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Lipopolysaccharide Stimulus Induces Gene Expression of Cytokines and Toll-Like Receptor 2/4 in Ovine Primary Alveolar Macrophages

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Abstract: In this study, Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) assays were performed with EvaGreen to investigate the dynamics of cytokine (Interleukin (IL)-1β, IL-8, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interferon (IFN) (γ) and Toll-Like Receptor 2/4 (*TLR2/4*) gene expression in ovine primary Alveolar Macrophages (AMs) following Lipopolysaccharide (LPS) stimulation. Expression of cytokine and TLR2/4 mRNA was quantified by comparison of Cycle threshold (C_T) values with a standard curve generated from plasmid DNA containing the target gene. Examination of LPS-stimulated ovine AMs revealed that cytokine mRNA expression peaked between 4 and 12 h with the exception of IFN-γ mRNA which peaked around 16 h post stimulation. Furthermore, TLR2 and TLR4 mRNA expression rapidly increased post-stimulation and peaked 20 min post-stimulation at a level which was maintained throughout the procedure. In summary, a sensitive and reliable real-time RT-PCR protocol was implemented for the analysis of ovine TLR2/4 and *cytokine* gene expression profiles.

Key words: Ovine, alveolar macrophage, cytokine, toll-like receptor 2/4, real-time reverse-transcription polymerase chain reaction, China

INTRODUCTION

Cytokines are low-molecular-weight proteins that are predominantly secreted by cells of the immune system and are involved in modulation of major immune responses. Cytokines initiate, mediate and propagate numerous cellular inflammatory responses (Biron, 1998; Del Prete and Romagnani, 1994; Rottman, 1999).

Toll-Like Receptors (TLRs) are a highly conserved group of proteins that provide the host with a means of discriminating non-self from self and as such function during the earliest stages of immune development. Therefore, an investigation of the gene expression profiles of cytokines and TLRs will facilitate an understanding of many of the processes underlying pathogenic infections and disease development and may provide some insight into the complex immune responses that are involved.

Lipopolysaccharide (LPS), a predominant glycolipid of the outer membrane of gram-negative bacteria, induces cellular responses by complex formation with circulating LPS-binding proteins (Hailman *et al*., 1994) which interact with signaling molecules belonging to the TLR family (Aderem and Ulevitch, 2000). This interaction stimulates monocytes, macrophages and neutrophils to produce cytokines, increase the expression of cell-adhesion molecules and induce secretion of proinflammatory mediators (Glauser *et al*., 1991; Rietschel and Brade, 1992).

Although, induction of cytokine and TLR expression by bacterial products (e.g., LPS) have been reported in monocytes, splenic macrophages, granulocytes, dendritic cells and endothelial cells in mammals (Caril *et al*., 2000; Matsuguchi *et al*., 2000a, b; Faure *et al*., 2001; Muzio *et al*., 2000; Sabroe *et al*., 2002; Akira, 2001), there is limited information available describing the effects of bacterial products on these proteins in ovine cells.

Several techniques are available for analysis of cytokine and TLR expression profiles in cattle and sheep. However, in domestic animals, the use of currently available techniques, including Enzyme-Linked Immunosorbent Assay (ELISA), ELIspot, flow cytometry and intracellular staining, rely on detection at the protein level and are limited by the availability of specific antibodies. Therefore, methods which assay cytokine and TLR expression at the mRNA level are used in preference. These include Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Keefe *et al*., 1997a, b; Yakobson *et al*., 2000), Quantitative-Competitive PCR (QC-PCR) (Pyeon *et al*., 1996; Pyeon and Splitter, 1998; Kabeya *et al*., 2001) and real-time PCR (Leutenegger *et al*., 2000; Waldvogel *et al*., 2000; Mena *et al*., 2002). RT-PCR and PCR are useful for the analysis of mRNA expression in various samples although the results obtained are not quantitative. QC-PCR can be used for quantitative analysis is relatively easy to perform and requires no specialized equipment. However, this technique it is labor

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intensive, time-consuming and requires large amounts of template for the multiple reactions necessary to determine the quantity of a single transcript. Therefore, a practical, rapid and more efficient method for quantification of cytokine and TLR mRNA expression in sheep is required.

Real-time PCR is a simple and rapid technique for the analysis of expression profiles of cytokines and TLRs in humans (Sumikawa *et al*., 2006) and other mammals (Menzies and Ingham, 2006; Konnai *et al*., 2003). Furthermore, the high sensitivity and reproducibility of this method allows detection of mRNA transcripts from a limited number of cells in which the corresponding protein is barely detectable. However, there is extremely limited information regarding the quantification of cytokine and TLR mRNA expression in sheep using real-time PCR. Quantification of cytokine and TLR expression is essential in order to develop effective vaccines that induce cell-mediated immune responses. Detailed knowledge of the levels of ovine cytokines and TLRs would provide valuable information about ovine immune responses and the pathogenesis of economically important ovine diseases. In this study, a quantitative RT-PCR (qRT-PCR) method using EvaGreen (like SYBRGreen) was developed and implemented for the detection of ovine cytokine and TLR mRNA expression in ovine primary AMs following LPS stimulation.

MATERIALS AND METHODS

Isolation of ovine Ams: Healthy lambs (aged 17-21 days) were humanely euthanatized by the intravenous administration of barbiturate. The trachea and lungs were removed and AMs collected by lavage using four aliquots (50 mL each) of sterile pre-chilled Phosphate Buffered Saline (PBS) at pH 7.4. The lavage fluid was filtered through sterile gauze and cellular components were separated by centrifugation at 800×g (4°C) for 10 min. The supernatant was discarded and the cells were washed twice in RPMI-1640 supplemented with 20% Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Cell counts and viability were determined by trypan blue exclusion. Cells were resuspended at 5×10^6 cells mL^{-1} in RPMI-1640/20% FCS, seeded (10^6) in 25 cm^2 tissue culture flasks and incubated at 37°C under 5% CO_2 for 40 min. Non-adherent cells were removed by washing twice with RPMI-1640 medium and adherent cells were used immediately for LPS treatment. The purity of AMs in culture was shown to be 95% by non-specific esterase staining.

LPS treatment: Purified ovine AMs in cell culture were treated with LPS (Sigma,USA) at a final concentration of 1 mM for 0 h (control), 20 min and 1, 2, 4, 8, 12, 16, 24 and 48 h. Subsequently, the culture medium was removed and the adherent cells were lysed in 2 mL TRIzol reagent (Invitrogen, USA).

RNA extraction: All solutions were treated with 0.1% Diethylpyrocarbonate (DEPC) and all glassware was baked at 180°C (4 h) before use to destroy RNase activity. Total cellular RNA was extracted from ovine AMs using TRIzol reagent according to the instructions provided by the manufacturer. Residual DNA was removed by treatment with 1 U DNase I (Promega, USA) at room temperature for 15 min followed by inactivation of the enzyme with 1 μL 25 mM Ethylene diaminetetra acetic Acid (EDTA) solution at 65°C for 10 min. RNA was quantified by measuring the optical density of a dilute RNA solution. RNA integrity was analyzed by 1% agarose/Ethidium Bromide (EtBr) gel electrophoresis and purity was evaluated by measurement of the OD_{260}/OD_{280} ratio. The total RNA yield was between 250 and 880 ng μL^{-1}.

Primers: GenBank sequence information was used to design specific oligonucleotide primers for amplification of genes encoding Interleukin (IL)-1β, IL-8, Interferon (IFN)-γ, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), TLR2/4 and the internal controls, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and α-tubulin. Details of all oligonucleotide primer sequences, predicted lengths of the amplified products, annealing temperatures and GenBank accession numbers are shown in Table 1.

Table 1: Sequences of the oligonucleotide primers and optimal amplification condition for real-time RT-PCR

Primer name	Sequence (5'-3')	Product size (bp) a	Annealing temperature (°C)	Accession N°b
GAPDH	Fw:AGCTCACTGGCATGGCCTT;Rv: AGGTCCACCTGTTGCT	300	55	U39091
α-Tubulin	Fw:TGTCCACCTTCCAGCAGAT Rv:ATCCCAGCCTCATAACCCT	245	56	AF251146
IL-1β	Fw:ATGGCAACCGTACCTGAACC; Rv: TTAGGGAGAGAGGGTTTCCATTC	799	55	NM_001005149
IL-8	Fw:AGAACGAGAGCCAGAAGA; Rv: CCCAGGAACTACCACAAT	464	55	S74436
IFN-γ	Fw:GCTACCGATTTCAACTACTC;Rv: GGCAGGAGAACCATTACA	538	55	X52640
GM-CSF	Fw:ATGTGGCTGCAGAAC;Rv:TTACTTCTGGACTGGACTGGTTCC	435	53	X53561
TLR2	Fw:ACTTCTCCCACTTCCGTCT;Rv:TGGAAAGCAGGCACATTG	167	55	NM_001048231
TLR4	Fw:ACTGACGGGAAACCCTATCC; Rv: CAGGTTGGGAAGGTCAGAAA	208	55	AY957615

Fw: Forward primer; Rv, Reverse primer; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; IL: Interleukin; IFN-γ: Interferon gamma; GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor; TLR: Toll-Like Receptor; a: Amplicon length in base pairs; b: GenBank accession number of ovine cDNA (available online at http://www.ncbi.nlm.nih.gov/)

Conventional RT-PCR and clone identification: For the amplification of the target genes by RT-PCR, total RNA was mixed with cell aliquots from different time points and cDNA was synthesized using a reverse-transcription reagents kit (Promega) following an optimized procedure as follows: Improm-II 5× reaction buffer, 4.0 μL; dNTP mix (2.5 mM), 0.5 μL; oligdT (0.5 μg reaction^{-1}), 0.5 μL; Im-reverse transcriptase, 1.0 μL; RNA inhibitor, 0.5 μL; total RNA (1 μg μL^{-1}), 1.0 μL; RNase-free water, 12.5 μL.

The thermocycling conditions for reverse transcription were as follows: 60 min at 42°C and 15 min at 70°C. The RT products were directly used for conventional PCR: 10x PCR buffer, 2.5 μL; dNTP mix (2.5 mM), 0.5 μL; Taq polymerase (10 U μL^{-1}), 0.5 μL; RT products, 2 μL; ddH_2O, 19.3 μL; specific sense primer (25 μM) and specific anti-sense primer (25 μM), 0.1 μL of each.

The PCR reaction conditions were as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 30 sec, 55°C (Annealing temperature adjusted from 50-60°C for different genes) for 30 sec and 72°C for 30 sec with a final extension incubation for 10 min at 72°C. PCR products were separated by 1.5% TAE (Tris-Acetate-EDTA) agarose gel electrophoresis and stained with EtBr. Amplified gene products were excised from the gel, purified and cloned into the pGM-T vector. Cloning was verified by sequence analysis.

Real-time qRT-PCR: Real-time qRT-PCR was performed using a SmartCycler II according to the instructions provided by the manufacturer. The quantitative PCR protocol was as follows: 20×EvaGreen, 0.6 μL; 2x PCR buffer for EvaGreen, 12.5 μL; specific sense primer (5 μM) and anti-sense primer (5 μM), 0.5 μL of each; RT product, 2.0 μL; Hot start Taq (5 U μL^{-1}), 0.3 μL; ddH_2O, 8.6 μL. A three-step PCR was performed using optimal conditions depending on the size of the amplified products.

The amplification protocol was adjusted for different target genes and the modified protocol was as follows: 60 sec at 95°C followed by 45 cycles of 15 sec at 95°C, 30 sec at 55°C and 45 sec at 72°C. Samples were quantified by comparison with internal standard curves.

RESULTS AND DISCUSSION

RT-PCR amplification of target genes: Sheep gene-specific RT-PCR was used to amplify mRNA encoding IL-1β, IL-8, IFN-γ, GM-CSF, TLR2, TLR4, GAPDH and α-tubulin. The sizes of PCR products were 799, 464, 538, 435, 167, 208, 300 and 245 bp, respectively and were with consistent with the predicted sizes (Fig. 1). PCR products were verified by sequence analysis.

Specificity, amplification efficiency and validation of qRT-PCR assays: Preliminary PCRs were performed using cDNA and pDNA (Plasmid clones containing the cDNA

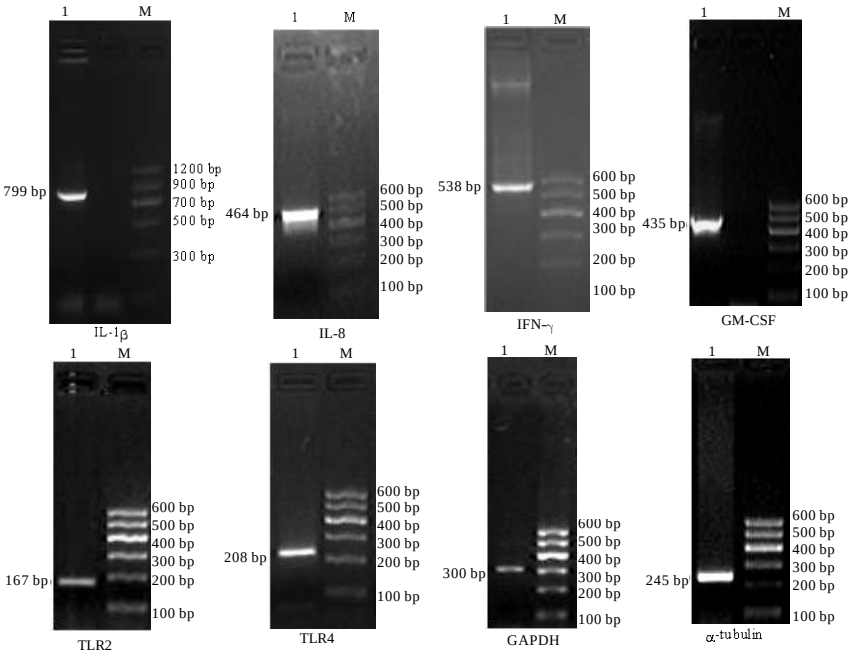

Fig. 1: RT-PCR amplification of all target genes. The expected fragments (ovine IL-1β, IL-8, IFN-γ, GM-CSF, TLR2, TLR4, GAPDH and α-tubulin) of RT-PCR amplification sized 799, 464, 538, 435, 167, 208, 300 and 245 bp, respectively

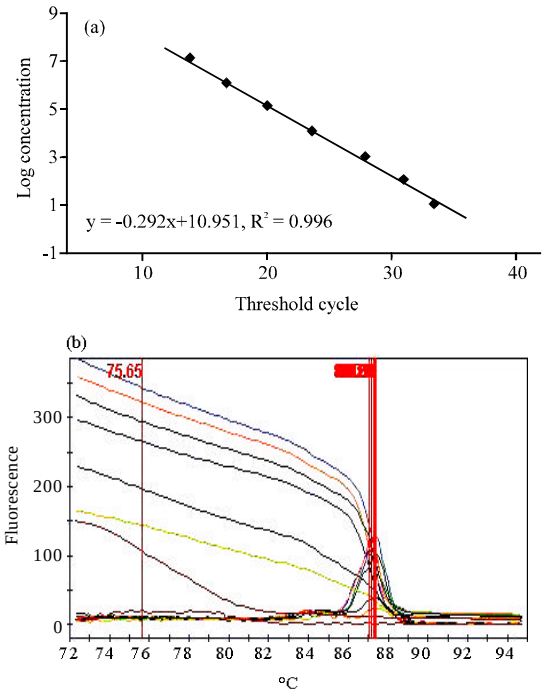

Fig. 2: Validation of ovine cytokines. Representative standard curve (a) and melting curve (b) of ovIL-1β. A 10 fold serial dilution of pIL-1β was used to produce the standard curve and amplified using a SmartCycler II PCR machine; (a) The standard curve is shown as the log concentration of the input copy number of plasmid DNA against the threshold cycle; (b) The melting curve is shown as a plot of temperature vs. change in EvaGreen fluorescence. No dimers were detected

of the target genes) of ovine AMs to confirm the specificity of the assay. The specificity of individual qRT-PCRs was determined with a SmartCycler II (Cepheid, USA) and EvaGreen (CapitalBio Corporation, Beijing) assays were used for detection. Melting curve analysis confirmed that no primer dimers had formed from the cDNA and pDNA (Fig. 2).

The accuracy of qRT-PCR is dependent on the linearity and efficiency of PCR amplification. The expression of all target genes was determined using a standard curve generated by a 10 fold serial dilution of cloned DNA (pDNA). The relationship between the threshold Cycle (C_T) value and the logarithm of the DNA copy number were studied using the correlation coefficient and the slopes calculated from the curves generated by the SmartCycler II sequence analysis software. The correlation coefficient (R^2 value) confirmed the linear relationship between the C_T value and the logarithm of the DNA copy number. The R^2 value was between 0.96 and 0.99 for all gene transcripts over several orders of magnitude of DNA copy number (Table 2). PCR efficiency is determined from the slope of the standard curve and calculated using the formula $E = 10^{a}-1$ (a = slope). Table 2 shows the average slope for each cytokine transcript from a 10 fold serial dilution assay. The efficiency for all gene transcripts was between 90 and 100%. For all transcripts examined, the upper limit was 10^7 gene copies per reaction and the lower limit was 10 gene copies per reaction (With the exception of IFN-γ for which the upper limit was 10^8 gene copies per reaction and the lower limit was 10^2 gene copies per reaction).

Table 2: Standard curve data obtained by real-time qPCR of plasmid DNA

Transcript	Detection limit (Gene copy number)	Range of C_T values	Mean R^2 value	Mean slope
IL-1β	10^7-10^1	13-34	0.996	-0.292
IL-8	10^7-10^1	12-37	0.976	-0.285
GM-CSF	10^7-10^1	15-40	0.994	-0.279
IFN-γ	10^8-10^2	12-37	0.984	-0.287
TLR2	10^7-10^1	16-44	0.995	-0.297
TLR4	10^7-10^1	13-36	0.997	-0.319
GAPDH	10^7-10^1	18-44	0.964	-0.307
α-tubulin	10^7-10^1	17-38	0.991	-0.304

Selection of housekeeping genes: In order to identify potential HKGs for normalization of the data in this study, a time-course experiment was performed using both unstimulated and LPS-stimulated ovine AMs. Sampling times varied from 0-48 h. Two potential ovine HKGs were analyzed: GAPDH and α-tubulin. Large quantities of both GAPDH and α-tubulin were expressed throughout the experiment although, levels of expression varied between unstimulated and LPS-stimulated samples when the data was normalized to the total RNA content (Fig. 3).

However, the data obtained from the standard curve of both genes fulfilled the requirements for the slope and correlation coefficient, suggesting that the PCR itself was successful. In addition, β-actin was tested. However, the qRT-PCR data obtained from the standard curve of this gene did not fulfill the criteria for the slope and correlation coefficient and hence, β-actin was not considered a suitable HKG. A stable HKG for normalization of cytokine and TLR2/4 expression was not identified in this study. Therefore, all data were normalized to the total RNA content.

LPS induces cytokine and TLR2/4 production in ovine AMs: In order to analyze ovine cytokine and *TLR2/4* gene expression following LPS stimulation, total RNA was obtained from unstimulated and LPS-stimulated AMs cultured for time periods ranging from 0-48 h. Cytokine (IL-1β, IL-8, GM-CSF and IFN-γ) and *TLR2/4* gene expression was analyzed by qRT-PCR with EvaGreen

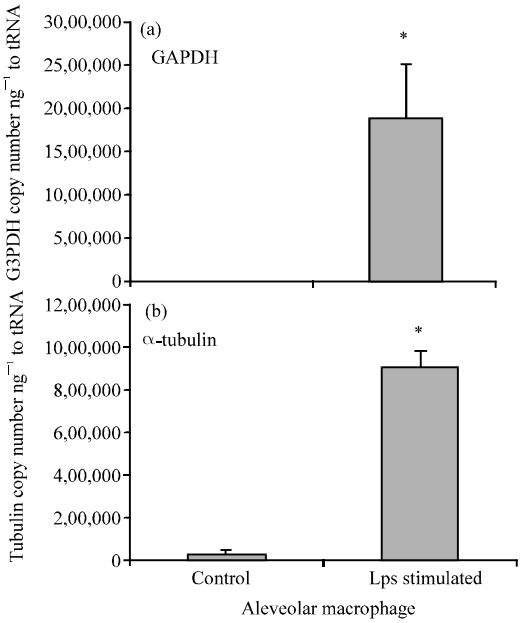

Fig. 3: Mean mRNA expression of GAPDH and α-tubulin in AMs with or without LPS stimulation. qRT-PCR data were normalized to the total RNA; *p<0.05. Standard error of the mean is shown by error bars

using the primer sets shown in Table 1. Expression of IL-1β, GM-CSF, IL-8 mRNA was upregulated. IL-1β mRNA expression peaked at 6 h and GM-CSF and IL-8 mRNA expression peaked between 4 and 12 h. The IFN-γ response was delayed with peak mRNA expression detected at 16 h. Expression of TLR2/4 mRNA rapidly increased, peaked at 20 min post-stimulation and remained high throughout the procedure (Fig. 4).

Cytokines and TLRs act as critical mediators in the complex network of the immune system, influencing the pathogenesis and outcome of many infectious diseases (Abdalla *et al*., 2003). However, the responses of cytokines and TLRs to LPS in ovine AMs are poorly described and their roles in pathogenic mechanisms remain to be elucidated. In this study, the mRNA expression kinetics of four cytokines (IL-1β, IL-8, GM-CSF and IFN-γ) and TLR2/4 were investigated in ovine LPS stimulated AM populations by real-time qRT-PCR.

A number of techniques have been developed to clarify the relationship between cytokine profiles and disease resistance and to elucidate the molecular mechanisms that modulate cell-mediated immunity. Such methods include qRT-PCR, Northern blotting and PCR (Anderson *et al*., 2001; Bailey *et al*., 2004; Kvarnstrom *et al*., 2004; Malec *et al*., 2004; Volman *et al*.,

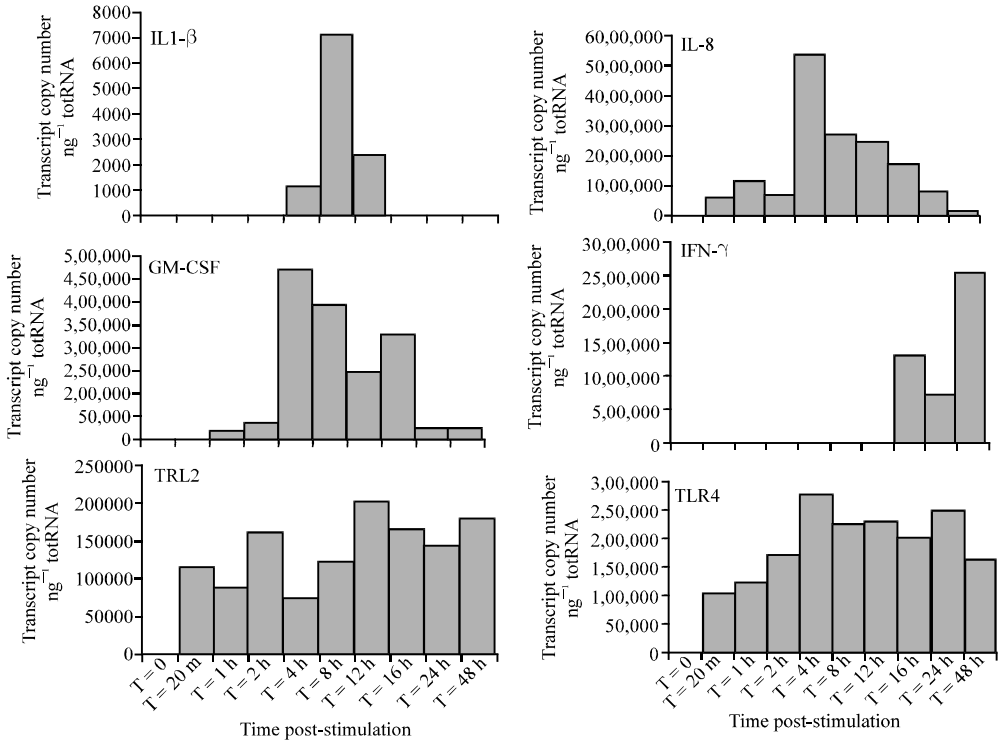

Fig. 4: Cytokine and TLR2/4 responses of ovine AMs stimulated with LPS. RNA samples were obtained from unstimulated and LPS-stimulated AMs that were cultured for different times (0 h; 20 min and 1, 2, 4, 8, 12, 16, 24 and 48 h) and analyzed by qRT-PCR. Results were normalized to the total RNA and are expressed as mRNA copy number per nanogram of the total RNA

2004). Northern blotting is laborious and the utility of this technique is limited by the requirement for large quantities of RNA. A number of studies have used qPCR and qRT-PCR to examine cytokine expression in important veterinary species including horses, cows, cats and pigs and many aquatic species (Colahan *et al*., 2002; Gil *et al*., 2003; Kaiser *et al*., 2000, 2003, Kipar *et al*., 2001; Leutenegger *et al*., 2000; Peuster *et al*., 2004; Werling *et al*., 2002). However, to date, analysis of ovine cytokine expression has been limited to Northern blotting, *in situ* hybridization and RNase protection assays.

In this study, a qRT-PCR method was developed to quantify *cytokine* gene expression in ovine AM populations. Accurate quantification of gene expression is achieved by normalization of data to a reference gene, the expression of which remains constant during the course of the experiment and between different experimental samples. Normalization allows for inter and intra-assay variations in samples with regard to cell number and efficiencies of RNA extraction and PCRs. The variation in PCR efficiency between samples is not a cause of concern in qRT-PCR since, the analysis is performed during the initial exponential stage of the reaction when the difference in efficiencies between samples is minimal. The amount of input material however, must be considered. In most cases, data are normalized to the expression of a HKG.

In order to accurately quantify gene expression, extensive preliminary experiments were performed to identify a HKG that did not alter its expression at any time under the experimental culture conditions. Four potential HKGs were examined, two of which were rejected due to generation of standard curves that did not meet the requirements for linearity of the PCR with differing amounts of template and for PCR efficiency. The expression of the two remaining genes, GAPDH and α-tubulin were examined in LPS-stimulated AMs (Fig. 2). For a gene to function as an HKG, the amplification between samples (Total RNA or cell number) should be approximately equal. This was not found to be true for either GAPDH or α-tubulin. In the absence of a reference gene, normalization to cell number or input total RNA is also acceptable. In this study, gene transcript data were normalized to the total RNA.

Sheep AMs are known to have potent phagocytic abilities and produce a variety of cytokines, including IL-1β, IL-8, IFN-γ and GM-CSF (Entrican *et al*., 1999). In this study, mRNA expression of IL-1β, IL-8, IFN-γ and GM-CSF was detected in ovine AMs following LPS stimulation. In accordance with many other studies on cytokine expression in veterinary species, expression of all the cytokines analyzed was also detected in unstimulated cells. Following LPS stimulation, the greatest increase was detected for IL-1β within 8 h (7.1×10^4 copies per nanogram total RNA). This is consistent with the upregulation of IL-1β expression detected in ovine Monocyte-Derived Macrophages (MDMϕ) stimulated with LPS (Budhia *et al*., 2006). Expression of GM-CSF and IL-8 was upregulated relative to the background mRNA level with peak expression detected between 4 and 8 h. In recent studies, high levels of IFN-γ mRNA have been identified in various murine, human and porcine macrophage populations (Choi *et al*., 2002; Frucht *et al*., 2001; Gessani and Belardelli, 1998) although, peak IFN-γ mRNA expression varies between species in different studies. In human studies, peak mRNA expression was detected within 6 h by Phytohaemagglutinin (PHA) stimulation. (Stordeur *et al*., 2002). However, in porcine Peripheral Blood Mononuclear Cells (PBMCs), peak cytokine mRNA expression (Including peak IL-4 and IFN-γ) was detected within 16 h of ConA stimulation and a similar pattern of expression was detected at the protein level (Verfaillie *et al*., 2001). These observations were reflected in this study with delayed IFN-γ mRNA expression reaching a peak at 16 h after LPS stimulation.

Recent research has focused on the ability of TLRs to recognize pathogens and initiate immune responses. Ten members of the TLR family have been identified in bovine and ovine tissues (Menzies and Ingham, 2006) although, to date, the availability of information regarding the expression of TLRs in ovine AMs is limited. The expression patterns of TLR2/4 have been intensively analyzed in human and murine systems. In this study, TLR2/4 mRNA expression was found to be upregulated in ovine AMs following LPS stimulation. TLR2/4 mRNA expression rapidly increased, peaked at 20 min post-stimulation and remained high throughout the experiment.

This result is consistent with observations from mouse peritoneal macrophages (Medvedev *et al*., 2000) and AMs (Oshikawa and Sugiyama, 2003).

CONCLUSION

In this study, the data demonstrate the development and implementation of a highly sensitive real time qRT-PCR assay for the quantification of ovine cytokine and TLR2/4 mRNA. Gene expression of TLR2/4 and the cytokines IL-1β, IL-8, IFN-γ and GM-CSF was upregulated in response to LPS. This method represents a powerful tool for the quantitative analysis of ovine gene expression thus, providing important information about ovine immune responses and the diagnosis and pathogenesis of economically important ovine diseases.

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