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Global Transcriptomic Profiling of Bovine Endometrial Immune Response In Vitro. I. Effect of Lipopolysaccharide on Innate Immunity[1]

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ABSTRACT

The dysregulation of endometrial immune response to bacterial lipopolysaccharide (LPS) has been implicated in uterine disease and infertility in the postpartum dairy cow, although the mechanisms are not clear. Here, we investigated whole-transcriptomic gene expression in primary cultures of mixed bovine epithelial and stromal endometrial cells. Cultures were exposed to LPS for 6 h, and cellular response was measured by bovine microarray. Approximately 30% of the 1006 genes altered by LPS were classified as being involved in immune response. Cytokines and chemokines (*IL1A*, *CX3CL1*, *CXCL2*, and *CCL5*), interferon (IFN)-stimulated genes (*RSAD2*, *MX2*, *OAS1*, *ISG15*, and *BST2*), and the acute phase molecule *SAA3* were the most up-regulated genes. Ingenuity Pathway Analysis identified up-regulation of many inflammatory cytokines and chemokines, which function to attract immune cells to the endometrium, together with vascular adhesion molecules and matrix metalloproteinases, which can facilitate immune cell migration from the tissue toward the uterine lumen. Increased expression of many IFN-signaling genes, immunoproteasomes, guanylate-binding proteins, and genes involved in the intracellular recognition of pathogens suggests important roles for these molecules in the innate defense against bacterial infections. Our findings confirmed the important role of endometrial cells in uterine innate immunity, whereas the global approach used identified several novel immune response pathways triggered by LPS in the endometrium. Additionally, many genes involved in endometrial response to the conceptus in early pregnancy were also altered by LPS, suggesting one mechanism whereby an ongoing response to infection may interfere with the establishment of pregnancy.

bovine, endometrium, gene expression, innate immunity, uterine disease

INTRODUCTION

Bacterial contamination of the uterus is observed in almost all dairy cows within the first two weeks after calving [1, 2]. *Escherichia coli* and *Trueperella pyogenes* were most prevalent in cows with uterine disease, followed by anaerobic bacteria, such as *Fusobacterium*, *Prevotella*, and *Bacteroides* species [3, 4]. Infection with *E. coli* predominated in the first few days after calving and appeared to promote subsequent infection with other bacteria [5]. The endometrium constitutes the first line of defense against such pathogens by mounting an innate immune response to eliminate the bacterial contaminants during uterine involution [6]. Following placental separation, the epithelium overlying the caruncles is eroded, exposing stromal as well as epithelial cells directly to the contents of the uterine lumen [7]. Similar to specialized immune cells, endometrial epithelial and stromal cells also possess pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), which detect pathogen-associated molecules like bacterial lipopolysaccharide (LPS) [8]. Activation of the TLRs initiates an inflammatory response typically characterized by the production of pro-inflammatory cytokines, type I interferons (IFNs), chemokines, and antimicrobial proteins to clear the infection [9]. Uterine immune function may, however, become compromised after calving, resulting in the development of inflammatory disease: clinical and subclinical endometritis are present in up to 20% and 30% of cows, respectively [10, 11]. Whereas a robust innate immune response is desirable to clear invading bacteria, the consequent inflammatory milieu may impair reproductive processes, predisposing affected cows to infertility and resulting in significant economic losses [10, 12].

The mechanisms of bovine uterine infection and disease have been studied using bacteria or bacterial ligands based on in vivo [13] and ex vivo models [14]. LPS is the main glycolipid component of the outer membrane of gram-negative bacteria that is capable of reproducing many of the features of an authentic gram-negative bacterial infection [15]. Bovine endometrial epithelial and stromal cells express the TLR4/CD14/MD2 receptor complex required for recognition of LPS [14] and respond to LPS by increased expression of inflammatory mediators, such as cytokines, chemokines, antimicrobial peptides (AMPs), and prostaglandins (PGs) [8, 16, 17].

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Studies to date of the endometrial immune response have largely been based on a candidate gene approach. A better understanding is needed of all the internal signaling pathways involved, however, so that the influences of other factors that may affect the ability of the uterus to clear an infection successfully can be established, such as metabolic status and concurrent viral disease. In the present study, we utilized an in vitro model involving whole-transcriptomic profiling to examine the influence of bacterial LPS on bovine endometrial immune function. This provides a deeper understanding of uterine immune functions that can influence further investigations and therapeutic strategies. In a companion paper [18], the additional effect of an ongoing viral infection with bovine viral diarrhea virus on the ability of the endometrium to respond to LPS is described.

MATERIALS AND METHODS

Bovine Endometrial Cell Culture

Fresh and apparently healthy bovine reproductive tracts from cows in the early luteal phase of the estrous cycle were obtained from the local abattoir. Primary mixed endometrial epithelial and stromal cell cultures were prepared based on methods described previously [17]. These contained both surface and glandular epithelium. Briefly, endometrial tissue was stripped off and chopped into 1-mm^3 pieces, followed by digestion for 90 min at 37°C in media containing 100 mg of bovine serum albumin (Sigma), 50 mg of trypsin III (Worthington), and 50 mg of collagenase A (Roche) per 100 ml of Hanks Balanced Salt Solution (HBSS; Sigma). Digested tissue was filtered through 100-μm sterile cell strainers (BD Falcon) and then washed by resuspending in HBSS containing 10% fetal bovine serum (FBS; PAA Laboratories) and 3 μg/ml of trypsin inhibitor (Sigma). After centrifugation at $100 \times g$ and 10°C for 10 min, the cell sediment was resuspended and the wash step repeated twice. Cell sediments were pooled together for each cow sample, and cell count/cell viability was evaluated by trypan blue exclusion (Sigma). The isolated mixed endometrial epithelial and stromal cells were resuspended in growth medium (GM), which comprised MEM (PAA Laboratories) containing 10% FBS and 1% antibiotic solution (100 IU/ml of penicillin and 100 μg/ml of streptomycin; Sigma), and then allocated at 5×10^5 cells/well to sterile 24-well plates (Nunc).

Validation of Endometrial Cell Culture Model

Six separate batches of cells, each obtained from an individual cow, were used in the main experiment. These cultures were established on four different days, but the same batches of reagents were used for all cultures to minimize variations in culture conditions. Endometrial cell cultures were validated using specific immunocytochemical staining as previously described [17]. Epithelial cells stained positive for cytokeratin, stromal cells for vimentin, and immune cells (e.g., macrophages and granulocytes) for CD172. The primary monoclonal mouse antibodies used were anti-human cytokeratin (clone AE1/AE3; Dako), anti-vimentin (clone V9; Dako), or anti-CD172a (DH59B; Monoclonal Antibody Center VM&P, Washington State University). The relative proportions of each cell type after 8 days of culture were evaluated using image-analysis software (ImageJ version 1.44; National Institute of Mental Health/National Institutes of Health). The stromal cells comprised 9.5% of the population present before LPS challenge (range, 7%–12% for cultures derived from $n = 6$ cows). Negligible contamination with immune cells occurred (<0.001%). No endothelial cells were present based on morphological appearance, although staining was not performed specifically for these cells.

Experimental Design

Cells were cultured in GM at 37°C and 5% CO_2 in a humidified incubator for 4 days with the medium changed after 2 days. They were then divided into the two treatment groups (control [CONT] and CONT+LPS) with six wells per treatment. The wells were replaced with 1 ml of maintenance medium (MM; the same composition as GM except using 5% FBS). The plates were incubated at 37°C and 5% CO_2 for a further 4 days, with the MM changed after 2 days. The culture media were discarded and the designated cultures treated with 100 ng/ml of ultrapure LPS from *E. coli* 0111:B4 strain (Invivogen) in warm MM for 6 h. The culture media were removed before RNA isolation.

Endometrial Cell Viability Assay

Evaluation of bovine endometrial cell viability following exposure to LPS was performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) following the supplied protocol. Briefly, approximately 50 000 mixed bovine endometrial epithelial and stromal cells were allocated per well in a 96-well plate and cultured at 37°C and 5% CO_2 for 4 days in GM and another 4 days in MM. This was followed by exposure to 100 ng/ml of LPS for 6 h in the specified wells before the cell viability assay was performed.

Isolation, Quantitative, and Qualitative Analysis of RNA

Isolation of total RNA from the endometrial cell cultures was performed using the RNeasy Mini Spin Column method (Qiagen) following the protocol supplied. Isolated RNA was quantified using a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies), showing a concentration of 230 ng/μl or greater for all RNA samples and ratios of absorbance at 260 and 280 nm within the range of 1.9 to 2.0. RNA integrity was further assessed using an Agilent 2200 TapeStation (Agilent Technologies). The RNA integrity numbers for all samples were nine or greater.

Microarray Hybridization and Analysis

Whole-transcriptomic gene expression of each sample was measured by Bovine Gene 1.1 ST 24-Array containing probes for 23 000 transcripts (Affymetrix). Microarray hybridization and scanning were performed by Edinburgh Genomics (The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, U.K.) using a one-round amplification (one-cycle target labeling) protocol with the GeneTitan instrument (Affymetrix) following their protocols (http://genomics.ed.ac.uk/resources/protocols). All arrays passed the GeneChip data quality assessment in the Affymetrix Expression Console.

Processing, normalization, and further analysis of the microarray data were performed using RMA16 built in GeneSpring GX software (version 12.5; Agilent Technologies) using the annotation files provided, which had annotations for more than 95% of the probes. Differences in gene expression between the treatment groups of bovine endometrial cells that met the cut-off ($-1.2 \leq$ fold-change ≥ 1.2) were compared using repeated-measures ANOVA and paired *t*-test with *P*-values adjusted for multiple comparisons using the Benjamini-Hochberg false-discovery rate method. The differentially expressed genes (DEGs) were considered to be significant based on an adjusted *P*-value of less than 0.05. A hierarchical cluster analysis of the DEGs was performed showing a heat map of the overall gene expression pattern by treatment group (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org).

Ingenuity Pathway Analysis

Array data were analyzed with Ingenuity Pathway Analysis (IPA; Qiagen). The selected DEGs were uploaded onto IPA to map onto the genomic database (annotation) and to analyze the pathways, biological processes, networks and upstream regulators, and so on using gene symbols as IDs and fold-changes and adjusted *P*-values as observations. Both direct and indirect interactions between genes were considered.

Real-Time RT-PCR Validation of Microarray Data

Following the microarray analysis, a total of 15 DEGs and two endogenous reference genes were selected for validation of array data by quantitative RT-PCR (qRT-PCR) (Table 1) using the methods established in our laboratory [19]. Specific oligonucleotide primers and target amplicons were designed for the selected genes using Primer3 (version 4.0) [20] with reference sequence templates derived from the GenBank database (National Center for Biotechnology Information). Primer specificity to target gene was evaluated using the web-based Primer-BLAST tool. Primers were first checked for optimum quality using OligoAnalyzer (version 3.1; Integrated DNA Technologies, Inc.) before they were synthesized by the manufacturer (Eurofins MWG Operon). The specificity of each primer pair was confirmed by PCR-gel electrophoresis. All qRT-PCRs were initially optimized and then performed using the CFX96 Real-Time Thermal Cycler (Bio-Rad) with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems). For each sample, 1 μg of total RNA was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega). All samples for each gene were run in duplicate in the same qRT-PCR assay using 5 μl of cDNA sample (equivalent to 50 ng of reverse-transcribed RNA) together with the no-template control and 10 known concentrations of the standard, ranging from 1×10^1 to 1×10^{-8} ng/ml. The mRNA expression values were calculated from the standard curves.

TABLE 1. Genes selected for qRT-PCR validation of microarray data.

Gene symbol	Primer direction	Primer sequence 5′→3′	Product size (bp)	GenBank accession no.
BST2	Forward	TGATCTACTTCGCTGTCATTGC	202	XM_002688577.3
	Reverse	TGGGTCTGTTCCTTCTTCAGAG		
AMIGO2	Forward	ACACTAGGCACTTCCATCAGGT	163	NM_001205786.1
	Reverse	GTATTTGCCCTACCAGTCTTGC		
C3	Forward	TGCAGGATTTCTTTATCGACCT	194	NM_001040469.2
	Reverse	GGCTGGGATTGTTATAGTCTGC		
CCL5	Forward	CTGCTGCTTTGCCTATATCTCC	159	NM_175827.2
	Reverse	ATGTACTCTCGCACCCACTTCT		
CX3CL1	Forward	CTGTCCTCTGCCATTTGGTT	198	XM_595523.6
	Reverse	CCTTTGGGTCAGCACAGAAT		
GBP5	Forward	CCATTGTCTCTTCATTCAGCAG	228	NM_001075746.1
	Reverse	AGACTTTCCATCAGCCTTTGTG		
IL1A	Forward	TGGATACCTCGGAAACCTCTAA	199	NM_174092.1
	Reverse	CTCTGGAAGCTGTAATGTGCTG		
ISG15	Forward	AGAAGATCAATGTGCCTGCTTT	161	NM_174366.1
	Reverse	CTTGTCGTTCCTCACCAGGAT		
PTGES	Forward	AAGTGAGGCTGCGGAAGAAG	162	NM_174443.2
	Reverse	AGTAGACAAAGCCCAGGAACAG		
MX2	Forward	AAAGTACATCCAGAGGCAGGAG	214	NM_173941.2
	Reverse	GCCCTTCTTGAGATGATAGGTG		
RND1	Forward	CAGATGTAAGCTCGTTCTGGTG	152	NM_001046016.1
	Reverse	GCTCCACTCTCTGTTCCTCTGT		
RSAD2	Forward	TATGCGCTTCCTGAACTGTAGA	150	NM_001045941.1
	Reverse	AGGTCTGCTTTGCTCCATACAT		
STAT1	Forward	CTCATTTGTGGTGGAAAGACAG	231	NM_001077900.1
	Reverse	ATGTTCATCACCTTCGTGTGAG		
TRIM56	Forward	CCGTGGATAAGAAAGGCTACAT	173	NM_001206574.1
	Reverse	GTTACTGAGGGACACGACCAG		
VCAM1	Forward	CCATTTGAAAGGCTGGAGATAG	207	NM_174484.1
	Reverse	TTTCCTTACTTTGGGTGGAGAA		
RN18S1	Forward	CGGCGACGACCCATTCGAAC	99	NR_036642.1
	Reverse	GAATCGAACCCTGATTCCCCGTC		
ACTB	Forward	GAAATCGTCCGTGACATCAA	182	NM_173979.3
	Reverse	AGGAAGGAAGGCTGGAAGAG		

Normalization and Analysis of qRT-PCR Gene Expression Data

The expression of the endogenous reference genes under the experimental conditions was evaluated. Statistical analysis using linear mixed-effects model built in SPSS for Windows (version 20; IBM) showed no significant differences of the mRNA expression of the two reference genes, *RN18S1* and *ACTB,* in bovine endometrial cells after exposure to LPS when compared to the control (data not shown). The mRNA expression values of the 15 selected genes were therefore normalized to those of *RN18S1* and *ACTB* by dividing the sample value for each gene with the corresponding sample normalization factor derived from geNorm (version 3.4) [21].

Data from qRT-PCR were evaluated using ANOVA with randomized block design via a linear mixed-effect model built in the SPSS software package. For each gene analysis, the mean qRT-PCR expression values were derived from each duplicate sample, and then the normalized data were compared between the CONT and CONT+LPS treatment groups. Treatments were taken as a fixed effect and cows as a random effect. Results were considered to be significant when $P < 0.05$.

RESULTS

Exposure of the cultured endometrial cells to 100 ng/ml of LPS for 6 h did not significantly alter the number of viable cells. The absorbance values at 490 mm in the cell viability assay were 2.0 ± 0.05 and 2.1 ± 0.05 in the CONT and CONT+LPS treatment groups, respectively (mean ± SD of n = 4 samples/group). The number of detectable probes (22 024) and their mean expression values (6.72; range, 4.2–13.4) were identical between CONT and CONT+LPS groups, confirming that the LPS treatment did not bias the results by altering the number of expressed genes. The array data are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71696.

Effects of LPS on Bovine Endometrial Response

Exposure to bacterial LPS for 6 h significantly altered the expression of 1006 genes compared with the control. Of these, 675 genes (67%) were up-regulated, whereas 331 genes (33%) were down-regulated (see Supplemental Table S1 and Supplemental Fig. S1). From the list of all DEGs, 919 genes were mapped and subsequently analyzed with IPA. The majority of the most up-regulated genes (12/16 with ≥4-fold increase) were associated with immune response, with five identified as being inducible by IFN (*RSAD2*, *MX2*, *OAS1Y*, *ISG15,* and *BST2*), whereas the eight most down-regulated genes (≥1.5-fold decrease) had a wide range of different functions (Table 2).

Diseases and Functions

The DEGs induced by LPS in bovine endometrial cells were significantly ($P < 0.05$) associated with 62 categories of diseases and disorders, molecular and cellular functions, and physiological system development and functions. The top 10 most significant disease processes and biological functions identified (all $P < 0.001$) were inflammatory response, cellular growth and proliferation, hematological system development and function, cell death and survival, cellular function and maintenance, cellular movement, immune cell trafficking, cell-to-cell signaling and interaction, infectious disease, and inflammatory disease (Table 3).

TABLE 2. Genes for which expression was most altered by LPS treatment in bovine endometrial cells.

Gene symbol	FC in expression[a]	Function
Top up-regulated (≥4-fold increase)		
RSAD2	9.7**	Interferon inducible, immune response
SAA3	8.5***	Acute phase response, inflammatory response
CX3CL1	7.0***	Chemokine activity, immune response
MX2	7.0*	Interferon inducible, GTPase activity
IL1A	6.9**	Cytokine factor, immune response
CXCL2	6.8**	Chemokine activity, immune response
CCL5	6.2**	Chemokine activity, immune response
CXCL3	6.0**	Chemokine activity, immune response
HS3ST1	5.6***	Biosynthesis of heparan sulfate
BCL2A1	5.1**	Anti-apoptotic inflammatory regulator
NPPC	5.0**	Natriuretic peptide hormone
OAS1Y	4.9*	Interferon inducible, immune response
ISG15	4.6*	Interferon inducible, protein modification
BST2	4.1**	Interferon inducible, signal transduction
USP18	4.1*	Protein modification, ISG15-specific protease
RND1	4.0***	GTPase activity
Top down-regulated (≥1.5-fold decrease)		
HECW1	1.6**	Protein modification
NPPB	1.5*	Natriuresis, cardiovascular homeostasis
CYP2C87	1.5**	Cytochrome P450 enzyme activity
FGFR2	1.5***	Fibroblast growth factor mediator
MID1IP1	1.5**	Regulation of lipogenesis in liver
PTGER3	1.5**	Mediates the activity of prostaglandin E_2
PTPDC1	1.5**	Signal transduction
DGAT2	1.5**	Synthesis of intracellular triglycerides

[a] Microarray analysis showing the fold-change (FC) in gene expression based on Benjamini-Hochberg adjusted *P*-value: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, paired *t*-test.

Canonical Pathways Analysis

Using IPA, we identified 351 canonical pathways containing one or more genes for which expression was significantly altered by LPS treatment ($P < 0.05$) in bovine endometrial cells. The eight most significant (all $P < 0.001$) are shown in Table 3 and Supplemental Table S2. Exposure to LPS up-regulated genes involved in the pathways for granulocyte adhesion and diapedesis, activation of IFN regulatory factor (IRF) by cytosolic PRRs, interleukin (IL) 10 signaling, communication between innate and adaptive immune cells, IL6 signaling, role of cytokines in mediating communication between immune cells, nuclear factor kappa B (NF-κB) signaling, and agranulocyte adhesion and diapedesis (see Supplemental Table S2 for the list of genes in each pathway).

Interaction Network Analysis

Using IPA, we identified 25 network functions associated with the 919 mapped DEGs with differential expression induced by LPS. The top 12 networks, each containing 22 or more focus molecules, are listed in Table 3. Six of these networks related to immune function, which was deemed most relevant to the experiment, were selected for further evaluation.

Network 1 (score = 43) contained 31 DEGs involved in infectious disease, antimicrobial response, and inflammatory response (Fig. 1). LPS induced the activation of IFN regulatory transcription factor (IRF1), which in turn up-regulated the tumor necrosis factor (TNF)-ligand cytokine *TNFSF10* and the IFN-inducible genes *IFIT1* and *1SG15*. The activation of the protein ligase transcription factor *TRIM25* was directly involved with the up-regulation of the tripartite motif factors (*TRIM5*, *TRIM8*, *TRIM21,* and *TRIM47*), ubiquitin-related factors (*UBE2l6*, *RNF125,* and *RNF144*), and *ISG15*. An increased expression of components of the poly(ADP-ribose) polymerase (PARP) enzyme complex (*PARP8*, *PARP9,* and *PARP14*) also was found.

Network 4 (score = 32) contained 26 DEGs involved in cell death and survival, antigen presentation, and cell-to-cell signaling and interaction (Fig. 2). In this network, LPS induced an up-regulation of the I-kappa-B kinase complex and the TNF receptor-associated factor (*TRAF1*), which were associated with the activation of the complex between NF-κB and the transcription factor RelB and its regulatory molecule, *CYLD*. In turn, NF-κB activation had direct interactions with the increased expression of several TNF-associated apoptotic factors (*CFLAR*, *RIPK1-3*, and *BIRC3*) that modulate inflammatory signaling and immunity.

Network 5 (score = 29) contained 24 DEGs involved in cell-to-cell signaling and interaction, inflammatory response, and cellular function and maintenance (Fig. 3). LPS induced an up-regulation of members of the S100 family of proteins containing calcium-binding motifs (*S100A8*, *S100A9,* and *S100A12*), which have prominent roles in the regulation of inflammatory processes and immune response.

Network 7 (score = 27) contained 23 DEGs also involved in cell-to-cell signaling and interaction, cellular movement, and hematological system development and function (Supplemental Fig. S2). LPS induced an increase in the expression of *TLR2,* which indirectly triggered activation of the NF-κB-mitogen-activated protein kinase kinase kinase B (MAP3KB) complex. In addition, the increased expression of *TLR2*, *IL1RL1* transmembrane receptor, *CD200* membrane glycoprotein immunoglobulin, and *VDR* transcriptional regulatory factor all interacted with the down-regulation of IL18 and up-regulation of IL1A and a large number of chemokines (*IL8*, *CCL4*, *CCL20*, *CXCL2*, *CXCL3*, *CSCL10*, *CXCL11*, *CCL3*, *CCL4*, *CCL11*, and *CCL20*) involved in inflammatory and immune processes. *TLR4* expression did not, however, alter significantly following 6 h of LPS treatment.

Network 10 (score = 24) contained 22 DEGs involved in developmental disorder, hereditary disorder, and immunological disease (Supplemental Fig. S3). LPS induced an increase in IFN alpha (IFNA) signaling, resulting in up-regulation of

TABLE 3. IPA network analysis of genes significantly altered in bovine endometrial cells treated with LPS for 6 h showing the top 10 disease processes and biological functions, the top 8 canonical pathways, and the top 12 network functions.

Parameter	*P*-value	No. of genes	Ratio[a]	Score[b]	Focus molecules
Disease processes and biological functions					
Inflammatory response	<0.001	280			
Cellular growth and proliferation	<0.001	413			
Hematological system development and function	<0.001	295			
Cell death and survival	<0.001	388			
Cellular function and maintenance	<0.001	229			
Cellular movement	<0.001	278			
Immune cell trafficking	<0.001	190			
Cell-to-cell signaling and interaction	<0.001	227			
Infectious disease	<0.001	233			
Inflammatory disease	<0.001	217			
Canonical pathways					
Granulocyte adhesion and diapedesis	<0.001		38/182		
Activation of IRF by cytosolic PRRs	<0.001		20/73		
IL10 signaling	<0.001		24/78		
Communication between innate and adaptive immune cells	<0.001		21/112		
IL6 signaling	<0.001		30/124		
Role of cytokines in mediating communication between immune cells	<0.001		16/55		
NF-kB signaling	<0.001		33/181		
Agranulocyte adhesion and diapedesis	<0.001		35/192		
Network[c]					
1. Infectious disease, antimicrobial response, inflammatory response				43[d]	31
2. Gene expression, embryonic development, organismal development				43	31
3. Molecular transport, lipid metabolism, small molecule biochemistry				37	28
4. Cell death and survival, antigen presentation, cell-to-cell signaling and interaction				32[d]	26
5. Cell-to-cell signaling and interaction, inflammatory response, cellular function and maintenance				29[d]	24
6. Cell death and survival, drug metabolism, small molecule biochemistry				28	24
7. Cell-to-cell signaling and interaction, cellular movement, hematological system development and function				27[d]	23
8. Cellular function and maintenance, molecular transport, hereditary disorder				25	22
9. Cell death and survival, immunological disease, inflammatory disease				25	22
10. Developmental disorder, hereditary disorder, immunological disease				24[d]	22
11. Infectious disease, cellular function and maintenance, inflammatory disease				24[d]	22
12. Cell morphology, digestive system development and function, endocrine system development and function				12	24

[a] Number of genes in the list of DEGs that participate in the canonical pathway divided by the total number of genes known to be associated with the pathway in the Ingenuity Knowledge Base.
[b] Networks were scored based on the number of the network-eligible molecules that were present in the list of DEGs. A higher network score corresponds to a lower probability of finding the observed number of the DEGs in a given network by chance.
[c] A limit of 35 genes was set for each generated network.
[d] Networks associated with a known immunological function that were selected for further evaluation.

IFNAR2 (a type I IFN receptor) and several IFN-inducible genes (*IFIT3*, *IFI35*, *IFIHI*, *IFI27*, and *BST2*). The increase in IFNA signaling also had indirect interactions with the up-regulation of membrane-associated antigen transport factors (*TAP1* and *TAP2*) via the major histocompatibility complex (MHC) class I complex and the up-regulation of the immunoproteasome complex (*PSMB9*, *PSMB10*, and *PSMA6*).

Network 11 (score = 24) contained 22 DEGs involved in infectious disease, cellular function and maintenance, and inflammatory disease (Supplemental Fig. S4). This showed that the LPS-induced increase in *IFNAR2* also indirectly interacted with the up-regulation of IFN-regulatory transcription factors (*IRF7* and *IRF9*), STAT1/STAT2-ISGF3 transcription activator complex, and IFN-inducible and antiviral factors (*RSAD2* and *DHX58*).

Upstream Regulator Analysis

The upstream analysis function of IPA was used to predict the activation state of upstream molecules and transcription factors that may be causing the observed changes in expression of the DEGs induced by LPS in bovine endometrial cells (Fig. 4). The top upstream regulators with predicted activation state ($P < 0.001$) were cytokines (TNF, IL1B, and IL1A), transmembrane receptors (TLR3 and TLR4), receptor-adaptor proteins (TICAM1 and MYD88), transcription regulators (STAT3 and RELA), NF-κB complex, PGE_2, and INFA (Fig. 5).

Validation of Microarray Gene Expression Data by qRT-PCR

The results of the 15 DEGs selected for validation of array data by qRT-PCR are shown in Table 4. A comparison of the array and qRT-PCR data showed similar patterns of changes in gene expression between the CONT and CONT+LPS treatment groups.

DISCUSSION

For optimum financial returns, cows should conceive again within 3 mo of calving. The postpartum cow must first undergo a period of uterine involution during which contaminating pathogens are eliminated and the disrupted uterine tissue repaired. A uterine inflammatory response is thus a normal component of involution. However, an increase in the expression of inflammatory genes and gene products is

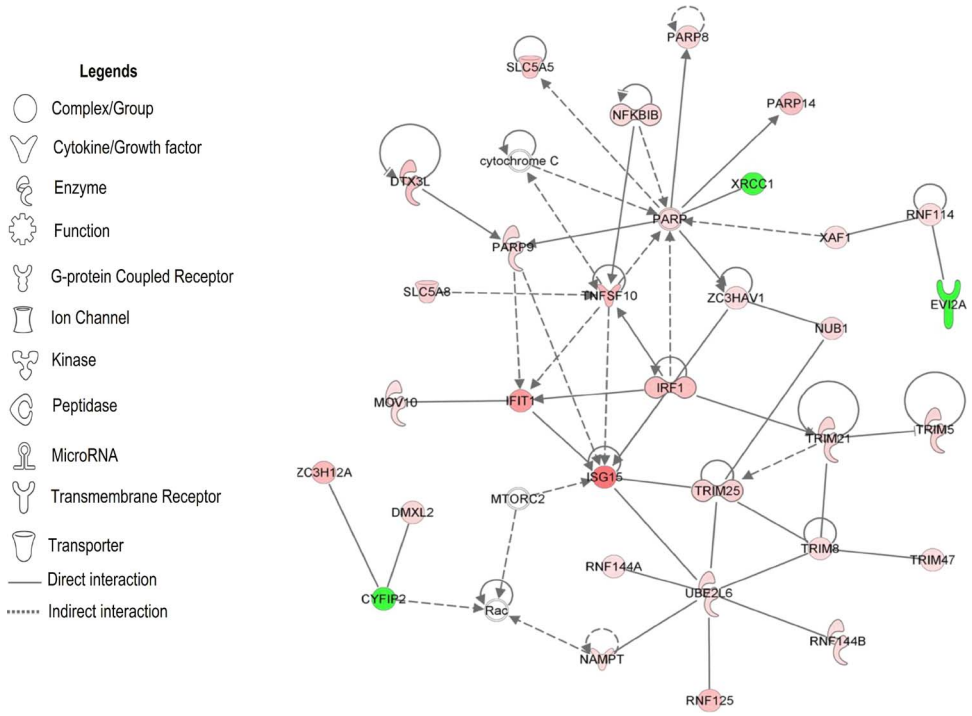

FIG. 1. IPA network 1. The genes significantly altered by LPS treatment in bovine endometrial cells were identified in networks involved in infectious disease, antimicrobial response, and inflammatory response (score = 43, with 31 focus molecules). The network describes the biological relationship between the DEGs as either a direct interaction (solid line) or an indirect interaction (dashed line). The intensity of the color indicates the level of up-regulation (red) or down-regulation (green) of the respective molecules.

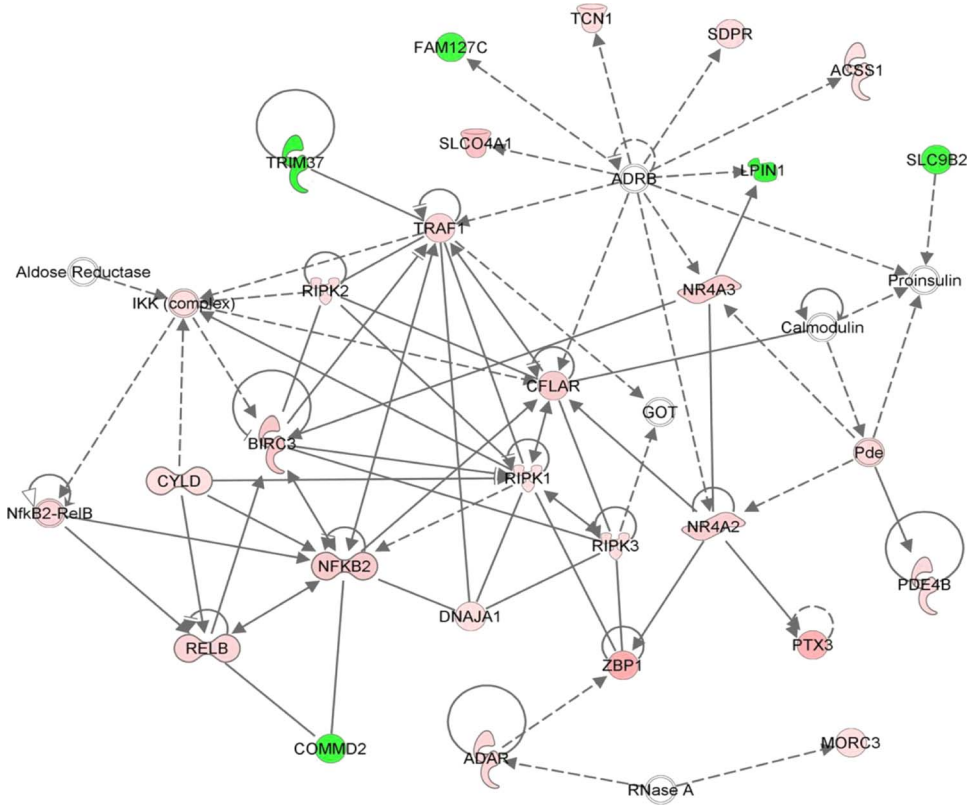

FIG. 2. IPA network 4. The genes altered by LPS treatment in bovine endometrial cells were identified in networks involved in cell death and survival, antigen presentation, and cell-to-cell signaling and interaction (score = 32, with 26 focus molecules). See Figure 1 for explanation of symbols.

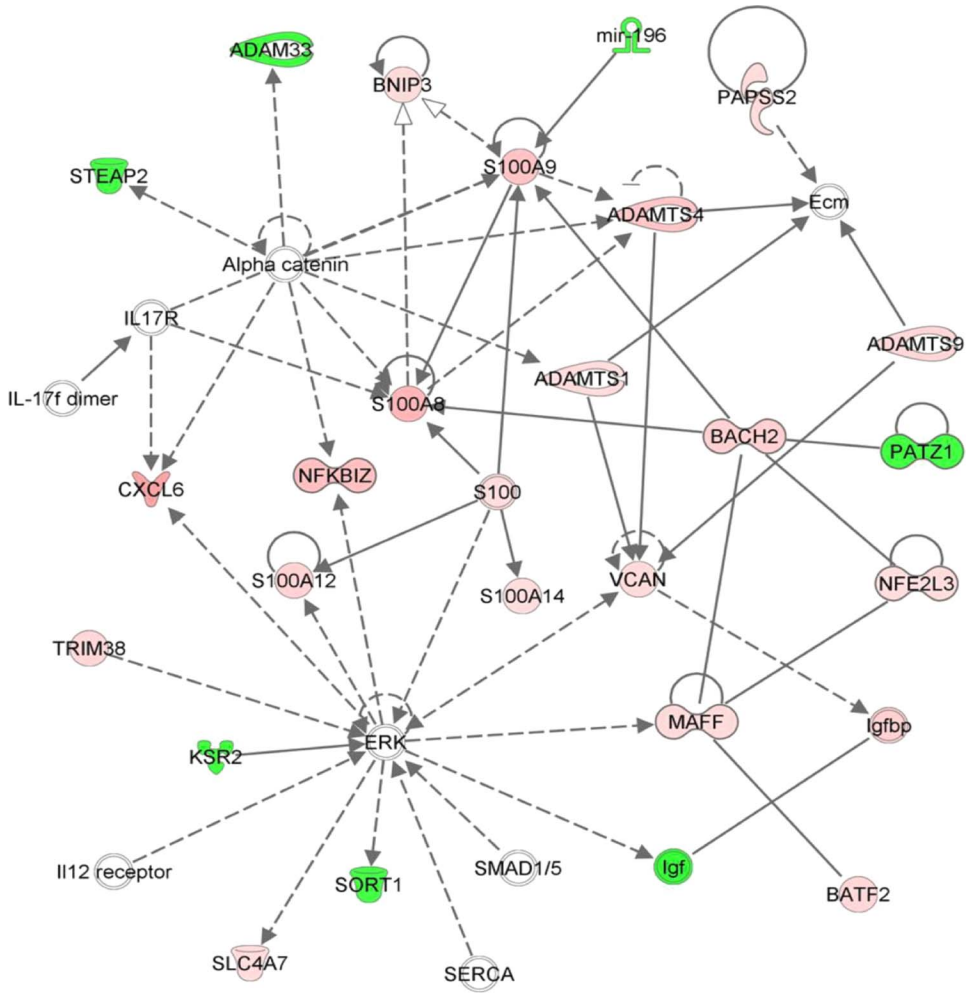

FIG. 3. IPA network 5. The genes altered by LPS treatment in bovine endometrial cells were identified in networks involved in cell-to-cell signaling and interaction, inflammatory response, and cellular function and maintenance (score = 29, with 24 focus molecules). See Figure 1 for explanation of symbols.

observed in cases of uterine disease, and a significant number of these cows fail to conceive [22, 23]. Pregnancy requires a delicate balance between proinflammatory and anti-inflammatory molecules to maintain maternal immune system integrity while preventing rejection of the embryo [24]. Therefore, TLR-mediated immune dysregulation in response to bacterial and viral ligands is capable of inducing adverse pregnancy outcomes [25].

The uteri of most cows are contaminated with bacteria and other pathogens after calving. Bacterial LPS is widely recognized to induce an innate immune response [15]. Here, we report whole-transcriptomic gene expression in bovine endometrial cell cultures exposed to bacterial LPS for 6 h. The results support those of previous studies based on a candidate gene approach [16, 17, 26] and extend these by identifying a number of other important immune response pathways that are triggered by LPS in the endometrium. Approximately 30% (280 genes) of the genes for which expression was altered by LPS in the present study are already known to be involved in inflammatory responses. The cultures contained very few immune cells (<0.001%), indicating that the responses were generated by the epithelial and stromal cells themselves, although it was not possible to attribute the responses between these two cell types. The caruncular tissue is disrupted after calving, allowing contaminant bacteria access to the submucosal tissue layers. Thus, stromal cells may have important roles in initiating an innate immune response in addition to the surface epithelia, and we included both cell types in our cultures, with the stromal cells comprising approximately 10% of the cell population at the time of testing with LPS. Stromal cells also secrete soluble growth factors that act on the epithelial cells to increase transepithelial resistance and barrier function [27], indicating that the interaction between both cells types has important physiologic roles.

Because of practical limitations imposed by microarray analysis, the response to LPS was only assessed at one time point. The time of 6 h was chosen based on previous time-course studies in endometrium. These showed increased expression of early response genes (e.g., *TNF*), which peaked by 1 h postexposure to LPS, followed by intermediate response genes (e.g., *IL1B*, *IL6,* and *IL8*), which peaked by 3–6 h, and late response genes (e.g., AMPs and *IFNB*), which peaked by 12–24 h; almost all the inflammatory gene changes had resolved to the baseline by 48 h after stimulation [17, 26]. The LPS dose used (100 ng/ml) was consistent with the range of endotoxin levels observed in the lochia of cows with uterine infection [5]. At this dose, LPS did not compromise cellular integrity, as observed from the cell viability assay.

Lipopolysaccharide is recognized by TLR4, which forms a complex with myeloid differentiation factor 2 (MD2) on the cell surface with the co-operation of additional proteins, such as LPS-binding protein and CD14 [28]. This triggers two

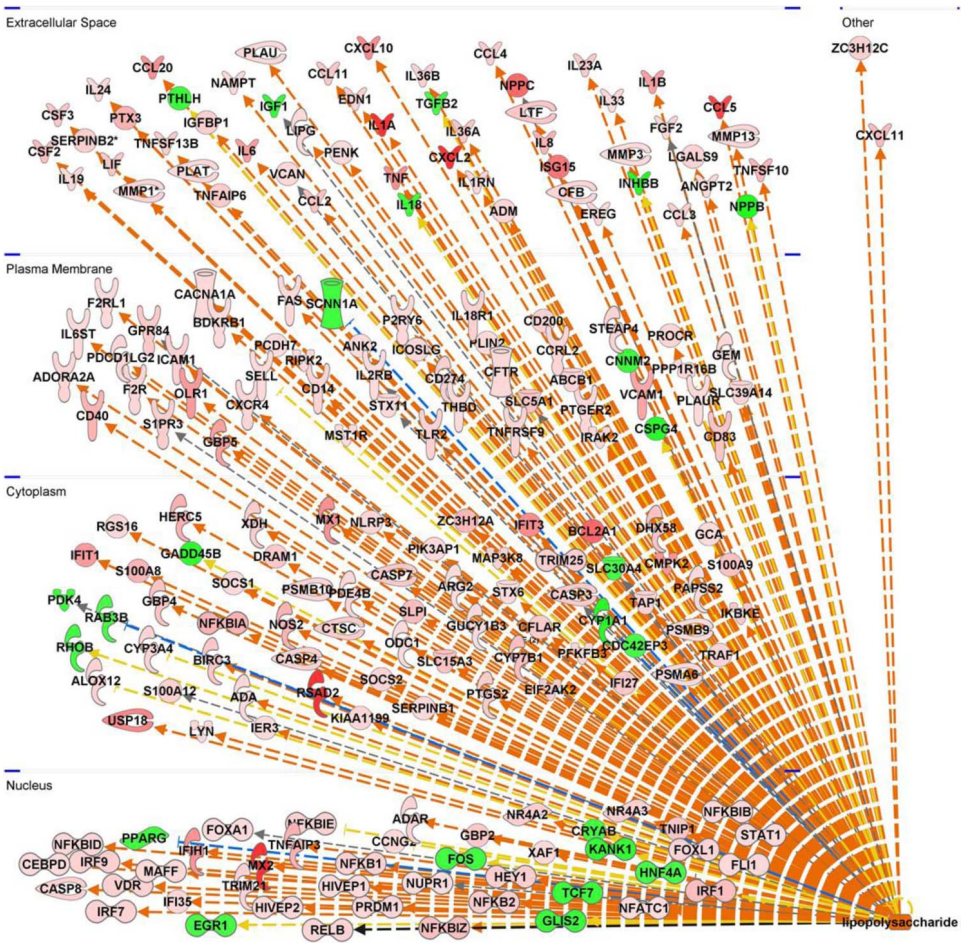

FIG. 4. IPA of the genes altered by LPS in bovine endometrial cells showing the predicted activation of LPS upstream regulator.

downstream signaling pathways: the MyD88-dependent pathway, which activates NF-κB and mitogen-activated protein kinase signaling and leads to the induction of inflammatory cytokines, and the TRIF-dependent pathway (MyD88-independent), which leads to the induction of type I IFNs via IRF3 activation and inflammatory cytokines via NF-κB activation [28]. Our data provide further evidence for the activation of the MyD88-independent pathway because LPS up-regulated the expression of several IRFs and type I IFN-inducible genes (Fig. 1, Supplemental Figs. S3 and S4, and Supplemental Table S1). Bovine endometrial cells express the TLR4/CD14/MD2 receptor complex [14]. Our finding of increased *CD14* expression but no change in *TLR4* or *MyD88* expression after 6 h of exposure to LPS is consistent with previous studies [17, 29]. In contrast, LPS up-regulated *TLR4* in another study utilizing only epithelial cell culture [26]. LPS was also shown previously to increase the expression of *TLR2* in bovine epithelial cells [26]. In the present study, we show that an LPS-induced increase in *TLR2* expression was linked to the activation of NF-κB-MAP3KB complex and the up-regulation of *IL1A* and many chemokines (Supplemental Fig. S2).

Apart from the membrane surface receptors, evidence is increasing that LPS is recognized intracellularly in a TLR-independent manner, triggering activation of inflammatory caspases [30]. In the present study, LPS up-regulated the expression of several molecules involved in the intracellular recognition of pathogens or their ligands, including *IFIH1, MDA5*, *DEXH* Asp-Glu-X-His box polypeptide 58 (*DHX58*), eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*), and adenosine deaminase, RNA-specific (*ADAR*). The RIG-I like receptors (RLRs) IFIH1 and DHX58 are RNA helicases that have a major role in the cytosolic recognition of virus-derived RNA [31]. Recently, RLRs have also been implicated in the detection of intracellular bacteria or bacterial ligands [32]. The protein kinase EIF2AK2 is a cytosolic sensor of viral double-stranded RNA but may also have an important role in bacterial immunity [33].

Activation of PRRs by LPS triggers an inflammatory response characterized by downstream signaling and transcription of both proinflammatory and chemotactic cytokines [9, 17]. These pleiotropic proteins regulate the cell death of inflammatory tissues, modify vascular endothelial permeability, recruit and activate inflammatory cells, and induce the production of antimicrobial proteins and acute phase proteins [9, 27]. Previously, the endometria of cows with uterine disease were observed to have increased mRNA expression of the proinflammatory cytokines *IL1A*, *IL1B*, *IL6,* and *TNF* and the chemokines *IL8* and *CXCL5* [34, 35]. The expression of these cytokines was also increased in endometrial cells stimulated with LPS in vitro [16, 17]. Our global approach identified a number of other proinflammatory cytokines and chemokines and their receptors, such as *CCL5* and *CXCR4* (Supplemental Table S1). Indeed, *IL1A* and several chemokines (*CX3CL1*, *CXCL2*, *CCL5,* and *CXCL3*) were among the genes most up-regulated by LPS (Table 2). Activation of PRR by the pathogen or its ligand initiates a series of signal transduction pathways via one or more of the IRF family of transcription factors, leading to the expression of IFNs [36]. IFNs bind their cognate

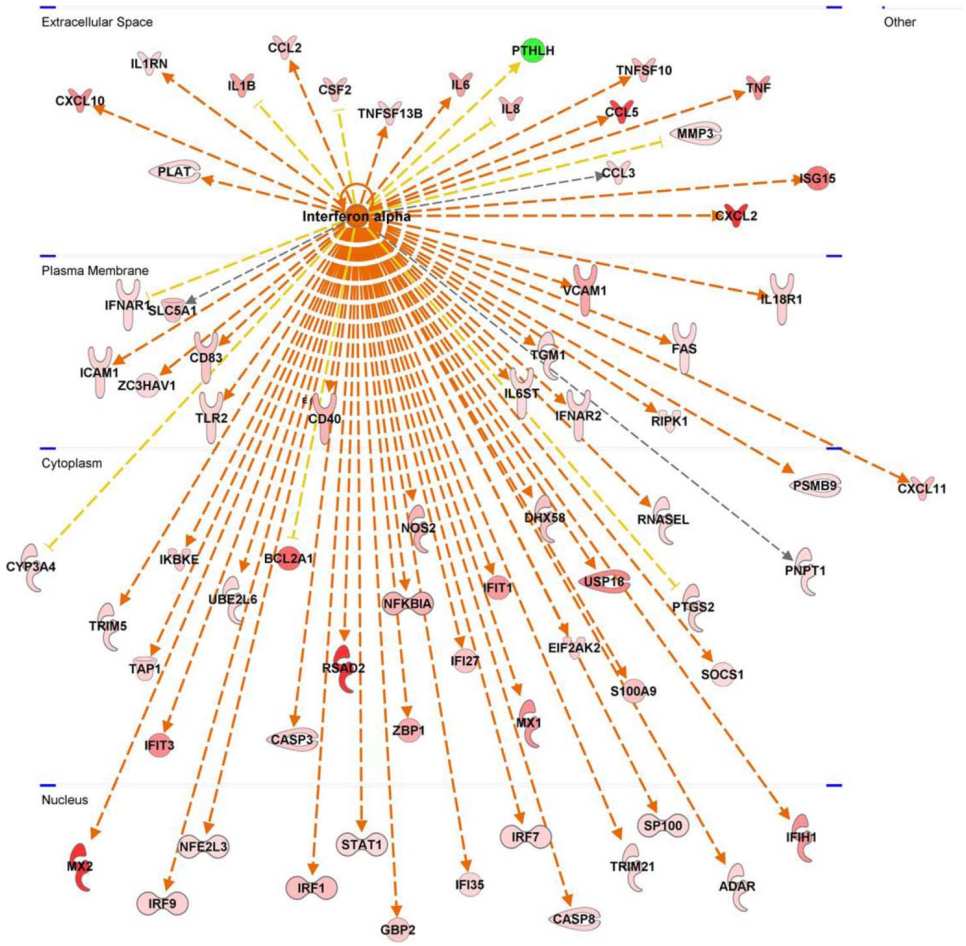

FIG. 5. IPA of the genes altered by LPS in bovine endometrial cells showing the predicted activation of IFNA upstream regulator. IFNA is a type I IFN group factor.

receptors, initiating signaling pathways that transcriptionally induce hundreds of IFN-stimulated genes (ISGs) [37]. Although the type I IFNs (IFNA and IFN beta [IFNB]) are typically considered to be most important in the host antiviral immune response, they are also induced by almost all bacterial pathogens via the TLR4-TRIF pathway [38]. LPS up-regulated the expression of *IRF3* and *IFNB* in bovine endometrial cells [26], and in the present study, several ISGs (*RSAD2*, *MX2*, *OAS1Y*, *ISG15*, and *BST2*) were among the genes up-regulated to the greatest extent by LPS (Table 2). In addition, LPS

TABLE 4. Quantitative RT-PCR analysis of selected genes for the validation of microarray data.

Gene	CONT[a]	CONT+LPS[a]	FC[b]	FC Microarray[b]
Up-regulated				
BST2	0.5 ± 0.28	2.7 ± 1.39	5.4*	4.1**
CCL5	1.6 ± 0.89	15.4 ± 6.59	9.6**	6.2**
CX3CL1	5.0 ± 1.68	62.3 ± 11.2	12.5***	7.0***
GBP5	0.4 ± 0.08	2.6 ± 0.99	6.5**	3.2*
IL1A	14 ± 3.07	122 ± 10.4	8.7***	6.9**
ISG15	1.1 ± 0.18	23.7 ± 9.59	21.6**	4.6*
MX2	2.3 ± 1.06	4.5 ± 1.25	2.0	7.0*
RND1	6.0 ± 1.04	21.5 ± 1.47	3.6***	4.0***
RSAD2	0.4 ± 0.13	9 ± 3.86	22.5**	9.7**
VCAM1	2.8 ± 0.84	6.2 ± 2.06	2.2*	3.0**
STAT1	9.5 ± 4.16	5.9 ± 1.47	0.6 (NS)	1.3* (up)
Down-regulated				
AMIGO2	7.3 ± 3.35	2.7 ± 0.78	2.7(*)	1.4***
No change				
C3	1.9 ± 0.24	2.2 ± 0.46	1.2	1.1
PTGES	2.0 ± 0.42	2.7 ± 0.40	1.4	1.0
TRIM56	0.8 ± 0.14	0.8 ± 0.15	1.0	1.1

[a] Gene expression values represent the mean ± SEM (n = 6) in arbitrary units after normalization to *RN18S1* and *ACTB*.
[b] Fold-change (FC) in expression; qPCR comparison by linear mixed-effects model; microarray comparison by paired *t*-test, Benjamini-Hochberg adjusted: (*)$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant.

induced an increase in the expression of the type I IFN receptors *IFNAR1* and *IFNAR2* and several IRFs (*IRF1*, *IRF7,* and *IRF9*), which were linked to the increased expression of IFNA-inducible genes and other ISGs (*IFIT3*, *IFI35*, *IFIHI*, *IFI27*, *IFIT1*, *ISG15*, *RSAD2,* and *DHX58*) (Supplemental Figs. S3 and S4). Of these, ISG15 is an ubiquitin-like modifier with antibacterial immune function [39] in addition to its antiviral activity, and RSAD2 (viperin) is similarly thought to possess both antiviral and antibacterial activity [40]. These findings indicate a major role for type I IFNs in the elimination of pathogenic bacteria in the bovine endometrium.

The endometrium can produce a wide variety of other proteins that contribute to innate immune defense mechanisms, and the present study confirms that LPS can up-regulate many of these. Mucins form a physical and antimicrobial protective layer of mucus above the epithelial cells of mucosal surfaces, including the female reproductive tract [41]. The expression of *MUC1* was significantly increased in the endometrium of cows with metritis and endometritis [23], and LPS up-regulated *MUC1* in bovine endometrial epithelial cells [8]. In the present, the expression of *MUC13* was increased by LPS. AMPs, including members of the defensin family, exhibit antimicrobial activity and are secreted by both epithelial and immune cells at the mucosal surface of the female reproductive tract [41]. Previous studies have shown increased expression of *TAP, LAP, DEFB1,* and *DEFB5* in endometrium of cows with severe inflammation or following LPS treatment in vitro [8, 17, 19, 22]. The effects on the expression of AMPs is a relatively late response (12–24 h), although LPS increased the expression of *TAP* and *SLPI* by 6 h in the present study. S100A calcium-binding proteins have inflammatory and innate immune activity against pathogens [42, 43] and are also secreted by activated or damaged cells in the extracellular space, where they function as damage-associated molecular pattern proteins and trigger immune response by binding to PRRs [44]. Our previous work has shown that the endometrial expression of *S100A8*, *S100A9,* and *S100A12* was markedly increased in cows with severe inflammation in the endometrium and was up-regulated by LPS [17, 19, 45]. The present study confirmed these reports in addition to showing the increased expression of *S100A14* (Fig. 3). Acute phase proteins are primarily synthesized by hepatocytes as part of the acute phase response to a variety of stimulants, including trauma and infection [46]. Concentrations of acute phase proteins, such as haptoglobin (*HP*), α_1-acid glycoprotein, and SAA3, in the plasma [47] and their endometrial expression were increased in postpartum cows undergoing inflammation and clearance of contaminating bacteria [48, 49]. In the present study, *SAA3* was the second most up-regulated gene by LPS, with an approximately 9-fold increase in expression (Table 2). The complement signaling cascade is another important immune defense mechanism that upon activation results in the opsonization of pathogens and their lysis or removal by phagocytes [50]. In the present study, the expression of complement factors *C2* and *CFB* were up-regulated by LPS. In addition, LPS increased expression of the guanylate-binding proteins *GBP2*, *GBP4,* and *GBP5,* which play a role in killing intracellular bacteria [51]. LPS also increased the expression of immunoproteasomes, including *PSMB9*, *PSMB10,* and *PSMA6* (Supplemental Fig. S3). These process damaged proteins from pathogenic sources to amplify peptide (ligand) supply for MHC class I antigen presentation and promote innate immunity by reducing oxidative stress and regulating proinflammatory cytokines [52].

The present results also provide evidence that LPS can regulate many genes involved in tissue turnover and remodeling. This included up-regulation of genes involved in cell death, including the caspases (*CASP3*, *CASP4*, *CASP7,* and *CASP8*), *RIPK1-3,* the antiapoptotic factor *BIRC3,* and FADD-like apoptosis regulator (*CFLAR*) (Fig. 2). A large number of vascular adhesion factors and cell-to-cell adhesion molecules were also up-regulated, including *SELL*, *SELP*, *VCAM1*, *ICAM1*, *VCAN*, *ALOX12*, *ITGB6,* and *FERMT1*. These have important functional roles in cell proliferation, tissue differentiation, and leukocyte migration during inflammatory processes and immune response [53]. Matrix metalloproteinases (MMPs) can degrade the extracellular matrix and also process proteins, such as cytokines and chemokines, to regulate leukocyte recruitment and inflammation [54]. In the present study, we showed increased expression of *MMP1*, *MMP3,* and *MMP13* in response to LPS, supporting results of our previous in vivo and in vitro studies [17, 55]. LPS also increased the expression of hyaluronan 2 (*HAS2*) and the fibroblast growth factors *FGF2* and *FGF18*. HAS2 is a member of the HA family of extracellular matrix polysaccharides with a variety of functions, including tissue repair and the activation of inflammation-related genes, such as *TNF*, *IL12*, *IL1B*, and *MMPs* [56]. FGF2 and FGF18 have potent angiogenic properties, and FGF2 can promote leukocyte recruitment to inflammatory sites by enhancing the expression of endothelial adhesion molecules [57]. Many members of the insulin-like growth factor (IGF) system are expressed in the postpartum bovine uterus, with *IGFBP4* expression influenced by the energy balance status [58] and *IGF1* and *IGFBP2* increased in the presence of clinical endometritis [23]. In the present study, the expression of *IGF1* was decreased, whereas that of *IGFBP1* was increased, by LPS. These changes may negatively impact immune responses [59]. Several other immune-related cytokines were also altered by LPS. Increased expression of leukemia inhibitory factor (*LIF*) and the colony-stimulating factors *CSF2* and *CSF3* was found, whereas transforming growth factor, beta 2 (*TGFB2*) was down-regulated.

Dysregulation of endometrial PG production appears to play an important role in uterine disease and infertility. Postpartum uterine infection was associated with prolonged luteal cycles in dairy cows [60], suggesting an impairment of luteolytic mechanisms. Relatively high concentrations of PGE_2 [61] or of PGE_2 and $PGF_{2\alpha}$ [62] were observed in the uterine fluid of cows with uterine disease. In addition, endometrial cells from cows with clinical endometritis secreted higher levels of PGE_2, $PGF_{2\alpha}$, and leukotrienes B_4 and C_4 [63]. $PGF_{2\alpha}$ and PGE_2 production in endometrial cells can be stimulated by proinflammatory cytokines and LPS [17, 64]. Evidence from an in vitro study [14] indicates that LPS can increase PGE_2 production to a greater extent than $PGF_{2\alpha}$ in both epithelial and stromal cells from bovine endometrium, although we did not find a significant change in the PGE_2:$PGF_{2\alpha}$ ratio in a previous experiment using mixed epithelial and stromal cells following 24 h of exposure to LPS [17]. In the present study, we did not measure actual PG secretion, but LPS increased the expression of the PG-endoperoxide synthase *PTGS2*, the cytosolic phospholipase A_2 (PLA2) enzyme *PLA2G4A,* and its family receptor *PLA2R1*. PTGS2 is the rate-limiting enzyme for the conversion of arachidonic acid (AA) into PGH_2 [65], and its expression was also increased in the endometrium of postpartum cows with severe inflammation [19]. PLA2G4A catalyzes the release of AA from membrane glycerophospholipids, another crucial step in PG biosynthesis [66]. In addition, LPS altered the expression of receptors that mediate the activity of PGE_2: *PTGER2* was up-regulated, whereas *PTGER3* was down-regulated. This supports an increasing role for PGE_2 during inflammation.

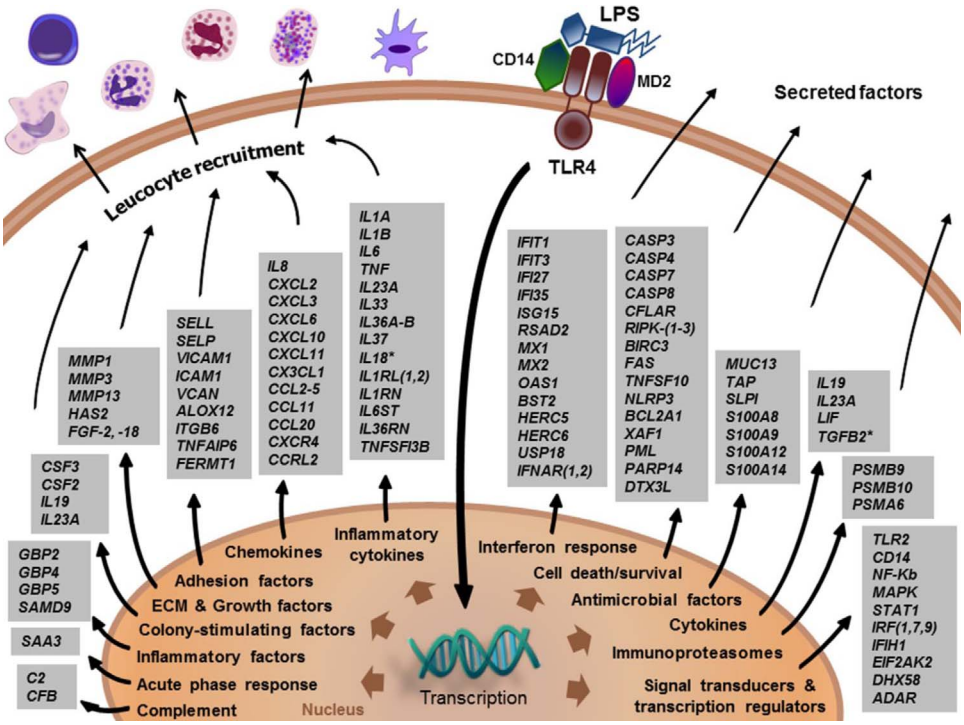

FIG. 6. Summary of the innate immune response in bovine endometrial cells exposed to bacterial LPS. TLR4 recognizes LPS on the cell surface in association with the coreceptor MD2 and additional proteins, such as CD14 and LPS-binding protein. This triggers downstream signaling, leading to the induction of type I IFNs and inflammatory cytokines [28]. LPS treatment for 6 h up-regulated the mRNA expression of many genes involved in inflammatory and innate immune response. *Genes down-regulated by LPS. ECM, extracellular matrix.

During expansion, the ruminant conceptus secretes the cytokine IFN tau, a key factor in the maternal recognition of pregnancy [67]. This induces a period of immune activation in the endometrium caused by the expression of IFN response genes [24, 68]. A combined total of 2218 genes from five different microarray studies were differentially expressed in the bovine endometrium on Days 15–20 of pregnancy [24, 69–72]. Interestingly, 286 (13%) of these genes were identified in the present study as being up-regulated by LPS, including *MX2*, *BST2*, *RSAD2*, *ISG15*, *OAS1*, *USP18*, *SAMD9*, *PLAC8*, *MX1*, *LGALS9,* and *GBP5*. Of these, *SAMD9*, *PLAC8,* and *LGALS9* were also up-regulated in early pregnancy. Other antimicrobial or immune-related genes, such as peptidase inhibitor 3 (*PI3*) and HECT domain and RLD 5 (*HERC5*), were down-regulated in early pregnancy but up-regulated by LPS. On the other hand, some genes involved in regulation of growth and cell death, such as caprin family member 2 (*CAPRIN2*) and growth arrest and DNA-damage-inducible, beta (*GADD45B*), were up-regulated in early pregnancy but down-regulated by LPS. This suggests one mechanism whereby an ongoing response to infection may interfere with the establishment of pregnancy.

In summary, our findings confirmed and extended those of previous studies showing that bovine endometrial epithelial and stromal cells are important in uterine innate immunity by detecting bacterial LPS and initiating a wide array of inflammatory response signaling (Fig. 6). Whereas this is very important for the initial clearance of invading bacteria in the postpartum uterus and serves as a vital link for the subsequent activation of an adaptive immune response, the consequential inflammatory activity may upset uterine function and constitute a hostile environment for ongoing reproductive processes.

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