Article

Differential Responses Between Monocytes and Monocyte-Derived Macrophages for Lipopolysaccharide Stimulation of Calves

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In this experiment Toll-like receptor expression pattern in monocytes and monocyte-derived macrophages by lipopolysaccharide (LPS) stimulation was examined. Jugular venous blood was collected from four Japanese calves, and the peripheral blood mononuclear cells (PBMCs) were isolated. The cells were directly used for collecting monocytes by magnetic cell sorting or cultured for 7 days to collect monocyte-derived macrophages in Repcell. Then we analyzed the mRNA expression pattern of TLRs and cytokines in monocytes and monocyte-derived macrophages after LPS stimulation for 24 h. LPS stimulation of both monocytes and monocyte-derived macrophages resulted in an increase in the levels of mRNA transcripts for TNF-α, IL-6 and IL-8. Moreover, TNF-α and IL-6 mRNA expressions were significantly augmented by LPS stimulation in monocyte-derived macrophages. TLRs mRNA expressions were unchanged after LPS stimulation of monocytes, while TLRs mRNA expressions in monocyte-derived macrophages were complicated. TLR1, 3, 5, 8 and 10 were significantly decreased after LPS stimulation and there were no differences in the mRNA expressions of TLR2, 4, 6 and 7 between the groups of control and LPS stimulation. Besides, no expression of TLR9 was found. As antigen presenting cells, monocytes and monocyte-derived macrophages respond differently to LPS, so they may have different functions in the innate immune system. *Cellular & Molecular Immunology*. 2009;6(3):223-229.

Key Words: Toll-like receptor, monocyte, monocyte-derived macrophage, bovine

Introduction

Immune response in higher organisms can be generally divided into innate and adaptive immune response. Together, they are activated in order to destroy intracellular or extracellular invaders such as viruses, fungi, bacteria and parasites. Until recently, the innate immunity acts as the first line of defense against infectious agents, which recognizes invading pathogens through germ-line encoded pattern recognition receptors (PRR). A lot of researches show that this recognition can mainly be attributed to the family of Toll-like receptors (TLRs) which have been identified in organisms such as insects (1) and mammals (2).

TLRs are type I *trans*-membrane proteins that are grouped in the same family in concern to their sequence similarities. Several leucine-rich repeats (LRR) are present in the extracellular domains of these molecules, being a universal property of cell-surface adhesion molecules (3). Recognition of foreign molecules is based on the detection of molecular patterns that are indicative of entities likely to cause the host harm. These pathogen associated molecular patterns (PAMPs) comprise molecules such as lipopoly-saccahride (LPS), flagellin, dsRNA and (unmethylated dinucleotides) CpG DNA.

Recently, the partial sequences for bovine TLR1-10 were published, and these sequences also seem to share a high homology to ovine TLRs (4). A report indicates that TLR family members are expressed differentially among immune cells and appear to respond to different stimuli, but their protein and mRNA expression patterns are still largely unknown (5). Surface expression seems to be extremely low: only a few hundred molecules on one immature dendritic cell (iDC) and a few thousand on monocytes. Antigen-presenting cells (APCs), such as monocytes, macrophages and dendritic cells (DCs), express TLRs on their surface, which recognize and bind these PAMP and initiate a signalling pathway that stimulates the host defenses. TLR-PAMP interaction also initiates adaptive immunity as it activates APC by inducing production of pro-inflammatory cytokines and up-regulating co-stimulatory molecules. Moreover, TLR signalling stimu-

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lates the maturation and migration of APCs to the draining lymph nodes in some species (i.e., mice), although this migration seems to be more constitutive in ruminants, but can be modulated (6). In this project we set up an *in vitro* experimental system and investigated TLRs mRNA expression patterns in professional APCs of calves to elucidate whether there are any regulations of these patterns upon LPS stimulation.

Materials and Methods

Animals

Four Japanese calves, housed at the Miyagi Agricultural University experimental farm, were selected for the study. All calves received humane care according to the criteria outlined in the guide for the Care and Use of Experimental Animals (Tohoku University Animal Care Committee), and were clinically normal during the study.

Cell preparation

Jugular venous blood (50 ml) was collected aseptically into heparinized test tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (1.077 g/L, Lympholyte-H, CEDARLANE, Hornby, Ontario, Canada) for 30 min at 1,200 g, 18ºC. The cells were washed three times with PBS and resuspended in RPMI-1640 supplemented with 10% FBS, 1% Glutamax-1 (Invitrogen), and 100 IU/ml penicillin and 100 μg/ml streptomycin.

Generation of monocytes is described before (7). Briefly, suspended PBMCs were adjust to 1×10^8 cells/ml with PBS and 100 μl of the cell suspension was added to each well of 96-well polystyrene V-bottom plate (NUNC, Roskilde, Denmark) incubated with CD14 mAbs (CAM36A, VMRD) for 30 min followed by the addition of magnetic beads conjugated with IgG1 for 30 min on ice. After washing off the excess beads, purified $CD14^+$ cells were passed through Auto MACS separation column (Miltenyi Biotec, Germany), according to the manufacturer's instructions. For generation of monocyte-derived macrophages, suspended PBMCs were transferred to Repcell^{TM} (Cellseed, Japan) and cultured at 37ºC with 5% CO_2 (Forma Scientific Incubator, Waltham, US). The medium was changed to remove non-adherent cells 24 h later, and the culture was kept under the same conditions for 7 days, changing the medium every 2 days. Seven-day is the least time required for differentiation of monocytes into macrophages (8). Therefore, after 7 days of culture, the cells formed a confluent monolayer, mainly as a result of cell spreading as observed by phase microscopy and May-Grunwald Giemsa coloration.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from monocytes and monocyte-derived macrophages by using TRIzol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol as previously described (9, 10). The first strand cDNAs were synthesized using the Superscript III reverse transcriptase (RT; Invitrogen, Carlsbad, CA) with the oligo (dT) primer. For PCR, oligonucleotide primers were used as previously described (4, 11, 12) and are listed in Table 1. PCR was performed using the TLRs and cytokine primers. PCR amplification consists of initial denaturation at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55-63ºC for 1 min, and extension at 72ºC for 1 min, and final extension at 72ºC for 7 min. PCR products were run on 2% agarose gels and stained with ethidium bromide, and then visualized by UV-transillumination. For mRNA analysis, the image of the gel was digitized using NIH image computerizd densitometry program and the results were normalized to G3PDH.

FACS analysis

FACS analysis was used to check the CD14 expression on monocytes and monocyte-derived macrophages, which

Table 1. Sequences of primers specific for bovine TLRs and cytokines used for RT-PCR analysis

Primer	Sequence 5'-3'		Size (bp)	Annealing temperature (ºC)
	Forward	Reverse		
TLR1	CTGCCCATATGCCAAGAGTT	AAACCAACTGGAGGATCGTG	421	60
TLR2	GCTCCTGTGACTTCCTGTCC	CCGAAAGCACAAAGATGGTT	501	60
TLR3	GAGGCAGGTGTCCTTGAACT	GCTGAATTTCTGGACCCAAG	327	60
TLR4	CTCTGCCTTCACTACAGAGA	CTGAGTCGTCTCCAGAAGAT	237	55
TLR5	AACGCTTTGCTCAAACACCT	ACCCTCTGATGGACTGATGC	301	60
TLR6	ACTGACCTTCCTGGATGTGG	GCACCACTCACTCTGGACAA	679	60
TLR7	ACTCCTTGGGGCTAGATGGT	GCTGGAGAGATGCCTGCTAT	180	60
TLR8	CTGGAGGAGCTGAACCTGAG	TGGTTGTAGGACAGCAGCAG	393	62
TLR9	GTTATGTTGGCTGCCCTGG	GTTCTCATCCATTAGAATATGC	320	62
TLR10	GGCACAGGGTTAGGAAAACA	GAGATTGTGGTGGGCAAAGT	303	55
IL-6	GAC GGATGCTTCCAATCTG	ACCCACTCGTTTGAAGACTGCATCTT	251	56
IL-8	CTGCAGTTCTGTCAAGAATGAGTAC	AGCATGTCCTACATGAACAATGTAC	453	56
TNF-α	CAGAGGGAAGAGTCCCCAGG	CTTTGGTCTGGTAGGAGACT	325	55
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	55

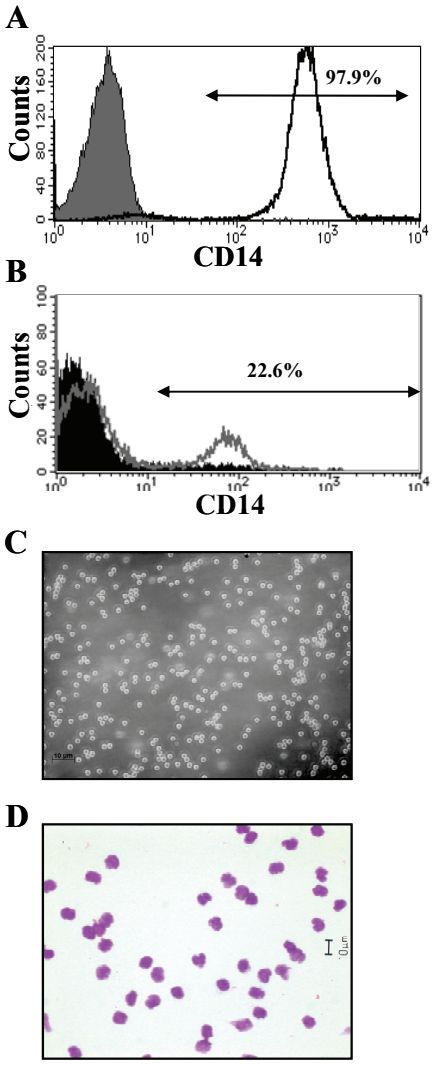

Figure 1. The characteristic of bovine monocytes. (A) The purity of monocytes isolated by magnetic cell sorting. PBMCs were stained with anti-CD14 mAb, followed by the addition of magnetic beads conjugated with mouse anti-IgG1. $CD14^+$ monocytes were positively selected by using magnetic cell separation. Then the cells were stained with PerCP-rat-anti-IgG1 for 30 min to examine the purity of the monocyte population by flow cytometry. The histogram covered with gray color was negative control. (B) The expression of CD14 on PBMCs. PBMCs were stained with anti-CD14 mAb and analyzed by flow cytometry. The histogram covered with black color was negative control which was stained with only secondary antibody. (C) The morphology of monocytes observed by phase-contrast microscopy after isolated from PBMCs by magnetic cell sorting. (D) Microscopic analysis of monocytes stained with May-Grunwald Giemsa coloration. Bar = 10 μm.

indicates the characteristic of these cells. The flow cytometer used was a Becton Dickinson FACSCalibur with CellQuest software program from the same manufacturer.

Statistics

Data of the metrical averages were calculated, and described as the mean ± SEM. Differences in TLRs and cytokine mRNA expressions of the monocytes and monocyte-derived macrophages between LPS stimulation and no stimulation were reported using one-way ANOVA and Turkey's multiple comparison method. Significance was accepted at $p < 0.05$.

Results

Different morphology and CD14 expressions between monocytes and monocyte-derived macrophages

Monocytes can differentiate into macrophages *in vitro* through incubation in the special plates for 6-8 days (13). The morphology of monocytes and peripheral blood monocyte-derived macrophages was analyzed by phase contrast microscopy and May-Grunwald Giemsa coloration (Figures 1 and 2). The monocytes showed a small and round morphology, while monocyte-derived macrophages spread on plastic surface and lost the circular shape. Monocyte-derived macrophages were larger than original monocytes and showed a higher capacity to adhere during the 7-day culture. These cells have bean shaped nuclear and finely granular cytoplasm containing phagocytic vacuoles and therefore exhibit features consistent with a macrophage morphology.

To ensure that adherent cells after 7-day culture were indeed enriched for a macrophage phenotype, expression of the monocyte/macrophage marker CD14 was assessed by flow cytometry. Monocytes were tested for high purity (97.9%) using $CD14^+$ selection markers, and peripheral blood monocyte-derived macrophages were tested for the expression of $CD14^+$ about 73.9% (Figures 1 and 2).

Different cytokines and TLRs mRNA expressions after LPS stimulation between monocytes and monocyte-derived macrophages

The mRNA expressions of cytokines in monocytes and monocyte-derived macrophages were shown in Figure 3. IL-6, IL-8 and TNF-α were relatively augmented after LPS stimulation for 24 h in monocytes, while in monocyte-derived macrophages the mRNA expressions of IL-6 and TNF-α were significantly increased to 2.02 ± 0.48 and 1.66 ± 0.39, respectively. The mRNA expressions of IL-8 were relatively augmented after LPS stimulation.

TLRs mRNA expressions in monocytes after 24 h-LPS stimulation were shown in Figure 4A and further analyzed semi-quantitatively using a densitometic method (Figure 4B). The data were conformed by repeated experiments. The results showed that TLR1, 2, 4, 7 and 8 were highly expressed at over 1.0 fold compared with the G3PDH mRNA expression, while TLR3, 5, 6 and 10 were relatively low. There was no alternation in the TLRs transcripts after LPS stimulation. Interestingly, no expression of TLR9 mRNA was found.

The difference of TLRs mRNA expressions in monocyte-derived macrophages between LPS stimulation and no stimulation were also analyzed (Figure 5). TLR1, 3, 5, 8 and 10 transcripts were significantly decreased after LPS stimulation, especially for the TLR5 and 10, which were

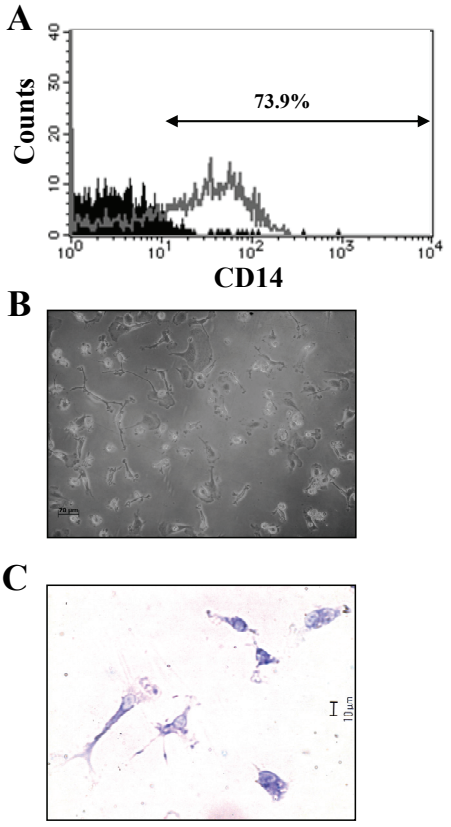

Figure 2. The characteristic of bovine monocyte-derived macrophages. (A) The expression of CD14 on monocyte-derived macrophages using flow cytometric analysis. Monocyte-derived macrophages were cultured from PBMCs for 7 days in Repcell^{TM}. The cells were stained with anti-CD14 mAb for 30 min and then stained with PerCP-rat-anti-IgG1 for 30 min. The histogram covered with black color was negative control which was stained with only secondary antibody. (B) The morphology of monocyte-derived macrophages observed by phasecontrast microscopy after 7-day culture. (C) Microscopic analysis of monocyte-derived macrophages stained with May-Grunwald Giemsa coloration. Bar = 10 μm.

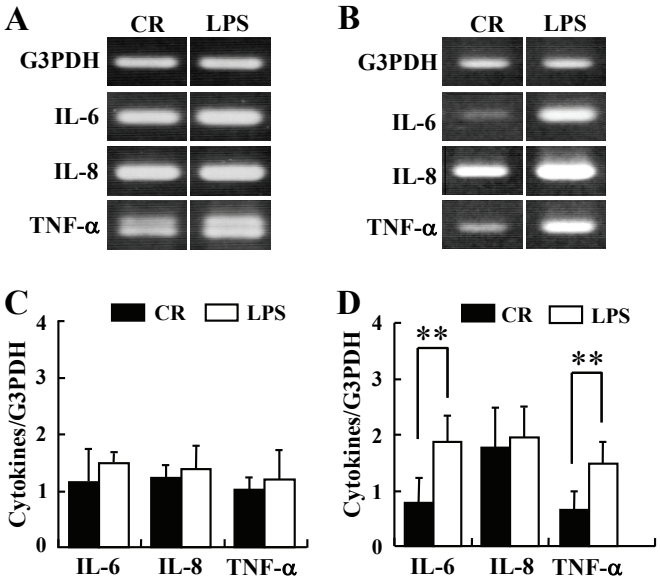

Figure 3. Cytokine expressions in bovine monocytes and monocyte-derived macrophages after LPS stimulation for 24 h. The profile of the cytokine mRNA expressions in monocytes (A) and monocyte-derived macrophages (B) with no stimulation or stimulated with 100 ng/ml of LPS for 24 h, respectively. Semi-quantitative RT-PCR analysis of cytokine mRNA expressions in monocytes (C) and monocyte-derived macrophages (D), respectively. The figures are representatives obtained from four calves. *$p < 0.05$; **$p < 0.01$. Data were shown as mean ± SEM from four experiments (n = 3).

significantly decreased to 0.13 ± 0.24 and 0.10 ± 0.16, respectively. There was no difference in the mRNA expressions of TLR2, 4, 6 and 7 between non-treatment and LPS stimulation groups. Besides, no expression of TLR9 was found.

Discussion

Until now, a lot of researchers have done the study on the mononuclear phagocytes of monocytes and macrophages (14-17), however, TLRs expression pattern differences between them were few reported. Previous studies have shown that bovine TLRs are differentially expressed in variety of tissues (4). Above all, this study examined a clear dissociation of either cytokines or TLRs mRNA expressions by TLR agonist (LPS) stimulation in bovine monocytes and monocyte-derived macrophages.

In order to collect the monocyte-derived macrophages, PBMCs were cultured as previous reports (8, 18) with a little change. Based on the morphology and cell surface marker expression analyses (19, 20), monocyte-derived macrophages in the culture system had an increase in size and granularity and highly expressed CD14 as previous reported (17, 21-23).

Signaling through TLR induces pro-inflammatory cytokine gene expressions, such as TNF-α, IL-8 and IL-6. In this study, LPS stimulation of monocytes resulted in an increase in the levels of mRNA transcripts for TNF-α, IL-6 and IL-8, which were the same to the results of human's monocytes (24, 25). Moreover, TNF-α and IL-6 mRNA expressions were significantly augmented by LPS stimulation in monocyte-derived macrophages which was the same as Weriling has done (26). In this study, monocytes and monocyte-derived macrophages have the different ability to produce pro-inflammatory cytokines that could be due to the biological differences between them.

TLRs mRNA expressions were unchanged after LPS stimulation in monocytes, while TLRs mRNA expressions in monocyte-derived macrophages were complicated. TLR1, 3, 5, 8 and 10 were significantly decreased; TLR2, 4, 6 and 7 were unchanged in monocyte-derived macrophages after LPS stimulation. In order to interpret our results we decided to create three TLR groups in relation to their specificity as

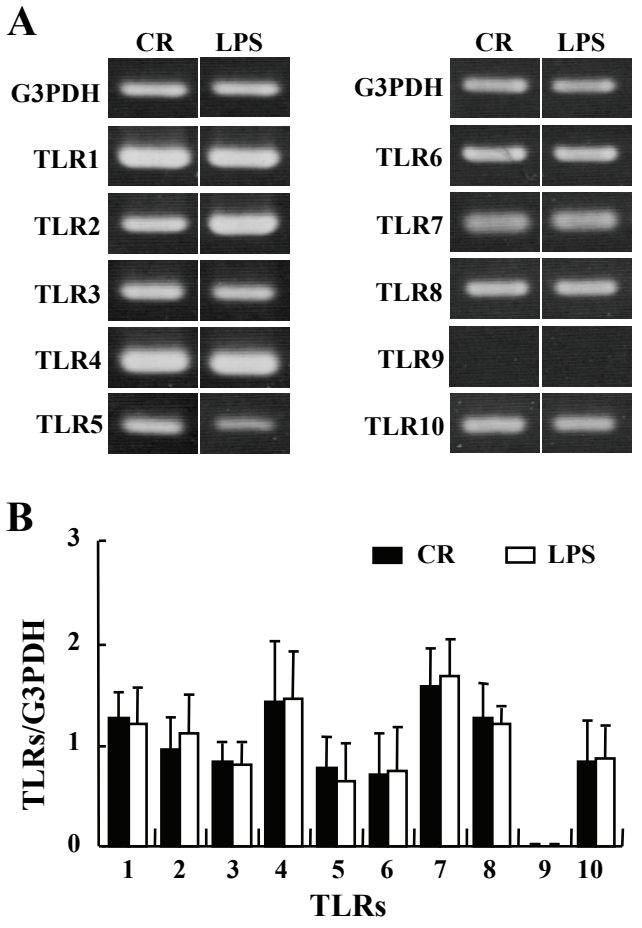

Figure 4. TLRs mRNA expressions in bovine monocytes after LPS stimulation for 24 h. (A) The profiles of the TLRs mRNA expressions in monocytes with no stimulation (CR group) or stimulated with 100 ng/ml of LPS for 24 h (LPS group). (B) Semi-quantitative RT-PCR analysis of TLRs mRNA expression in monocytes. Data were shown as mean ± SEM from four experiments (n = 3).

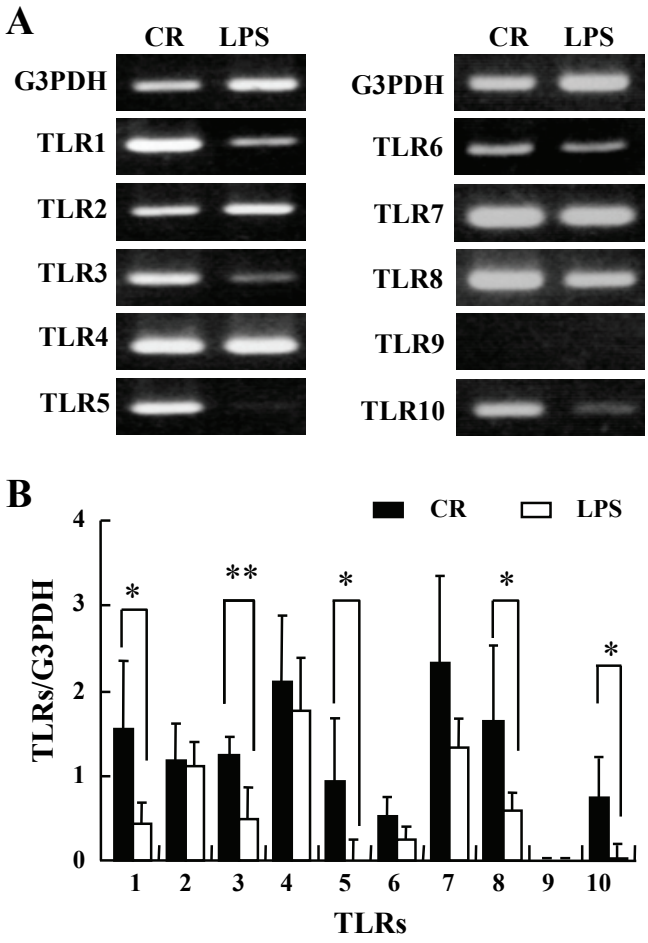

Figure 5. TLRs mRNA expressions in bovine monocyte-derived macrophages after LPS stimulation for 24 h. (A) The profiles of TLRs mRNA expressions in monocyte-derived macrophages with no stimulation (CR group) or stimulated with 100 ng/ml of LPS for 24 h (LPS group). (B) Semi-quantitive RT-PCR analysis of TLRs mRNA expression in monocyte-derived macrophages. *$p < 0.05$, **$p < 0.01$. Data were shown as mean ± SEM from four experiments (n = 3).

Kokkinopoulos has used (27). This specificity lies on TLR ligands of viral and bacterial-LPS nature, and a special group that comprises TLR with increased specificity to conserved bacterial molecules. The first group, named "Bacterial-LPS" TLRs groups involves TLR1, 2, 4, 6, 10. This group carries the characteristics of being able to recognize bacterial LPS.

LPS (other than *E. coli*) initiates TLR1/2 hetrodimerisation (28), while *E. coli* LPS is recognized only by TLR4 (29). Our data reveal that TLR2 and TLR4 mRNA expressions remained the same, while TLR1 and TLR10 transcripts decreased in monocyte-derived macrophages upon LPS challenge. The intracellular signaling event of TLR1 has not been elucidated yet (30). Since it heterodimerise with TLR2, it seems that it depends on whether the latter will be expressed or not. Bovine TLR10 has the similar expression profiles with TLR1 and TLR6 (22), in our experiments it was abolished in monocyte-derived macrophages upon LPS challenge. This result could lead to several assumptions such as LPS tolerance. Its implication in allergic asthma has been recently reported (31).

TLR2 and TLR4 mRNA expressions were unchanged after *E. coli*-LPS stimulation. There are several possible interpretations: (a) mRNA measurement is not adequate for assessing TLR2 and TLR4 protein levels. However, it is a plausible conclusion, but cannot be tested before a quantitation of bovine TLR protein is possible (32, 33); (b) there is some protein involves in the TLR4 receptor complex. MD-2 is needed to activate TLR4 upon LPS addition. The concentrations of soluble CD14 (sCD14) and LPS binding protein (LBP) are essential in establishing a stable TLR4/MD-2/CD14/LPS structure, but at this point it is not possible to explain how and why (34). Assuming that this protein was missing from the serum, TLR4 is hyporesponsive to *E. coli* LPS.

The "viral" TLRs group, comprises of TLR3, 7, and 8, all

documented to mainly being involved in recognizing viral particles, like dsRNA or mimics of it. TLR3 responds to dsRNA, a replication intermediate among many RNA viruses, and a byproduct of transcription among DNA viruses (35-37). Bovine TLR3 transcripts were detectable upon LPS stimulation (38), which was the same as the data obtained from our study in general. We observed no changes in the TLR3 mRNA expression after 4 h stimulation with LPS in monocyte-derived macrophages. However, our data demonstrated that after 24 h stimulation, TLR3 mRNA expression level was significantly down-regulated. By expanding our hypothesis to the protein level, TLR3 might be in a ready-made stage intracellularly, to immediately recognize any intracellular invaders. Since LPS gives the signal for an extracellular infection, it can be assumed that these viral recognition receptors are not in need or inhibited (24).

TLR7 recognizes ribonucleic acid homologs like imiquimod and resiquimod, and synthetic single-stranded RNA (ssRNA) oligonucleotides rich guanosine or uridine derived from ssRNA virus (39, 40). TLR8, like TLR7, also recognizes viral ssRNA and synthetic imidazoquinolines. TLR7 and TLR8 mRNA expressions were the same as the data reported by Lee SR (14). TLR7 mRNA expressions were down-regulated and TLR8 transcripts were significantly decreased by LPS stimulation. This decrease might indicate the need for other innate immune signals, through similar intracellular pathways (41), apart from specific viral compounds (42). In conclusion, the viral TLRs group showed a very consistent abolition due to LPS challenge, which implies that the bacterial TLR group was activated exclusively by LPS. Until that stage, LPS was in part involved in down-regulation of human TLRs that recognize viral-like structures.

The final group, the "bacterial" TLR group, involves TLR5 and TLR9 that recognize flagellin structure and CpG motifs, respectively (43, 44). Our data showed a decreased alternation in TLR5 transcript in monocyte-derived macrophages, while no expression of TLR9 was found in monocytes or monocyte-derived macrophages. These results are similar to the data that Werling (26) and Hornung (45) have published, respectively. TLR9 mRNA expression was always detectable in pDC. Currently, there are no published experimental data that could explain these differences. *E. coli* species contain variants possessing flagellum (46), and CpG unmethylated regions within their nuclear DNA.

In conclusion, there is a high complexity of innate immune recognition in mammals. In our study, monocytes have a low response to LPS stimulation, however, monocyte-derived macrophages have an increased response to LPS stimulation with down-regulation of some TLRs. Taken together, we hypothesize that stimulation of macrophages through the TLR signaling cascade can trigger other signaling pathways to help the cell to have an increased reaction when viral infection, such as activation of Notch signaling, which in turn regulated gene expression patterns involved in pro-inflammatory responses, through activation of NF-κB (47). The delicate function of different stages cells will give us the best therapeutic for disease.

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