in the mammary gland is crucial for effective clearance of the pathogen. It is the predominant defense strategy during the early stages of an infection, as it targets a wide range of microorganisms, is ubiquitous, and is short acting (Gray et al., 2005). Mammary epithelial cells and macrophages initiate the immune response at the site of infection (Rainard and Riollot, 2003; Goldammer et al., 2004; Strandberg et al., 2005) to attract neutrophils and other immune cells for phagocytosis and killing of invading bacteria. Chemokine receptors 1 (*CXCR1*) and 2 (*CXCR2*) are G-protein-coupled IL-8 receptors (Murdoch and Finn, 2000). On neutrophil surfaces, recognition of chemokines by *CXCR1* and *CXCR2* induces β_2 -integrin upregulation, chemotaxis, enhancement of reactive oxygen species generation, and phagocytosis of pathogens (Rambeaud et al., 2006). β_2 -Integrins are surface receptors that facilitate firm adhesion of neutrophils to ligands on vascular endothelial cells, followed by extravasation to the site of infection. A polymorphism in *CXCR1* [*CXCR1*-777 (G/C)] within the protein-coding region of the gene results in a gluta-

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mine to histidine substitution at AA 245 located within the third intracellular loop of CXCR1 (Youngerman et al., 2004b). In vitro, this polymorphism associates with impaired neutrophil migration, attenuated adhesion molecule upregulation (Rambeaud and Pighetti, 2005), and decreased reactive oxygen species generation (Rambeaud et al., 2006). This polymorphism also associates with a susceptibility to subclinical mastitis (Youngerman et al., 2004a) and decreased milk fat composition (Leyva-Baca et al., 2007).

Previous studies have demonstrated the variability of the bovine innate immune response to different mastitis pathogens (Bannerman et al., 2004a,c; Oviedo-Boyso et al., 2007; Bonnefont et al., 2011; Schukken et al., 2011). However, little is known of the specific immune response to *Strep. dysgalactiae*. The aims of this study were to (1) characterize the bovine innate immune response to a deliberate udder challenge with *Strep. dysgalactiae* and (2) investigate the functional effects of CXCR1 genotypes on the immunological response to this pathogen attack.

MATERIALS AND METHODS

Animal Selection

Nine Holstein-Friesian cows, in their first or second lactation, were selected for the deliberate challenge based on their CXCR1 genotype and their health status. The animals were primarily grass-fed (100% perennial grass) spring calving cows located at Teagasc research stations in the south of Ireland. Genotyping analysis of CXCR1-777, using the method described by Goertz et al. (2009), determined that 6 of the cows selected were homozygous for the G allele (GG), whereas 3 were homozygous for the C allele (CC). The cows were considered healthy before deliberate challenge (i.e., time zero) as judged by the following: (1) the healthy appearance of udders and milk, (2) SCC in the milk were <200,000 cells/mL, (3) no pathogens were isolated, by microbiological methods, from quarter milk samples for 7 consecutive days before deliberate challenge, and (4) no recent history of subclinical or clinical mastitis. The milk samples were screened for the presence of pathogens by streaking 100 μ L onto esculin blood agar plates containing blood agar base no. 2 (Oxoid Ltd., Basingstoke, Hampshire, UK), supplemented with 7% citrated whole calf blood (vol/vol) and 0.1% esculin (vol/vol; Sigma, St. Louis, MO) and incubating overnight at 37°C. Milk SCC was quantified using a Somacount 300 (Bentley Instruments Inc., Chaska, MN) somatic cell counter. Infusions as well as milk and blood sampling were performed under license from the Irish Department of Agriculture and Food, and the cows' health

was subsequently monitored by trained farm staff and veterinary personnel.

Intramammary Challenge and Sampling

Streptococcus dysgalactiae DPC 5435, isolated originally from a subclinical mastitis case on a Teagasc research farm, was grown at 37°C in tryptic soy broth (Difco Laboratories Inc., Detroit, MI). Overnight cultures of the bacteria were serially diluted and plated to determine the number of colony-forming units. A 2-mL suspension, containing 2,500 cfu of *Strep. dysgalactiae* in maximum recovery diluent, (Oxoid Ltd.) was prepared from the overnight culture. Preliminary trials indicated that 2,500 cfu of *Strep. dysgalactiae* were sufficient to cause infection. Immediately following the morning milking, 1 quarter from each animal was infused with this suspension into the teat sinus via the streak canal. The contralateral quarter was infused with 2 mL of diluent and served as a within-animal control. Following challenge, daily aseptic milk samples were collected from all infused and control quarters. To determine bacterial load, 100 μ L of each milk sample was serially diluted, plated on esculin blood agar plates, which are capable of supporting the growth of *Strep. dysgalactiae*, and incubated at 37°C overnight. Somatic cell count was also determined. Molecular fingerprinting of representative isolates from each cow was performed by pulsed-field gel electrophoresis as described previously using *Sma*I restriction endonucleases (Wang et al., 1999). Total quarter milk, (up to a 2-L volume), was also collected from the infused quarter and the control quarter immediately before infusion and at 7, 24, and 72 h, and 7 and 14 d postinfusion (pi) for RNA extraction from milk somatic cells (MSC). In addition, 10 mL of blood was collected at the time points specified from the tail vein by caudal venipuncture, using tubes containing potassium EDTA (EDTA K3E 15%, 0.12 mL; BD Vacutainer Systems, BD Vacutainer Pre-analytical Solutions, Plymouth, UK). Blood samples were placed immediately on ice for subsequent RNA extraction. For animal welfare purposes, intramammary-challenged cows received antibiotic treatment (Leo Yellow; Boehringer Ingelheim, Berkshire, UK) as soon as clinical signs of infection were observed. For all cows, the time of antibiotic treatment, relative to deliberate challenge with *Strep. dysgalactiae*, was recorded.

Harvesting MSC and Blood Leukocytes for RNA Isolation

To harvest total RNA from MSC, 1 mL of 0.5 M EDTA (Sigma-Aldrich, Ireland Ltd., Dublin) was added per li-

ter of milk (Boutinaud et al., 2002) and milk samples (2 L) were then centrifuged at $1,500 \times g$ for 30 min at 4°C. After the fat layer and the supernatant were discarded, the cell pellets were washed twice in PBS (Sigma) pH 7.4 with EDTA at a final concentration of 0.5 mM. The washed cell pellets were then resuspended in 1 mL of TriPure isolation reagent (Roche Diagnostics Ltd., Bell Lane, East Sussex, UK) and RNA was extracted using TriPure (Roche Diagnostics Ltd.) according to the manufacturer's instructions.

Blood samples were combined with 40 mL of erythrocyte lysis buffer from Qiagen Ltd. (Qiagen House, Crawley, West Sussex, UK) and placed on ice for 15 min. Following centrifugation at $3,000 \times g$, the supernatant was decanted and the leukocytes were washed in an additional 20 mL of ELB. The cells were resuspended in 1 mL and RNA was extracted using TriPure (Roche Diagnostics Ltd.) according to the manufacturer's instructions.

Ribonucleic acid was quantified using optical density readings at 260 nm and the integrity was analyzed following electrophoresis through glyoxyl gels (Ambion; Applied Biosystems Ltd., Warrington, UK). One microgram of total RNA was DNase treated and reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen Ltd.) according to manufacturer's instructions in a final volume of 20 μ L.

Quantification by Real-Time PCR

The panel of immune genes investigated consisted of toll-like receptors (*TLR2* and *TLR4*), cluster of differentiation 14 (*CD14*), interleukins (*IL-1 β* , *IL-8*, *IL-10*, and *IL-12*), tumor necrosis factor α (*TNF- α*), nuclear factor-kappa B1 (*NF- κ B1*), *CXCR1*, and β_2 -integrins (*CD11b* and *CD18*). Primers were designed for real-time PCR across intron/exon boundaries where possible, to minimize amplification of genomic DNA. The primers were designed using data available in the GenBank database, and accession numbers and primer sequences are listed in Table 1. A housekeeping gene, ubiquitin-conjugating enzyme (*E2D2*), was also included for analysis as an internal control. Quantitative analysis of the genes of interest was performed in a LightCycler 480 instrument (Roche Diagnostics Ltd.) using a dilution series of external plasmid DNA standards (Pfaffl and Hageleit, 2001). Plasmid standards were created for each gene by cloning a cDNA PCR product into pCR TOPO (Invitrogen, Life Technologies, Carlsbad, CA). Cloning was confirmed by sequencing. One microliter of template (serially diluted plasmid standards or cDNA extracted from MSC) was used per 10- μ L LightCycler reaction. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics Ltd.) was

used for quantification according to the manufacturer's instructions using 0.5 μ M forward and reverse primer. Each program began with initial denaturation at 95°C for 10 min, followed by 50 cycles of quantification consisting of 5-s denaturation at 95°C, 10-s annealing, and 25-s elongation at 72°C. The annealing temperatures for each gene are given in Table 1. Melting curve analysis was performed on each product by heating from a temperature 5°C above the annealing temperature to 95°C in the continuous fluorescence acquisition mode to ensure specificity of LightCycler products. For each gene, LightCycler runs were performed in triplicate.

Statistical Analysis

For statistical analysis, gene expression data and SCC data were transformed by obtaining the natural logarithm. The SCS variable is the transformed variable of SCC. Although all immune gene expression and SCS data for all cows were monitored over a 2-wk period, only data obtained before antibiotic treatment was included in statistical analysis. A hierarchical mixed model (PROC MIXED; SAS Version 9.1; SAS Institute Inc., Cary, NC) was used to quantify the effect of treatment (infused or control) on SCS and gene expression. The dependent variable was transformed gene expression or SCS. Fixed effects included in the model were time since the start of the study, treatment, and time by treatment interaction. Where significant ($P < 0.05$), a covariate, which was the gene expression or SCS for the control and infused quarters at time zero, was included as a fixed effect. Time relative to the start of experiment was included as a repeated effect within the udder quarter, and cow was included as a random effect. Least squares means were extracted from the analysis and differences between the control and infused quarters were considered significant at $P < 0.05$. To investigate the effect of *CXCR1* genotype on gene copy number, the genotype of the cows (homozygous G or homozygous C) was included as a fixed effect in the analysis. Fold change was determined as the difference between peak gene expression and preinfusion levels divided by preinfusion expression for that gene.

RESULTS

Bacterial Growth in Infected Quarters

Within 24 h of a deliberate udder challenge with 2,500 cfu of *Strep. dysgalactiae* DPC 5435, viable gram-positive bacteria were recovered from all quarters challenged with this pathogen in 9 Holstein-Friesian lactating cows. Bacteria were confirmed as *Strep. dysgalactiae* DPC 5435 isolates by pulsed-field gel electro-

Table 1. Primers, amplicon product size, and annealing temperature used for real-time PCR assays

Gene ¹	Accession no.	Primer ²	Product size (bp)	Annealing temperature (°C)
<i>IL-1β</i>	NM_174093	IL1β-591 F: 5'-TGG GTA TCA AGG ACA AGA ATC-3' IL1β-772 R: 5'-CCA GTT AGG GTA CAG GAC AGA C-3'	182	51
<i>IL-8</i>	NM_173925	IL8-305 F: 5'-CTA AAC CCC AAG GAA AAG TG-3' IL8-693 R: 5'-CAA GAT TAA CAA AAA CCG AAA ACA-3'	389	50
<i>IL-10</i>	NM_174088	IL10-373 F: 5'-CGC TGT CAT CGC TTT CTG-3' IL10-482 R: 5'-AAC TCA CTC ATG GCT TTG TAG-3'	110	53
<i>IL-12</i>	U11815	IL12-789 F: 5'-GAG CAC CCC GCA TTC CTA CTT C-3' IL12-974 R: 5'-GAC ACA GAT GCC CAT TCA CTC CAG-3'	186	57
<i>TNF-α</i>	AF011926	TNF α-2394 F: 5'-TAA CAA GCC GGT AGC CCA CG-3' TNF α -2985 R: 5'-GCA AGG GCT CTT GAT GGC AGA-3'	277	64
<i>NF-κB1 (p50)</i>	AY849381	NFκB-719 F: 5'-ACC CTA TGA GCC AGA GTT T-3' NFκB-1216 R: 5'-AAG GCA TTG TTC AGT ATC C-3'	171	54
<i>TLR-2</i>	NM_174197	TLR2-2236 F: 5'-CAT TCC TGG CAA GTG GAT TAT C-3' TLR2-2433 R: 5'-GGA ATG GCC TTC TTG TCA ATG G-3'	198	61
<i>TLR-4</i>	NM_174198	TLR4-132 F: 5'-TCT CTA CAA AAT CCC CGA CAA CAT-3' TLR4-369 R: 5'-AGA AAA GGC TCC CCA GGC TAA ACT-3'	238	57
<i>CD14</i>	NM_174008	CD14-156 F: 5'-CCT GCG AGC TGG ACG ACG ACG AT-3' CD14-354 R: 5'-CGA ACG CGC AGA GCC TTG ATT GTG-3'	199	61
<i>CXCR1</i>	U19947	CXCR1-648 F: 5'-CAA TAC AAC GAA ATG GCG GAT GAT-3' CXCR1-849 R: 5'-CAG GTT GTA GGG CAG CCA GCA GAG-3'	202	60
<i>E2D2</i>	XM_582519	E2D2-48 F: 5'-CAG GGG TGG AGT ATT TTT CTT GA-3' E2D2-339 R: 5'-AGT CCA TTC CCG AGC TAT TCT GTG-3'	291	57
<i>CD11b</i>	NM_001039957	CD11b-2459 F: 5'-TCT TCT ACC CGT CTG GCT TGT CCT-3' CD11b-2730 R: 5'-GTT CCC CGG CAT GTT GTT CTC ACT-3'	272	58
<i>CD18</i>	NM_175781	CD18-2141 F: 5'-GCG ACG GGC GGG ACA GAT ACG A-3' CD18-2511 R: 5'-GAG GCA GCG GGG TCA CGG TC A G-3'	371	63

¹TNF = tumor necrosis factor α; NF = nuclear factor; TLR = toll-like receptor; CD = cluster of differentiation; CXCR = C-X-C chemokine receptor; E2D2 = ubiquitin conjugating enzyme.

²F = forward; R = reverse.

phoresis analysis (data not shown). Bacterial load from pathogen-infused quarters peaked in 2 animals at 24 h pi and in 4 animals at 48 h pi. The remaining animals peaked at 7 d pi or later. Once animals received antibiotic treatment, numbers of viable bacteria recovered decreased dramatically.

IMI with *Strep. dysgalactiae* Induces an Increase in MSC and a Subsequent Clinical Manifestation of Disease

Following infusion of experimental quarters, 8 of 9 animals had increased SCS within 24 h of challenge; however, only 1 of these animals displayed physical symptoms of disease (i.e., a swollen hard udder quarter and clots in milk). This animal was immediately treated with antibiotic. A further 4 animals presented with swollen udders and milk clots within 48 h of infusion and were treated with antibiotic. By 72 h pi, 1 additional animal displayed signs of mammary infection and required antibiotic treatment. Of the remaining animals, 2 displayed clotted milk and swollen udders at 7 d pi, at which stage they received antibiotic treatment. One animal failed to show any increase in SCS or physical symptoms of infection for the duration of the trial. However, at the end of the challenge period, the

pathogen-infused quarter of this animal continued to shed bacteria and as such, this animal was treated with antibiotics to clear the udder of bacteria.

Statistical analysis of SCS data demonstrated that SCS of infused quarters were elevated compared with control quarters within 24 h of infusion ($P < 0.001$; Figure 1) and continued to be significantly different to control quarters up to 72 h pi ($P < 0.01$). A threshold SCS of 12.2 (~200,000 cells/mL) was used to determine healthy quarters and was exceeded in the infused quarters from 24 h pi to 7 d pi.

Intramammary Challenge with *Strep. dysgalactiae* Induces Cytokine Expression in MSC from Infused Quarters

Messenger RNA levels of the genes coding for bacterial recognition membrane receptors *TLR2* and *TLR4* were significantly increased (Table 2) in MSC from infused quarters, compared with control quarters at 24 h pi ($P < 0.05$), with an 8- and 43-fold increase, respectively, compared with preinfusion levels. Transcript abundance of *TLR4* in MSC from infused quarters also remained significantly different from control quarters until 48 h pi ($P < 0.01$) but no significant difference was observed at 7 d pi. Messenger RNA levels of *CD14*,

Figure 1. Somatic cell score (ln SCC) $\pm$ 95% confidence intervals in milk from quarters infused with *Streptococcus dysgalactiae* DPC5435 compared with milk from control quarters infused with diluent. *** $P < 0.001$; ** $P < 0.01$; n = number of animals measured at each time point; dark, horizontal line denotes threshold value of 12.2 SCS (198,789 somatic cells/mL), where quarters with values below this were considered healthy and free of infection.

whose gene product acts as a co-receptor for LPS, differed significantly in MSC from test quarters (Table 2) compared with control quarters at 48 h pi ($P < 0.05$).

Transcript levels peaked in MSC from test quarters at 48 h pi with a 10-fold increase observed at this time. Levels of *NF- κ B1*, whose gene product functions as a transcription factor, increased at 24 h pi ($P < 0.01$; Table 2) and peaked at 48 h pi ($P < 0.001$, 5-fold increase) in MSC from infused quarters. A second increase was observed at 7 d pi ($P < 0.01$) before returning to non-significant levels at 14 d pi (data not shown).

Transcript levels of *IL-1 β* and *TNF- α* (Table 3), whose gene products are potent inducers of the acute-phase response, were significantly higher at 24 h pi ($P < 0.05$) in MSC collected from test quarters but did not peak until 48 h pi, where a 35- and 16-fold increase, respectively, were observed compared with preinfusion levels ($P < 0.05$). Transcript abundance of *IL-1 β* had a second, albeit lesser, peak at 7 d pi ($P < 0.01$) in MSC from test quarters, but no significant difference was observed in MSC from pathogen-infused and control quarters at 14 d pi (data not shown).

For the chemokine receptor gene *CXCR1*, transcript abundance was increased in MSC from infused quarters compared with control quarters from 24 h pi ($P < 0.05$; Table 3). Transcript abundance peaked 48 h pi ($P < 0.001$) with a 10-fold increase observed. Levels of *IL-8*

Table 2. Transcript levels per 50 ng of total RNA in milk somatic cells (MSC) shed from *Streptococcus dysgalactiae*-infused quarters compared with control quarters (50 ng of mRNA)¹

Time ² (n)	Control		Infused		P-value
	Average value	95% CI	Average value	95% CI	
<i>TLR2</i>					
0 h (n = 9)	819	555–1,717	992	671–2,074	NS
7 h (n = 9)	890	620–2,047	234	164–546	NS
24 h (n = 9)	2,083	1,411–4,367	9,009	6,093–18,827	<0.05
48 h (n = 8)	3,111	2,176–7,236	12,003	8,364–27,584	NS
72 h (n = 4)	1,545	1,261–6,855	1,024	883–6,392	NS
168 h (n = 3)	603	517–3,633	1,856	1,591–11,154	NS
<i>TLR4</i>					
0 h (n = 9)	105	76–280	101	73–269	NS
7 h (n = 9)	167	124–490	117	87–343	NS
24 h (n = 9)	290	211–772	4,420	3,215–11,794	<0.05
48 h (n = 8)	209	156–616	3,002	2,242–8,856	<0.01
72 h (n = 4)	117	100–688	349	311–2,900	NS
168 h (n = 3)	77	69–644	602	538–5,055	NS
<i>CD14</i>					
0 h (n = 9)	4,999	3,396–10,589	5,611	3,811–11,885	NS
7 h (n = 9)	4,546	3,088–9,630	1,889	1,283–4,002	NS
24 h (n = 9)	10,564	7,176–22,379	38,685	26,279–81,950	NS
48 h (n = 8)	12,516	8,503–26,515	60,876	41,355–128,961	<0.05
72 h (n = 4)	25,627	17,409–54,288	44,770	32,409–117,380	NS
168 h (n = 3)	7,774	5,281–11,289	9,306	6,322–19,714	NS
<i>NF-κB1</i>					
0 h (n = 9)	4,429	2,552–6,024	5,128	2,955–6,975	NS
7 h (n = 9)	12,010	7,161–17,736	4,880	2,901–7,154	NS
24 h (n = 9)	8,655	5,160–12,781	28,158	16,228–38,302	<0.01
48 h (n = 8)	12,671	7,303–17,236	40,733	23,475–55,406	<0.001
72 h (n = 4)	8,948	5,157–12,171	12,091	7,442–19,356	NS
168 h (n = 3)	5,338	3,076–7,261	15,748	9,076–21,421	<0.01

¹Values are given as the exponential of transformed data $\pm$ 95% CI.

²TLR = toll-like receptor; CD = cluster of differentiation; NF = nuclear factor.

Table 3. Transcript levels per 50 ng of total RNA, in milk somatic cells (MSC) shed from *Streptococcus dysgalactiae*-infused quarters compared with control quarters¹

Time ² (n)	Control		Infused		P-value
	Average value	95% CI	Average value	95% CI	
<i>IL-1β</i>					
0 h (n = 9)	4,925	3,659–14,242	4,122	3,068–11,991	NS
7 h (n = 9)	15,446	11,766–49,393	9,983	7,606–31,952	NS
24 h (n = 9)	14,152	10,516–40,927	159,468	118,674–463,911	<0.01
48 h (n = 8)	5,819	4,435–18,646	178,136	135,788–571,199	<0.001
72 h (n = 4)	3,403	2,940–21,606	23,546	21,188–211,577	NS
168 h (n = 3)	4,810	4,323–42,717	234,756	211,209–2,105,679	<0.01
<i>TNF-α</i>					
0 h (n = 9)	5,232	3,585–11,388	4,708	3,233–10,313	NS
7 h (n = 9)	3,541	2,499–8,493	2,800	1,978–6,739	NS
24 h (n = 9)	9,668	6,625–21,043	42,387	29,101–92,845	<0.05
48 h (n = 8)	17,429	12,311–41,927	86,977	61,414–208,956	<0.05
72 h (n = 4)	10,000	8,360–50,989	9,002	7,798–58,332	NS
168 h (n = 3)	8,080	6,983–51,470	27,095	23,431–173,295	NS
<i>CXCR1</i>					
0 h (n = 9)	4,504	2,618–6,253	4,985	2,898–6,925	NS
7 h (n = 9)	12,079	7,289–18,380	4,662	2,809–7,064	NS
24 h (n = 9)	8,744	5,277–13,310	27,367	15,913–38,020	<0.05
48 h (n = 8)	11,970	7,218–18,181	49,365	29,776–75,033	<0.05
72 h (n = 4)	7,785	5,823–23,101	7,020	5,673–29,573	NS
168 h (n = 3)	3,672	2,778–11,412	15,837	11,984–49,263	NS
<i>IL-8</i>					
0 h (n = 9)	14,200	11,374–57,139	15,231	12,194–61,147	NS
7 h (n = 9)	14,264	11,674–64,285	4,701	3,851–21,278	NS
24 h (n = 9)	59,326	47,517–238,720	160,043	128,128–642,514	NS
48 h (n = 8)	44,016	36,046–199,153	141,634	115,955–639,574	NS
72 h (n = 4)	20,366	18,525–204,925	46,649	43,489–730,584	NS
168 h (n = 3)	14,438	13,540–217,776	27,343	25,643–412,531	NS

¹Values are given as the exponential of transformed data $\pm$ 95% CI.²TNF = tumor necrosis factor; CXCR = C-X-C chemokine receptor.

mRNA, whose gene product functions to enable neutrophil migration to sites of local infection and inflammation, was not significantly different in MSC from control quarters throughout the challenge (Table 3). A significant decrease in transcript levels of the β_2 -integrin gene *CD18* was observed in MSC from infused quarters within 7 h of deliberate challenge (Table 4; $P < 0.05$). However, no difference existed in levels of the β -integrin gene *CD11b* in the MSC of control and infused quarters over the course of the experiment (Table 4).

No increase occurred in the transcript abundance of the antiinflammatory cytokine gene *IL-10* or the proinflammatory gene *IL-12* in MSC from infused quarters (Table 4). Finally, no significant change was observed in mRNA abundance of the housekeeping gene *E2D2* in MSC from infused quarters compared with control quarters over the course of the experiment (data not shown).

Cytokine Expression in Control Quarters and Blood Compared with Preinfusion Levels

Transcript levels of the chosen cytokine genes did not vary in MSC from control quarters at the various time

points (Tables 2 to 4). In addition, mRNA levels of these cytokine genes remained unchanged in leukocytes harvested from the blood of any animal in this study (data not shown).

Effect of Genotype on Immune Response

To investigate the effect of the CXCR1-777 G/C polymorphism on immune gene expression during a *Strep. dysgalactiae* assault, mRNA data from MSC of 6 CXCR1-777 homozygous GG cows were compared with 3 CXCR1-777 homozygous CC cows. Cows homozygous for the G allele had significantly higher copy numbers of *IL-8* and *CXCR1* mRNA in MSC of pathogen-infused quarters at 24 h pi than cows homozygous for the C allele (Figure 2). No significant difference was observed at any other time point, including preinfusion levels. No difference was observed in mRNA levels of any other genes investigated, including the β -integrin genes (data not shown). In addition, no difference was observed in mRNA levels of the chosen genes in MSC from control quarters of different genotypes following *Strep. dysgalactiae* infusion. Finally, no significant difference was observed

Table 4. Transcript levels per 50 ng of total RNA in milk somatic cells (MSC) shed from *Streptococcus dysgalactiae*-infused quarters compared with control quarters¹

Time ² (n)	Control		Infused		P-value
	Average value	95% CI	Average value	95% CI	
<i>CD18</i>					
0 h (n = 9)	1,277	831–2,381	1,163	759–2,186	NS
7 h (n = 9)	1,591	1,088–3,437	431	288–870	<0.05
24 h (n = 9)	1,975	1,286–3,683	1,536	1,003–2,887	NS
48 h (n = 8)	3,549	2,366–7,096	2,591	1,728–5,189	NS
72 h (n = 4)	2,636	2,011–8,479	1,455	1,242–8,489	NS
168 h (n = 3)	3,545	2,847–14,452	5,107	4,101–20,816	NS
<i>CD11b</i>					
0 h (n = 9)	14,278	8,025–18,321	13,581	8,332–21,562	NS
7 h (n = 9)	7,165	4,209–10,201	4,168	2,698–7,654	NS
24 h (n = 9)	36,323	21,336–51,711	14,142	8,677–22,453	NS
48 h (n = 8)	29,998	17,598–42,573	28,251	18,266–51,679	NS
72 h (n = 4)	16,687	11,466–36,650	15,308	13,931–154,970	NS
168 h (n = 3)	16,113	11,915–45,724	31,195	23,139–89,601	NS
<i>IL-12</i>					
0 h (n = 9)	14,274	11,435–57,487	15,256	12,216–61,316	NS
7 h (n = 9)	14,209	11,636–64,252	4,559	3,738–20,755	NS
24 h (n = 9)	59,629	47,768–240,148	160,300	128,363–644,284	NS
48 h (n = 8)	43,183	35,375–195,660	137,324	112,467–621,337	NS
72 h (n = 4)	21,801	19,834–219,812	42,856	40,334–685,318	NS
168 h (n = 3)	14,317	13,422–214,789	25,900	24,281–388,468	NS
<i>IL-10</i>					
0 h (n = 9)	801	542–1,675	1,042	704–2,167	NS
7 h (n = 9)	856	595–1,950	288	201–2,167	NS
24 h (n = 9)	2,038	1,378–4,261	1,502	1,014–3,124	NS
48 h (n = 8)	2,119	1,434–4,432	1,286	869–2,676	NS
72 h (n = 4)	1,347	911–2,816	1,851	1,329–4,707	NS
168 h (n = 3)	601	417–1,368	1,965	1,327–1,087	NS

¹Values are given as the exponential of transformed data $\pm$ 95% CI.²CD = cluster of differentiation.

in SCS between animal genotypes at any of the time points analyzed (data not shown).

DISCUSSION

Although milk in healthy cows consists of several somatic cell types (neutrophils, macrophages, lymphocytes, and epithelial cells), it is dominated by macrophages, with neutrophils accounting for only 5 to 20% (Paape and Capuco, 1997; Bannerman, 2009; Pisoni et al., 2010). However, during both acute and chronic mastitis conditions, not only a dramatic increase in SCC occurs, but also a dramatic alteration in MSC composition, with neutrophils accounting for as much as 95% of the leukocytes present (Rainard and Riollet, 2003).

Following intramammary challenge with *Strep. dysgalactiae*, mRNA abundance of *TNF- α* and *IL-1 β* , potent inducers of the acute phase response, in MSC peaked at 48 h pi. This differs from the udder response to other mastitis pathogens with *Staphylococcus aureus* infection failing to significantly increase *TNF- α* mRNA and protein levels in milk (Riollet et al., 2000; Bannerman et al., 2004b; Lee et al., 2006). Riollet et al. (2000)

also reports that *IL-1 β* is not detectable in milk secreted from glands experimentally infected with *Staph. aureus*. However, Bannerman et al. (2004b) reported an increase in *IL-1 β* protein abundance in milk, 32 h after *Staph. aureus* infusion, which may suggest a strain-

Figure 2. Chemokine receptor 1 (*CXCR1*) and *IL-8* mRNA levels per 50 ng of total RNA in milk somatic cells (MSC) of *Streptococcus dysgalactiae*-infused quarters of CXCR1-777 GG animals compared with CC animals at 24 h postinfection. Values are given as the exponential of transformed data $\pm$ 95% confidence intervals. ** P < 0.01; * P < 0.05.

specific bovine response to *Staph. aureus* (Kim et al., 2011). *Streptococcus uberis*-infected quarters showed an increase in IL-1 β and TNF- α in milk, but at later time points in the infection, at 60 and 66 h, respectively (Bannerman et al., 2004a; Moyes et al., 2009). In contrast, IL-1 β and TNF- α responded much more quickly to gram-negative pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Pseudomonas aeruginosa*), where an increase in protein abundance of both of these cytokines was observed in milk within 20 h of challenge (Bannerman et al., 2004a,b,c, 2005). Results from the same animal studies revealed that gene copy number of TNF- α was increased in MSC within 8 h of *E. coli* infusion (Lee et al., 2006).

Interestingly, TNF- α inhibits *IL-12* transcription in human monocyte-derived macrophages, independently of IL-10 (a major IL-12 synthesis inhibitor), NF- κ B, and interferon regulatory factor-1 (major IL-12 activators; Ma, 2001). The tight control of *IL-12* expression by TNF- α may benefit the host through preventing excessive inflammatory responses, but conversely may provide an opportunity for *Strep. dysgalactiae* to establish a chronic infection in the mammary gland. The absence of *IL-12* induction by *Strep. dysgalactiae* in MSC is similar to a *Staph. aureus* challenge, where Lee et al. (2006) reported that *IL-12* mRNA abundance does not increase in MSC. In contrast, Alluwaيمي et al. (2003) observed an *IL-12* mRNA increase in milk cells at 24 h following intramammary challenge with *Staph. aureus*, with a sharp decrease within 32 h of infection. Increases in IL-12 protein were detected in milk within 32 h of *E. coli* or *Staph. aureus* challenge, but returned to nonsignificant levels by 48 h pi (Bannerman et al., 2004c). Two peaks in NF- κ B1 RNA abundance were observed in our study, at 48 h pi and 7 d pi. Although the p50 subunit is known to act in stimulation of the proinflammatory response in early infection, it also has inhibitory functions that appear to prevent extended inflammatory responses and immune-mediated host damage (Sha et al., 1995; Artis et al., 2002). The NF- κ B1 double peak observed may be reflective of the dual function of NF- κ B1 in modulating the local innate immune response.

No increase in transcript abundance of *IL-10*, a potent inhibitor of *IL-12* transcription (Del Vecchio et al., 2007), was observed in MSC following intramammary challenge with *Strep. dysgalactiae*. This is similar to *Staph. aureus* infections where Bannerman et al. (2004b) failed to induce expression of IL-10 protein in milk. However, Riollet et al. (2001) reported an IL-10 increase in milk cells following *Staph. aureus* experimental challenge. Interleukin 10 is produced by an assortment of cell types, including T lymphocytes, B cells, eosinophils, mast cells, and monocytic cells

(Asadullah et al., 2003). Interleukin 8 mRNA levels did not increase in MSC shed from quarters infused with *Strep. dysgalactiae*. This failure to observe an increase in *IL-8* and *IL-10* may indicate that cells producing IL-8 and IL-10 were lowly represented in MSC from infected quarters. For instance, bovine neutrophils, the predominant cells in MSC of infected quarters (Rainard and Riollet, 2003), are not a major source of IL-8 or IL-10 (Matsukawa et al., 2000; Asadullah et al., 2003). Milk somatic cells typing, in parallel with gene expression studies, would be able to ascertain levels of cytokine expression within individual cell types. Indeed, as bovine neutrophils are not attracted to bacteria directly but require recruitment by mammary epithelial cells via signals such as IL-8 (Rainard and Riollet, 2006), this observed lack of IL-8 response in MSC by *Strep. dysgalactiae* may provide an opportunity for this pathogen to lower the efficiency of neutrophil recruitment. In agreement, *Strep. uberis* initiates minimal and delayed IL-8 protein increases in milk of infected animals (Bannerman et al., 2004a). In contrast, mastitis caused by either *Staph. aureus* or *E. coli* results in an increase in IL-8 levels (Lee et al., 2006).

The *CXCR1* transcript peaked 48 h pi, with a 10-fold increase in gene copy number. This peak in *CXCR1* correlated with an increase in SCS, indicating a flood of neutrophils into the mammary gland and subsequently into the milk. Without quick and efficient neutrophil migration, infections can persist and lead to acute clinical mastitis, chronic infections and severe tissue damage. Cows homozygous for the G allele of *CXCR1*-777 had significantly higher *IL-8* and *CXCR1* levels at 24 h pi compared with their CC homozygous counterparts. A previous study by Rambeaud and Pighetti (2005) demonstrated that neutrophils harvested from CC cows had less β_2 -integrin than those harvested from GG cows after IL-8 stimulation. The glutamine to histidine substitution at AA 245 is located within a region implicated in neutrophil mobilization and calcium signaling during IL-8 stimulation (Youngerman et al., 2004a), potentially affecting *CXCR1* signaling and consequent neutrophil function in dairy cattle. Reduced *CXCR1* signaling in neutrophils of CC cows is hypothesized to be responsible for differences in β_2 -integrin surface levels (Rambeaud and Pighetti, 2005). However, in this study, although differences in *IL-8* and *CXCR1* levels were observed, no differences in levels of β_2 -integrin genes (*CD11b* and *CD18*) were observed between *CXCR1* genotypes. In addition, SCS did not differ between *CXCR1* genotypes following *Strep. dysgalactiae* challenge. In agreement, no associations were identified between *CXCR1*-777 polymorphism and SCS in a population of over 800 Irish Holstein-Friesian bulls (Beecher et al., 2010).

Messenger RNA abundance of *TLR2*, *TLR4*, and *CD14* peaking locally at 48 h after *Strep. dysgalactiae* infusion is similar to increases reported for other gram-positive mastitis pathogens (Goldammer et al., 2004; Yang et al., 2006; Petzl et al., 2008; Moyes et al., 2009). The observed individual cow variation to *Strep. dysgalactiae* attack may suggest that cell turn over and neutrophil diapedesis differed between animals that showed (1) immediate clinical signs of mastitis and (2) a weak or delayed response, or both (Bonnetfont et al., 2011). Messenger RNA abundance of *TNF- α* and *IL-1 β* remained elevated after antibiotic treatment. For example *TNF- α* gene copy number was significantly higher in MSC of infused quarters compared with control quarters (10,094 vs. 1,071; $P < 0.001$). This may indicate the development of endotoxin tolerance in an effort to prevent subsequent or secondary infections (Petzl et al., 2011; Günther et al., 2012).

CONCLUSIONS

Overall, the study provided key insights into *Strep. dysgalactiae* infection in the bovine udder, with distinct *TNF- α* and *IL-1 β* expression patterns in MSC when compared with other mastitis pathogens. Future work would include counting and typing of cells shed from *Strep. dysgalactiae*-infected quarters.

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