Genomic analysis of between-cow variation in dermal fibroblast response to lipopolysaccharide

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ABSTRACT

The innate immune response plays a major role in defense against mastitis-causing pathogens. Identification of existing variation in innate immune signaling among cows and the underlying molecular causes for the variation may help in design of new mastitis control strategies. The dermal fibroblast has been used as a model cell type to explore between-cow variation in the ability of cells to produce IL-8 in response to lipopolysaccharide (LPS) treatment, and this response appears related to an animal's ability to respond to in vivo challenge with LPS or *Escherichia coli* mastitis. In this study, primary dermal fibroblast cultures of cows and microarray-based genomic analysis were used to investigate the cause(s) for the variable response to LPS. Fibroblast cultures from 2 cows, one with a low response phenotype (LR_{array}) and another with a high response phenotype (HR_{array}), were selected from our collection of fibroblast cultures established from 88 cows. The LR_{array} fibroblast culture produced approximately 5-fold less IL-8 and IL-6 protein in response to 24-h LPS treatment than the HR_{array} fibroblast culture. Genomic analysis of RNA obtained from 3 replicates of the 2 cultures before and after 8-h LPS treatment revealed a combined LPS-induced differential expression of 321 transcripts, indicating the robust response capability of the fibroblast cell. Under basal conditions, the microarray analysis revealed 2-fold less expression of toll-like receptor 4 (*TLR4*) in the LR_{array} fibroblasts compared with the HR_{array} fibroblasts, and this was associated with a marked reduction in expression of genes regulated by the TLR4-MyD88-dependent and TLR4-TRIF-dependent pathways (*IL-8*, *IL-6*, *SAA3*, *CCL20*, *MX1*, *IRF1*, and *ISG20*). The between-culture differential expression of *TLR4* was confirmed and extended by quantitative PCR analysis (QPCR) that revealed a 33-fold lower expression of *TLR4* in the LR_{array} fibroblast culture. After LPS treatment, the difference

in *TLR4* expression increased to almost 50-fold and was associated with more than 8-fold lower expression of *IL-8* and *IL-6*. No DNA sequence variations were identified in the proximal 1,300-bp promoter region of the *TLR4* gene, and microarray analysis did not reveal a molecular explanation for the reduced *TLR4* expression under either basal conditions or following exposure to LPS. The attenuated innate immune response of the LR_{array} fibroblast culture to LPS may be caused by reduced *TLR4* receptor expression. Also, the primary dermal fibroblast cells can be used to examine underlying causes for between-cow variations in key immune response pathways.

Key words: dairy cow, toll-like receptor pathway, interleukin-8

INTRODUCTION

Mastitis often leads to heavy loss of milk production, poor milk quality, damage to mammary tissue, and sometimes premature culling of affected cows. Enhancing host resistance to mastitis through selective breeding is being considered as a method to reduce mastitis incidence (Detilleux, 2009), and some progress is being made (Heringstad et al., 2007). However, several factors impose limitations on the speed of genetic gains that can be made from traditional breeding strategies based on analysis of large data sets containing pedigree information and phenotypic measurements (Berry et al., 2011). Among the limiting factors are difficulties in accurately defining the phenotype without a bacterial challenge, the poor recording of mastitis incidence, the inability to determine the phenotype in males or preparturient females, and the low heritability of clinical mastitis (Detilleux, 2009; Berry et al., 2011). Marker-assisted selection (MAS) also has great potential in selecting cows with enhanced mastitis resistance but is still dependent on defining the appropriate disease-resistant phenotype (Griesbeck-Zilch et al., 2009). The accuracy of MAS could be improved if it were possible to locate functional polymorphisms in the actual genes affecting mastitis resistance.

Protective mammary gland defense and resolution of infection are dependent on the appropriate innate im-

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immune response that occurs immediately after bacterial penetration of the gland (Bannerman, 2009). Failure to induce an adequate innate immune response is thought to be the main factor in the development of persistent mammary gland infections (Yang et al., 2008; Günther et al., 2011). Deficiency in the innate immune response is implicated in delayed influx of neutrophils from blood into the mammary gland and impaired pathogen clearance. Burvenich et al. (2007) proposed that a 1-h delay in neutrophil transmigration during *Escherichia coli* infection of the mammary gland would cause an 8-fold increase in bacterial number and a subsequent high level of endotoxin release. It seems apparent that rapid detection of pathogens and subsequent induction of a contained innate immune response is essential for effective defense and quick resolution of infection.

Pathogen recognition and induction of the innate immune response after an infection is a complex process. The innate immune response is mainly initiated through a class of pattern recognition receptors called toll-like receptors (**TLR**; Medzhitov, 2007). Once receptors have been activated, downstream signaling occurs through the myeloid differentiation primary response gene (88) (**MyD88**)-dependent and toll/IL-1 receptor (**TIR**)-domain-containing adapter-inducing interferon- β (**TRIF**)-dependent pathways, with activation of associated kinases leading to translocation of nuclear factor- κ B transcription factors and activated interferon regulatory factor 3 (**IRF3**) into the nucleus (Zhang and Ghosh, 2001; Boulanger et al., 2003; Kawai and Akira, 2010). Subsequent expression and secretion of several innate immune response mediators play important roles in the host response by potentiating the recruitment and activation of neutrophils, the secretion of antibacterial factors, and the production of a febrile response in the animal (Bannerman, 2009).

Many previous studies have reported the existence of substantial between-animal variation in the severity of the response to intramammary challenge with *E. coli* (Van Werven et al., 1997; Burvenich et al., 2003; Kornalijnslijper et al., 2004). A portion of this variation is likely due to environmental factors affecting the immunological status of the animals at the time of infection (Wenz et al., 2006), but genetic differences between cows also likely affect mastitis susceptibility. Genetic variation in innate immune signaling pathways can be identified by using a relevant cell model under laboratory conditions. Dermal fibroblast cells have been used to locate and study key defects in pathogen detection and signaling pathways in humans (Picard et al., 2003; Davidson et al., 2006; von Bernuth et al., 2008). In one study, von Bernuth et al. (2008) discovered MyD88 mutations in 9 patients with invasive pyogenic bacterial

disease that resulted in reduced production of IL-6 and IL-8 by the patient's dermal fibroblasts. Using bovine dermal fibroblasts, we recently examined the relationship between an animal's dermal fibroblast response to LPS in vitro and the animal's in vivo response to either an intravenous injection of LPS (Green et al., 2011) or an intramammary challenge with *E. coli* (Kandasamy et al., 2011). In fibroblasts obtained from 15 heifers at 5, 11, and 16 mo of age, the relative ranking between animals in the amount of IL-8 produced by their cultured fibroblasts in response to LPS was stable (Green et al., 2011). After being challenged with intravenous LPS at 14 mo of age, the 4 highest-ranking animals developed higher plasma concentrations of IL-8 and tumor necrosis factor- α (**TNF- α**) than the 4 lowest-ranking heifers. Kandasamy et al. (2011) obtained fibroblast samples from 43 cows in mid lactation and found considerable between-animal variation in the amount of fibroblast production of IL-8 in response to LPS. Four of the high-ranked and 4 of the low-ranked animals were then challenged with intramammary *E. coli* and, although both groups cleared the resulting infection in a similar fashion, the high-ranked animals had more BSA in milk and a delayed resolution of inflammation, as indicated by longer periods of elevated SCC and lower milk production. These studies suggest that dermal fibroblasts may be used as a model in comparative basal and LPS-activation state analysis to detect genetic differences between animals in their innate immune response potential. In the current study, we used microarray-based genomic analysis and dermal fibroblast cells of selected cows in an attempt to locate functional differences in components of the LPS detection and response pathways.

MATERIALS AND METHODS

Dermal Fibroblast Cell Cultures

Primary dermal fibroblast cell cultures were selected from our collection of cultures that have been established and cryopreserved from 88 Holstein cows. The cells were derived by collagenase digestion of skin biopsies obtained from the shoulder region of lactating cows, as previously described (Kandasamy et al., 2011). Cows from the University of Vermont herd and a local collaborator's herd were selected for sampling based on their calving date without regard for milk production or previous disease history. All fibroblast cultures were screened for their ability to produce IL-8 in response to LPS (described below). Based on this initial screening assay, we selected 3 cultures from the lowest responding 10% and 3 from the highest responding 10% for fur-

ther analysis. Confirmation of the initially determined low- and high-response phenotypes was done as follows. Fourth-passage dermal fibroblast cells were seeded at a density of 2×10^5 cells/well in 6-well culture plates in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum, 1% insulin-transferrin-selenium supplement (Invitrogen), and penicillin-streptomycin-Fungizone (Thermo Scientific, Waltham, MA) and cultured for 48 h. Subsequently, cells in one well were treated with LPS (100 ng/mL; L4391, Sigma, St. Louis, MO), recombinant bovine IL-1 β (10 ng/mL; AbD Serotec, Raleigh, NC), or vehicle (endotoxin-free PBS) alone as control. After 24 h, the conditioned medium was centrifuged and supernatant stored at -20°C for IL-8 and IL-6 protein analysis using ELISA. This experiment was replicated 3 times.

IL-8 and IL-6 ELISA

The concentration of IL-8 in conditioned medium was determined by sandwich ELISA as previously described (Kandasamy et al., 2011) using an anti-bovine IL-8 monoclonal antibody (a gift from Samuel K. Maheswaran, University of Minnesota, St. Paul) as the capture antibody, recombinant bovine IL-8 (Thermo Scientific) as standard, biotinylated anti-bovine IL-8 polyclonal antibody (BAF208, R&D Systems, Minneapolis, MN) as the detection antibody, and streptavidin-horseradish peroxidase (Sigma-Aldrich) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich) for detection. The bovine IL-6 assay was similarly performed using bovine IL-6-specific capture and detection antibodies (Thermo Scientific) and recombinant bovine IL-6 (Thermo Scientific) as standard. All samples were assayed in one assay/protein, and the minimum detectable concentration of both assays was approximately 100 pg/mL.

Gene Expression Analysis

One low-responding (**LR**_{array}) and one high-responding (**HR**_{array}) fibroblast culture were selected for genome-wide expression analysis using bovine genome arrays (Affymetrix, Santa Clara, CA). These 2 cultures were recovered from cryopreservation, expanded, and seeded at 1×10^6 cells per 25-cm² flask and incubated at 37°C for 24 h. The cells were then treated with LPS (100 ng/mL) or vehicle alone for 8 h, after which the medium was collected for protein analysis and the adherent cells were lysed using cell lysis buffer for isolation of total RNA (PerfectPure RNA Cultured Cell Kit, 5 PRIME, Gaithersburg, MD). On-column

DNase digestion was performed to remove DNA during the RNA isolation procedure. Quality and quantity of the isolated RNA samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and a NanoDrop spectrophotometer (Thermo Scientific), respectively. Subsequently, gene expression analysis was conducted at the University of Vermont's core microarray facility. Twelve microarray slides were probed (2 fibroblast cultures $\times$ 2 treatments $\times$ 3 replicated experiments). Hybridization mixes were prepared and hybridized to the arrays according to the manufacturer's recommendations using the Affymetrix 450 fluidics station and scanned with the Affymetrix 3000-G7 scanner.

Microarray Analysis

Probe statistics (CEL files) were calculated from scanned images using Affymetrix GCOS software. All other calculations were performed using R software (R Development Core Team, 2009) and Bioconductor (Gentleman et al., 2004; Gentleman, 2005) tools. Probe set and sample matrix expression statistics were calculated using the Robust Multichip Average method (Bolstad et al., 2003), implemented in the *aroma.affymetrix* package (Bengtsson et al., 2008). Quality statistics were calculated using the *simpleaffy* (Wilson and Miller, 2005) and *affyQCReport* packages. Linear modeling was performed using the method developed by Smyth (2005) available in the Bioconductor *limma* package. Contrasts examined included the main effects fibroblast culture (**LR**_{array} minus **HR**_{array}) and treatment with LPS (LPS minus control), the effects of either fibroblast culture or treatment in the context of one of the other variables (LPS minus control for **LR**_{array}; LPS minus control for **HR**_{array}; **LR**_{array} control minus **HR**_{array} control; **LR**_{array} LPS minus **HR**_{array} LPS), and the interaction between the fibroblast cultures and the treatment. For all contrasts, differentially expressed genes that passed the false discovery rate ($P \leq 0.01$) and P -value/fold-change ($P \leq 0.01$ and ≥ 1.5 fold change) thresholds were selected for further analysis. A P -value (for each probe set) was calculated for each pair-wise comparison (e.g., LPS minus control). Fold-change values represent the ratio of responses between treatments or cultures. For ratios less than 1, the (negative) reciprocal is reported (i.e., a ratio of 0.5 is a 2-fold reduction or a -2.0 -fold change). Several genes are represented by redundant probe sets on the microarray chip. In such cases, the reference sequence of each probe set was subjected to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and then the probe set correspond-

ing to the reference sequence with highest bit score was selected to represent the particular gene.

Validation of Microarray Results by Quantitative Reverse-Transcription PCR

The RNA samples were converted into complementary DNA using RevertAid kit (Promega, Madison, WI). Subsequently, quantitative PCR (QPCR) was performed for *IL-8*, *IL-6*, *TLR4*, and β -actin genes using gene-specific primers (Table 1). The QPCR was performed using 1 $\times$ PerfeCTa SYBR Green SuperMix, Low ROX (Quanta BioSciences, Gaithersburg, MD) and 1 μ L of cDNA. The reactions were performed using the 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA) and cycling conditions were as follows: initial denaturation at 95°C for 2 min, and then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Melt curve analysis was performed to check amplification of the desired gene product. The β -actin gene was used as reference gene for normalization procedure. Threshold cycles (Ct) generated using the real-time PCR experiment for each sample were analyzed using the delta Ct method.

Microarray-based gene expression pattern was analyzed in only 2 fibroblast cultures. Presence of a similar gene expression pattern in other fibroblast cultures with similar phenotypes was analyzed as follows using QPCR. A new set of 4 LR and 4 HR cultures was selected from our bank of primary dermal fibroblast cultures. Fourth-passage fibroblast cells were cultured as previously described and treated with either LPS (100 ng/mL) or vehicle alone as control. After 8 h, the adherent cells were lysed using cell lysis buffer for isolation of total RNA. Synthesis of cDNA and QPCR were performed as previously described. Five genes were selected for gene expression analysis using QPCR with the primer sequences presented in Table 1: *IL-8*, myxovirus (influenza virus) resistance 1 (*MX1*), chemokine (C-X-C motif) ligand 12 (*CXCL12*), *TLR4*, and somatostatin (*SST*).

TLR4 Sequence Analysis

Genomic DNA was isolated from skin tissue or dermal fibroblast cells of the LR_{array} and HR_{array} cows using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The proximal promoter and 5' untranslated regions (UTR) of the *TLR4* gene (−1,301 to +565) were amplified and sequenced using gene-specific primers (Table 1). The *TLR4* exons were also amplified and sequenced using respective gene-specific primers (Table 1).

RESULTS

IL-8 Response of Selected LR and HR Fibroblast Cultures to LPS or IL-1 β

Among the preselected LR and HR fibroblast cultures, we observed an expected difference in both IL-8 (Figure 1A) and IL-6 (Figure 1B) secretion in response to LPS treatment. The LR fibroblast cultures produced approximately 5-fold less ($P < 0.05$) IL-8 protein compared with the HR cultures. A similar difference ($P < 0.05$) between LR and HR cultures was also observed in the IL-6 response to LPS.

The fibroblast cultures from cow #1 (LR) and cow #5 (HR) were selected for further analysis in an attempt to determine the cause of the differential IL-8 response to LPS. Stimulation with an alternate cytokine-inducing ligand, IL-1 β , also revealed significant differences ($P < 0.05$) in IL-8 secretion between the 2 cultures (Figure 2A). However, in contrast to the response to LPS, we observed no significant between-culture differences in IL-1 β -induced production of IL-6 (Figure 2B).

Genomic Analysis of Dermal Fibroblast Response to LPS Treatment

Genomic analysis of the 2 selected cultures in response to LPS was characterized by differential expression (fold change >1.5 , $P < 0.01$) of 395 transcripts in the LR_{array} fibroblasts, 585 transcripts in HR_{array} fibroblasts, with changes in 321 transcripts being common to both cultures (Supplementary Tables 1 and 2; available online at <http://www.journalofdairyscience.org/>). Surprisingly, LPS treatment did not induce any upregulation of TNF- α or IL-1 β transcripts in either LR_{array} or HR_{array} fibroblast cells, although IL-1 α was induced in both. In addition to stimulation of proinflammatory genes, LPS treatment did cause an upregulation of the gene encoding the potent antiinflammatory IL-1 receptor antagonist (*IL1RN*).

Genes Differentially Expressed Between LR and HR Fibroblast Cells Under Basal Conditions

Differential gene expression (>1.5 fold, $P < 0.05$) between LR and HR fibroblasts was determined during the basal state and after LPS exposure. During the basal state, 209 transcripts were upregulated and 179 transcripts were downregulated in LR_{array} fibroblasts relative to HR_{array} fibroblasts (Supplementary Table 3; available online at <http://www.journalofdairyscience.org/>). In general, relatively few immune-related genes were differentially expressed between the LR_{array} and

Table 1. Details of primer sequences used for quantitative (Q)PCR experiments

Gene symbol	Forward (top) and reverse (bottom) primer sequences (5'→3')	Amplicon length (bp)	Accession number	Reference
Primers used for QPCR				
β-actin	5'-GCAAATGCTTCTAGGCGGACT-3' 5'-CAATCTCATCTCGTTTCTGCG-3'	85	BT030480	Pareek et al. (2005)
IL-8	5'-GCTGGCTGTTGCTCTCTTG-3' 5'-AGGTGTGGAATGTGTTTTATGC-3'	118	EU276073	Pareek et al. (2005)
IL-6	5'-TGAGGGAAATCAGGAAAATGT-3' 5'-CAGTGTGTTGTGGCTGGAGTG-3'	110	EU276071	Pareek et al. (2005)
TLR4	5'-ACTGCAGCTTCAACCGTATC-3' 5'-TAAAGGCTCTGCACACATCA-3'	190	AB056444	Ibeagha-Awemu et al. (2007)
CXCL12	5'-ATTTAGGCCATGGAGGCTCT-3' 5'-GAAACTGTGCTGTGGCTTCA-3'	117	NM_001113174	—
SST	5'-TGGAGCCTGAAGATTTGTCC-3' 5'-GAAATTCCTTGCAGCCAGCTT-3'	122	NM_173960	—
MX1	5'-ACAGATGCGTCAGTGGTCAG-3' 5'-TGGAGAGAGTTCAGGGATGG-3'	133	NM_173940	—
Keratin 5	5'-CAAGGTCCTGGACACCAAGT-3' 5'-TCCAGCTGTCTCCTGAGGTT-3'	114	NM_001008663	—
TLR4 amplification and sequencing primers				
TLR4 promoter (amplification)	5'-CAAGATTTACAAAAATTGGACAGTGCC-3' 5'-TACCTGTACGCAAGGGTCCCAA-3'	1,870	NC_000070	—
TLR4 promoter (sequencing)	5'-TCCCTTGGACTGCAAGGAGATCCA-3' 5'-AGGCACAGGGAGAAGACAGCCA-3'	—	NC_000070	—
TLR4 exon 1	5'-CCTCTCACTTCCTCTCCTCCCGTAAC-3' 5'-CCTCGGGCTGCCTGTTAATGC-3'	434	NM_174198	—
TLR4 exon 2	5'-GTGACTTATGATTGGATGGA-3' 5'-GTAAGATGTCTGTTGAGTGA-3'	458	NM_174198	—
TLR4 exon 3	5'-ATATCTGTGTGGAGACCTAGATGACTGGG-3' 5'-GAACACGCCCTGCATCCATCTG-3'	2,414	NM_174198	—

Figure 1. Dermal fibroblasts cells isolated from mid-lactating cows were exposed to LPS (100 ng/mL) for 24 h and the concentrations in medium of *IL-8* (A) and *IL-6* (B) were determined by ELISA. The data are from fibroblast cells of 6 separate cows, with each bar in the chart representing the mean $\pm$ SE of triplicate aliquots of fibroblasts recovered separately from cryopreservation. Based on the *IL-8* and *IL-6* response to LPS, the lowest responding fibroblast culture (*LR_{array}) and one of the highest responding fibroblast cultures (†HR_{array}) were selected for microarray-based gene expression analysis.

HR_{array} cultures under basal conditions (Table 3). Of genes represented on the array related to the TLR/IL-1 β response pathway, one prominent difference between the LR and HR fibroblasts under control conditions was in the expression of *TLR4*. The expression of this gene was 1.9-fold less in the LR fibroblasts compared with the HR fibroblasts (Table 3). The LR fibroblast

cells also had significantly lower expression levels of *CXCL10*, *CXCL11*, and *CCL19* relative to that in HR fibroblast cells. Other notable genes that were expressed at a significantly lower level in LR relative to HR fibroblast under basal conditions were *TLR3*, *MX1*, and 2'-5'-oligoadenylate synthetase 2 (*OAS2*; Table 3). The MX1 and OAS2 proteins are involved in antiviral

Figure 2. The selected fibroblast cultures (see Figure 1) with the low responder (LR_{array}) and high responder (HR_{array}) phenotypes were exposed to IL-1 β for 8 or 24 h and the concentrations in medium of *IL-8* (A) and *IL-6* (B) were determined by ELISA. Each bar in the chart represents the mean $\pm$ SE of triplicate aliquots of fibroblasts recovered separately from cryopreservation. * P < 0.05 LR versus HR.

Table 2. Differential expression (fold change ≥ 1.5 ; $P \leq 0.01$) of selected immune-related genes between control and LPS-stimulated (100 ng/mL; 8 h) fibroblast cultures¹

Probe ID	Gene symbol	Fold change		
		LR fibroblast	HR fibroblast	Mammary epithelial cell response ²
Upregulated				
Bt.2408.1.S1_s_at	<i>CCL2</i>	10.1	13.9	7
Bt.9560.1.S1_at	<i>CCL20</i>	10.3	47.7	137
Bt.552.1.S1_at	<i>CCL5</i>	28.9	43.8	175
Bt.13130.1.S1_at	<i>CD40</i>	3.9	7.8	10
Bt.5494.1.S1_at	<i>CD44</i>	1.6	1.7	—
Bt.15742.1.S1_at	<i>CD47</i>	1.5	1.6	—
Bt.3841.1.S1_at	<i>CD83</i>	3.1	4.3	—
Bt.20259.1.S1_at	<i>CDC42EP2</i>	1.7	2.3	—
Bt.13542.1.S1_at	<i>CFB</i>	21.0	79.4	10
Bt.1275.1.S1_at	<i>CX3CL1</i>	5.7	14.4	7
Bt.16966.1.S1_at	<i>CXCL10</i>	5.0	1.8	—
Bt.610.1.A1_at	<i>CXCL2</i>	87.4	175.3	144
Bt.7165.1.S1_at	<i>CXCL6</i>	5.1	7.3	18
Bt.22021.1.S1_at	<i>IFI16</i>	2.5	2.4	8
Bt.20785.2.S1_at	<i>IFI44</i>	3.5	6.0	20
Bt.24098.1.A1_at	<i>IFIH1</i>	15.0	14.5	11
Bt.234.1.S1_at	<i>IL18</i>	1.5	1.5	—
Bt.29883.1.A1_at	<i>IL18R1</i>	1.9	2.0	—
Bt.191.1.S2_at	<i>IL1A</i>	1.5	3.9	5
Bt.15910.1.S1_at	<i>IL1F6</i>	4.7	8.2	—
Bt.4199.1.S1_at	<i>IL1RN</i>	2.6	2.4	—
Bt.3686.1.S1_at	<i>IL6</i>	12.1	35.0	13
Bt.155.1.S1_at	<i>IL8</i>	64.4	223.4	103
Bt.10077.1.S3_at	<i>IRF1</i>	2.0	3.1	3
Bt.5768.1.S1_at	<i>IRF7</i>	1.6	2.4	4
Bt.12304.1.S1_at	<i>ISG15</i>	36.7	33.0	28
Bt.22275.1.A1_at	<i>ISG20</i>	2.0	3.0	7
Bt.4675.1.S1_a_at	<i>MX1</i>	10.6	11.2	20
Bt.20288.1.S1_at	<i>NFKB2</i>	1.5	2.1	3
Bt.9027.1.S1_at	<i>NFKBIA</i>	7.8	11.3	11
Bt.8227.1.S1_at	<i>NFKBIZ</i>	1.8	2.9	8
Bt.20891.1.S1_at	<i>OAS2</i>	19.8	17.7	18
Bt.10398.1.S1_at	<i>PTX3</i>	6.3	21.7	—
Bt.24467.1.S1_at	<i>RSAD2</i>	15.9	13.1	57
Bt.278.1.S1_at	<i>SAA3</i>	6.3	29.2	299
Bt.27681.1.A1_at	<i>TICAM2</i>	1.6	2.0	—
Bt.7043.2.S1_a_at	<i>VCAM1</i>	8.4	12.4	—
Downregulated				
Bt.13934.1.S1_at	<i>CD3G</i>	−2.1	−2.9	—
Bt.22699.1.S1_at	<i>NFIA</i>	−1.7	−1.9	—
Bt.21763.3.S1_at	<i>NFIB</i>	−1.5	−1.9	—

¹Data were obtained by microarray analysis and represent mean fold change (control vs. LPS) from triplicate replicates of a low responding (LR_{array}) and a high responding (HR_{array}) culture (indicated in Figure 1).

²Corresponding differential gene expression by bovine mammary epithelial cells following 6 h of exposure to heat-killed *Escherichia coli* from Günther et al. (2011; not significantly regulated).

response, and expression of these genes is regulated through the TRIF-dependent pathway.

Several genes were upregulated in LR_{array} fibroblasts relative to HR_{array} fibroblasts under basal conditions. The upregulated immune-related genes included *CXCL12*, *CCL5*, *SST*, peroxisome proliferator-activated receptor gamma (*PPARG*), *CD36*, and *IL1RN*. Although the Affymetrix GeneChip used does not represent the entire bovine genome, it does contain appropriate probe sets to enable detection of many genes involved in the

TLR4 signaling pathway, including *TLR4*, *CD14*, myeloid differentiation protein-2 (*MD-2*), *MYD88*, interleukin-1 receptor-associated kinase (*IRAK*)-4, *IRAK1*, toll-like receptor adaptor molecule 2 (*TICAM2*), TNF receptor-associated factor 6 (*TRAF6*), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α (*NFKBIA*), v-rel reticuloendotheliosis viral oncogene (*REL*) homolog A (*RELA*), *RELB*, nuclear factor of kappa light polypeptide gene enhancer in B-cells (*NFKB*) 1 (*NFKB1*), and *NFKB2*. Of these, only

Table 3. Differential expression (fold change ≥ 1.5 ; $P \leq 0.01$) of innate immune-related genes between low responding (LR_{array}) and high responding (HR_{array}) fibroblast cultures maintained under control conditions¹

Probe ID ²	Gene symbol	Fold change
Downregulated		
Bt.26655.1.S1_at	<i>CCL19</i>	-1.6
Bt.28393.1.S1_at	<i>CD55</i>	-1.5
Bt.16966.1.S1_at	<i>CXCL10</i>	-3.1
Bt.18368.1.S1_at	<i>CXCL11</i>	-1.7
Bt.4675.1.S1_a_at	<i>MX1</i>	-2.1
Bt.20891.1.S1_at	<i>OAS2</i>	-2.2
Bt.5054.3.S1_at	<i>TGFB3</i>	-1.5
Bt.12298.1.S1_at	<i>TLR3</i>	-1.6
Bt.9030.1.S1_at	<i>TLR4</i>	-1.9
Bt.12694.1.S1_at	<i>TNFRSF21</i>	-1.6
Bt.8400.1.A1_at	<i>TOX</i>	-1.7
Upregulated		
Bt.26983.1.S1_at	<i>CASP1</i>	1.7
Bt.552.1.S1_at	<i>CCL5</i>	1.6
Bt.5392.1.S1_at	<i>CD36</i>	2.0
Bt.7425.1.S1_at	<i>CXCL12</i>	3.9
Bt.610.1.A1_at	<i>CXCL2</i>	1.7
Bt.26695.1.S1_at	<i>ICOSLG</i>	1.5
Bt.19889.1.A1_at	<i>IGSF1</i>	1.5
Bt.15910.1.S1_at	<i>IL1F6</i>	5.1
Bt.4199.1.S1_at	<i>IL1RN</i>	1.7
Bt.3862.1.S1_a_at	<i>PPARG</i>	2.2
Bt.456.1.S1_at	<i>SST</i>	3.6

¹Data were obtained by microarray analysis and represent mean fold change of 3 replicated experiments.

²Genes downregulated or upregulated in low responder relative to high responder.

TLR4 was differentially expressed (downregulated in LR) between LR and HR fibroblast cells during basal state.

Apart from the above gene list, greater expression of several keratin gene transcripts was observed in HR_{array} fibroblast cells relative to LR_{array} fibroblast cells under both basal and LPS-stimulated states. This indicates that the HR fibroblast cells had some keratinocyte contamination. However, we observed no morphological difference between the cultures, and the characteristic spindle shape morphology of the cells, combined with their intense staining for vimentin, suggests that the majority of the cells were fibroblasts. Subsequently, we examined whether keratinocyte contamination had any effect on either basal TLR4 mRNA expression or IL-8 response of our fibroblast cultures. To do this, a new set of 8 LR and 8 HR fibroblast cultures were selected and keratinocyte levels of those fibroblast cultures were indirectly determined by quantifying keratin 5 mRNA by QPCR. Subsequent analysis revealed no significant association or correlation between keratinocyte level and basal TLR4 mRNA expression ($P > 0.05$, $r = 0.06$) or IL-8 response phenotype ($P > 0.05$, $r = 0.06$; data not shown).

Genes Differentially Expressed Between LR_{array} and HR_{array} Fibroblast Cells After LPS Treatment

Following LPS treatment, 448 genes were differentially expressed between LR_{array} and HR_{array} fibroblast cultures (Supplementary Table 4; available online at <http://www.journalofdairyscience.org/>). For many TLR-regulated genes, such as *IL-8*, *IL-6*, *SAA3*, and *CCL20*, their expression following LPS treatment was several-fold less in the LR_{array} fibroblasts than in the HR_{array} fibroblasts (Table 4). As in the basal state, a lower expression of TLR4 following LPS treatment was observed in LR_{array} fibroblasts compared with HR_{array} fibroblasts.

Immune-related genes that were more expressed in the LR_{array} fibroblasts than the HR_{array} fibroblasts following LPS treatment included *CXCL12*, *IL1F6*, *IL1RN*, *PPARG*, and *SST*. All of these genes were also upregulated in LR_{array} fibroblasts under basal conditions.

Table 4. Differential expression (fold change ≥ 1.5 ; $P \leq 0.01$) of innate immune-related genes between low responding (LR_{array}) and high responding (HR_{array}) fibroblast cultures following exposure to LPS (100 ng/mL; 8 h)¹

Probe ID ²	Gene symbol	Fold change
Downregulated		
Bt.2408.1.S1_s_at	<i>CCL2</i>	-1.5
Bt.24326.1.S1_at	<i>CCL11</i>	-2.0
Bt.26655.1.S1_at	<i>CCL19</i>	-1.6
Bt.9560.1.S1_at	<i>CCL20</i>	-4.2
Bt.13130.1.S1_at	<i>CD40</i>	-1.8
Bt.50.1.S1_at	<i>CD8A</i>	-1.7
Bt.18368.1.S1_at	<i>CXCL11</i>	-1.5
Bt.7671.1.S1_at	<i>IFITM3</i>	-1.5
Bt.5508.1.S1_at	<i>IFNAR2</i>	-1.7
Bt.3686.1.S1_at	<i>IL6</i>	-2.5
Bt.155.1.S1_at	<i>IL8</i>	-3.6
Bt.4675.1.S1_a_at	<i>MX1</i>	-2.2
Bt.20891.1.S1_at	<i>OAS2</i>	-2.0
Bt.12148.1.S1_at	<i>PPARGC1A</i>	-1.6
Bt.357.1.S1_at	<i>S100A12</i>	-1.5
Bt.5970.1.S1_a_at	<i>S100A2</i>	-2.0
Bt.278.1.S1_at	<i>SAA3</i>	-5.3
Bt.8945.1.S1_at	<i>TLR2</i>	-1.9
Bt.12298.1.S1_at	<i>TLR3</i>	-1.5
Bt.9030.1.S1_at	<i>TLR4</i>	-2.2
Bt.26969.1.A1_at	<i>TNFAIP8L3</i>	-1.5
Bt.12694.1.S1_at	<i>TNFRSF21</i>	-2.3
Upregulated		
Bt.5392.1.S1_at	<i>CXCL12</i>	1.9
Bt.7425.1.S1_at	<i>IL1F6</i>	3.0
Bt.15910.1.S1_at	<i>IL1RN</i>	2.9
Bt.4199.1.S1_at	<i>PPARG</i>	1.9
Bt.456.1.S1_at	<i>SST</i>	4.3

¹Data were obtained by microarray analysis and represent mean fold change of 3 replicated experiments.

²Genes downregulated or upregulated in low responder relative to high responder.

Table 5. Messenger RNA expression profiles of *IL-8*, *MX1*, *CXCL12*, *TLR4*, and *SST* genes in an additional set of 4 low responding (LR) and 4 high responding (HR) fibroblast cultures; RNA was collected from cells that were exposed to either medium alone or LPS (100 ng/mL) for 8 h¹

Gene	Control		LPS		P-value		
	LR	HR	LR	HR	Treatment (LPS)	Group	Interaction
<i>IL-8</i>	20.7 ± 0.5	20.9 ± 0.3	9.3 ± 0.2	6.2 ± 0.1	0.0001	0.001	0.0003
<i>MX1</i>	19.6 ± 0.9	19.0 ± 1.2	13.1 ± 0.3	9.2 ± 0.1	0.0001	0.014	0.048
<i>CXCL12</i>	13.1 ± 0.9	14 ± 0.8	11.9 ± 0.4	12.1 ± 0.5	0.046	0.484	0.647
<i>TLR4</i>	12.0 ± 0.5	11.6 ± 0.1	11.3 ± 0.2	11.1 ± 0.5	0.118	0.412	0.916
<i>SST</i>	3.8 ± 0.6	5.2 ± 0.5	4.7 ± 0.8	5.5 ± 0.5	0.345	0.078	0.624

¹Gene expression changes are expressed as delta cycle threshold (Ct; mean ± SE).

Validation of Microarray Results by QPCR

The QPCR analysis of the same RNA samples used for the microarray confirmed that basal expression of *TLR4* was reduced in the LR_{array} fibroblasts compared with the HR_{array} fibroblasts (Figure 3C). However, the more sensitive QPCR analysis revealed that the magnitude of the differential *TLR4* expression (33-fold) was much greater than that found by microarray analysis (1.8-fold). This difference in *TLR4* expression between the cultures further increased to 47-fold following LPS stimulation. Expression of *IL-8* was very low (Δ Ct >36) in control conditions in both cultures. After LPS treatment, we found a marked induction of *IL-8* in both cultures, with expression in the HR_{array} being 14-fold greater than that in the LR_{array}. Similar results were obtained for *IL-6* expression with an 8-fold between-culture differential expression following LPS treatment.

The LR and HR fibroblast cultures were also treated with IL-1 β (10 ng/mL) for 8 h. Analysis by QPCR revealed a significant difference (2-fold) in *IL-8* mRNA between IL-1 β -treated LR_{array} and HR_{array} fibroblast cells (Figure 3A). Expression of *IL-6* differed between the IL-1 β -stimulated cultures (Figure 3B), whereas treatment with IL-1 β had little effect on the differential expression of *TLR4* (Figure 3C) observed in control conditions between the LR_{array} and HR_{array} fibroblasts.

The expression pattern of selected genes (*IL-8*, *MX1*, *CXCL12*, *TLR4*, *SST*) in a new set of 4 LR and 4 HR fibroblast cultures from our collection was also determined (Table 5). The QPCR analysis revealed that expression of *IL-8* and *MX1* was significantly affected by the LPS treatment and by the low versus high grouping. In both instances, we found a significant interaction that was likely due to the group effects being much more apparent in the LPS-stimulated state. The LPS

Figure 3. Expression of *IL-8* (A), *IL-6* (B), and toll-like receptor 4 (*TLR4*; C) mRNA in the low-responding (LR_{array}) and high-responding (HR_{array}) fibroblast cultures that were exposed to medium alone, LPS (100 ng/mL), or IL-1 β (10 ng/mL) for 8 h. Results are from 3 replicates of cryopreserved cells/cow thawed on separate days (mean ± SE). **P* < 0.05 LR versus HR.

treatment also caused a significant increase in *CXCL12* expression in both LR and HR groups. Expression of *TLR4* and *SST* genes was not different between the LR and HR groups and was not affected by LPS treatment. Of note is that the expression of *TLR4* in this group of 8 fibroblast cultures was similar to that of the HR_{array} fibroblast culture and much greater than that of the LR_{array} fibroblasts (Figure 3C).

Partial DNA Sequencing of the *TLR4* Gene

No sequence differences were found in the proximal promoter or coding regions of the *TLR4* gene between the LR_{array} and HR_{array} fibroblasts.

DISCUSSION

Selection of cows with enhanced disease resistance is an evolving strategy to control economically important diseases, including mastitis (Heringstad et al., 2007). However, direct selection for disease resistance is difficult because of confounding environmental effects that limit the ability to assign an accurate phenotype. Some progress is being made with MAS (Rupp and Boichard, 2003; Kuhn et al., 2008), and improvements in this strategy may result from locating polymorphisms in key innate immune response genes to further improve the accuracy of selection. Many studies have reported associations of specific polymorphisms in such genes with the incidence of clinical mastitis or average SCC (Sharma et al., 2006; Sugimoto et al., 2006; Galvão et al., 2011).

The low heritability of mastitis-related traits indicates the major role that environmental influences have on mastitis incidence, which negatively affects the ability to detect a mastitis-resistant phenotype. A more accurate assignment of a mastitis-resistant phenotype resulting from well-controlled challenge studies would aid in the search for causative genetic polymorphisms. However, the ability to contain environmental influences during bacterial challenge studies with lactating cows is limited by factors such as the animal's disease history and immunological status at the time of challenge, and the availability of a housing facility that would enable large numbers of repeatable challenges. Another approach to facilitate the detection of functional genetic differences between animals is to perform in vitro challenge studies under well-defined conditions on a model cell type. In our previous studies, we observed substantial between-cow variation in the fibroblast response to LPS, using production of IL-8 as a marker of the innate immune response, and found that this variation was related to between-cow variation in the response to an experimental mastitis challenge (Kandasamy et al., 2011) or

intravenous infusion of LPS (Green et al., 2011). In a related strategy, mammary epithelial cells have been used to investigate molecular causes for differences between cows selected based on a QTL affecting SCS on BTA18 (Griesbeck-Zilch et al., 2009). In this strategy, epithelial cells were obtained from mammary tissue from 6 animals in early lactation that had inherited a paternal chromosome region associated with decreasing SCS (MAS-Q) and compared with cells obtained from 5 animals that had inherited the chromosomal region associated with increasing SCS (MAS-q). Following in vitro stimulation for 6 or 24 h with heat-inactivated *E. coli* or *Staphylococcus aureus*, a markedly higher expression of innate immune response genes, such as *IL-8*, *IL1 β* , and *TNF- α* , was observed in the cells from the MAS-Q animals. Thus, enhanced production of innate response genes is the presumptive cause for the effects of the QTL on SCS, but the mechanisms leading to the differential expression remain to be defined. In the current study, we used genome-wide microarray analysis in an attempt to locate a cause for the differential ability of selected low- and high-responding fibroblast cultures to produce IL-8 in response to LPS.

A major goal of the current study was to analyze the potential cause of the attenuated response of fibroblasts from a particular cow (LR_{array}) to LPS. The fibroblasts from this cow consistently showed the lowest production of IL-8 and IL-6 in response to LPS stimulation out of our 88-cow collection. The coordinate attenuation of IL-8 and IL-6 production suggests reduced signal transduction via the TLR4 pathway. This lowered responsiveness was further examined using IL-1 β as the stimulatory ligand in an attempt to narrow the molecular possibilities for the attenuated response to LPS. Although IL-1 β induces its response through the IL-1 receptor, and LPS acts through the TLR4 receptor, both receptors contain a TLR/IL-1 receptor domain and signal through the MyD88-dependent signaling pathway (Arend et al., 2008). However, IL-1 β does not require the TLR4 accessory proteins (CD14, lipopolysaccharide-binding protein, MD-2) and does not appear to signal through the TLR4-TRIF dependent pathway (von Bernuth et al., 2008). We observed that, as with LPS stimulation, the LR_{array} fibroblasts produced less IL-8 than the HR_{array} fibroblasts when challenged with IL-1 β . However, in contrast to LPS, the LR_{array} and HR_{array} fibroblasts produced similar amounts of IL-6 in response to IL-1 β . The similar IL-1 β -induced production of IL-6 between the cultures is not easily explained given the disparate IL-1 β -induced production of IL-8.

Results of the microarray analysis revealed a significant reduction in *TLR4* gene expression in LR_{array} fibroblasts compared with that of HR_{array} fibroblasts, in both the basal state and after LPS treatment. The

more sensitive QPCR assay indicated that *TLR4* expression in the LR_{array} fibroblasts was around 40-fold less than in either the HR_{array} fibroblast culture or the other 8 fibroblast cultures examined in this study. This reduction in *TLR4* expression might have contributed to the attenuated response of the LR_{array} fibroblasts to LPS. However, the reason for the decreased *TLR4* receptor expression is yet to be determined. The proximal promoter region of the *TLR4* gene has several transcription factor binding sites, including AP-1, c-Ets-1, AP-4, ADR1, MZF1, Oct-1, and CRE-binding protein (Sharma et al., 2006). Any changes in these sites may cause altered DNA binding capacity of the transcription factors and thus lead to altered *TLR4* expression. However, DNA sequence analysis of the proximal 1,301 bp of the promoter region of the *TLR4* gene revealed no differences between the LR_{array} and HR_{array} genes. More extensive sequencing may have been informative because it has been reported that human *TLR4* expression is affected by combinations of 8 SNP contained between -4,038 and -1,607 bp relative to the translation start site (Ragnarsdóttir et al., 2010). Unfortunately, genomic analysis of differential gene expression in the current study did not reveal obvious candidates that could explain the differing expression of the *TLR4* gene between the LR_{array} and HR_{array} fibroblasts.

In contrast to a general reduction in chemokine expression profiles, a higher level of *CXCL12* expression was observed in LR_{array} fibroblasts compared with HR_{array} fibroblasts in the presence or absence of LPS. It is known that *CXCL12* is constitutively expressed in human dermal fibroblasts (Avniel et al., 2006) and gingival fibroblasts (Hosokawa et al., 2005) and it is involved in B-cell lymphopoiesis (Nagasawa et al., 1996) and homing of mononuclear cells (Bleul et al., 1996). The underlying cause and the functional consequences of the differential *CXCL12* expression between LR_{array} and HR_{array} fibroblast cultures remain to be determined. Similar to *CXCL12*, basal expression of *SST* was significantly higher in LR_{array} fibroblasts compared with HR_{array} fibroblasts. Expression of *SST* in B and T lymphocytes has also been reported (Lichtenauer-Kaligis et al., 2004), but its functional significance in immune function is not known.

A second goal of the current study was to evaluate the robustness of the response of LPS-stimulated dermal fibroblasts using a microarray-based approach. Numerous immune-related genes were induced following the 8-h exposure to LPS, suggesting the usefulness of these cells to evaluate innate immune response pathways. Furthermore, the expression profile of the fibroblast response to 8-h LPS treatment was highly reflective of that of primary cultures of bovine mammary epithelial cells exposed to heat-killed *E. coli* for 6 h and examined

with the same microarray chip (Günther et al., 2011). Some data from Günther et al. (2011) are included in Table 2 for illustrative purposes. Of note is the finding that all genes upregulated by more than 5-fold in LPS-treated HR_{array} fibroblasts were also upregulated by more than 5-fold in the *E. coli*-stimulated epithelial cells. Fibroblast microarray analysis did not detect induction of either *TNF- α* or *IL-1 β* after 8 h of LPS treatment. However, it is possible that transient induction of these key mediators of inflammation occurred before our observation time point. A more acute, transient upregulation of *TNF- α* and *IL-1 β* was observed after 1 and 3 h of *E. coli* treatment of mammary epithelial cells (Günther et al., 2011).

Thus, even though fibroblasts are not considered sentinel cells in the response to mammary infection, they do seem to model the acute infection process to gram-negative bacteria. The fibroblasts may also model response to gram-positive bacteria because they produce similar quantities of IL-8 in response to LPS or the synthetic diacylated lipopeptide Pam2CSK4 that activates TLR2-dependent signaling in an analogous fashion to gram-positive bacteria (Buwitt-Beckmann et al., 2005; Farhat et al., 2010; Kandasamy et al., 2011).

CONCLUSIONS

Dermal fibroblasts have a robust innate immune response to LPS. An approximately 5-fold difference between 2 fibroblast cultures in the production of IL-8 in response to LPS may have been caused by a greater than 30-fold difference in the expression of *TLR4*. Future studies will explore this possibility through a complementation approach with transfection of a functional *TLR4* expression plasmid.

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