Tropical Animal Health and Production (2021) 53: 65
https://doi.org/10.1007/s11250-020-02491-4

REGULAR ARTICLES

Effect of LPS and LTA stimulation on the expression of TLR-pathway genes in PBMCs of Akkaraman lambs in vivo

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Received: 9 March 2020 / Accepted: 2 December 2020
 / Published online: 3 January 2021

Abstract

This is the first study investigating the changes in some gene expressions related to the TLR pathway in vivo in sheep. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) molecules were administrated separately and in combination to the Akkaraman lambs via intranasal route. For this purpose, 28 lambs were distributed into four groups (LPS, LTA, LPS + LTA, and control, $n = 7$). Blood samples were collected to isolate the peripheral blood mononuclear cells (PBMCs) at 24 h and on day 7. Expression levels of *TLR2*, *TLR4*, *MyD88*, *TRAF6*, *TNF-α*, *IL-1β*, *IL-6*, IL-10, *NF-κβ*, and *IFN-γ* genes were determined by qRT-PCR. Increases were determined in the expression data of *TLR2* [LPS ($P < 0.05$) and LTA + LPS ($P < 0.01$)], *TLR4* [LTA + LPS ($P < 0.05$)], *TNF-α*, *IL-10* [LTA + LPS ($P < 0.05$)], and *IFN-γ* genes in all groups in the mRNA expression analysis of PBMCs isolated at 24 h whereas decreases were determined in the expression levels of these genes on day 7. The combination of LPS + LTA stimulated lamb PBMCs more effectively than separate administration of LPS and LTA at 24 h. Therefore, this article may contribute to the understanding the host-pathogen interaction of respiratory-transmitted bacterial diseases concerning PBMCs at 24 h and on day 7. Also this study may contribute to the dose adjustment for bacterial vaccine studies in sheep. Experimental application doses will be helpful for in vivo and in vitro drug and vaccine development studies in the fields of pharmacology and microbiology.

Keywords Lamb · Lipopolysaccharide · Lipoteichoic acid · Gene expression · Peripheral mononuclear blood cell

Introduction

Pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs) present in different microbial agents play a central role in the formation of immune response (Delves et al. 2011). Rapid progress has been made in understanding the molecular mechanisms of the immune system following the discovery of Toll-like receptors (TLRs) in mammals (Kawai and Akira 2010). TLRs that make up a significant portion of PRRs in host immune cells play a critical role both in the formation of the innate immune response to infectious agents and in the initiation of the acquired immune response (Beutler and Rietschel 2003; Delves et al. 2011; Takeda et al. 2003). Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) are important pathogenic agents present in Gram-negative and Gram-positive bacterial cell walls that stimulate the natural immune system (Heumann et al. 1998; Wakabayashi et al. 1991). It has been reported that Toll-like receptor 2 (TLR2) reacts with LTA. On the other hand, Toll-like receptor 4 (TLR4) responds to LPS of different types of bacterial agents (Beutler and Rietschel 2003; Hans and Hans 2011).

TLR stimulation via LPS or LTA agents activates the signaling pathway associated with NF-κβ to promote the release of proinflammatory cytokines (Akira et al. 2006; Song et al. 2007). Myeloid differentiation primary response 88 (MyD88) is an adapter protein that transmits the signal generated by extracellular TLR receptors into the cell (Takeda and Akira 2004). In addition to MyD88, TNF receptor-associated factor 6 (TRAF6) and Interleukin-1,4 receptor-associated kinases (IRAK1,4) lead to the release of proinflammatory cytokines and interleukins via adapter molecules by activation of NF-κβ-associated signaling pathway (Takeda and Akira

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2004). Several in vitro and in vivo studies were conducted on different species to understand the role of LPS and LTA. These molecules are effective on activating innate and adaptive immune system and the function of TLRs in these immune systems (Carroll et al. 2012; Deng et al. 2012; Gao et al. 2010; Islam et al. 2012; Nefefe et al. 2017; Passmore et al. 2016; Xu et al. 2017). The innate immune system is known to be rapidly activated within 0–4 h to eliminate foreign molecules in the body, give early stimulated response at the 4–96 h (innate-adaptive immunity), and transfer the task to the adaptive immune cells after the 96 h (Murphy 2014).

This is the first study investigating the changes of some gene expressions, which related to the TLR pathway, in vivo on sheep. For this purpose, lipopolysaccharide (LPS) and lipoteichoic acid (LTA) molecules were administrated intranasally via separately and in combination to the Akkaraman lambs. In this study, we selected *TLR2*, *TLR4*, *MyD88*, *TRAF6*, *Tumor necrosis factor-α (TNF-α)*, *Interleukin-1ß (IL-1ß)*, *Interleukin-6 (IL-6)*, *Interleukin-10 (IL-10)*, *Nuclear factor-kß (NF-κß)*, and *Interferon gamma (IFN-γ)* genes. Therefore we aimed to understand the host-pathogen interaction of separately and combined LPS and LTA stimulation in PBMCs at 24 h and on day 7.

Materials and methods

Animal material

The ethical permission to conduct this study was obtained from The Local Ethics Committee for Animal Experiments of Erciyes University dated 15 February 2017 and numbered 17/009. A total of 28 clinically healthy 3-month-old male Akkaraman lambs were purchased from Erciyes University Agricultural Research and Application Centre (ERUTAM). They were grown in the same farm under the same care and feeding conditions.

Experimental application

The Akkaraman lambs were selected for experimental application, and they were divided into four groups as LPS ($n = 7$), LTA ($n = 7$), LPS + LTA ($n = 7$), and control ($n = 7$). The live weights of all animals were recorded (Table 2), and the dosages of LPS, LTA, and LPS + LTA were adjusted according to their weights. The lambs were distributed to the groups randomly. Lyophilized LPS and LTA were diluted with phosphate-buffered saline solution (PBS, Sigma-Aldrich, Germany). The dosage calculation for LPS was selected based on previous studies (Van Gucht et al. 2004; Wyns et al. 2015). The dosage calculation for LTA was selected based on previous study (Atanasova et al. 2011). In the treatment groups, LPS (L2880-100 mg-*E. coli* 055-B5, Sigma-Aldrich, Germany) at a dose of 20 μg/1 kg live weight and LTA (L2515-25mg-*S. aureus*; Sigma-Aldrich, Germany) at a dose of 50 μg/1 kg live weight were applied to each lamb via intranasal route. The same dosages were administered together in the LPS + LTA group (LPS + LTA). PBS (250 μl) was administered intranasally to the control group through a micropipette. After the application, rectal temperature (RT) and blood pressure (BP) data were taken from the animals at 24 h and on day 7 with an electronic thermometer (Microlife, Taiwan) and stethoscope respectively. All the data were recorded.

Blood collection and PBMC isolation

Blood samples were collected from *Vena jugularis* in K_3EDTA tubes at 24 h and day 7. As Uddin et al. 2012) reported, PBMC isolation was calculated according to 15-ml falcon tubes (Sigma, Cat. 10771). Trizol was added on to the isolated PBMC pellet and stored at − 80 °C freezer until RNA isolation.

RNA isolation and cDNA synthesis

RNA isolation was performed, with some modification, according to the guanidium-acid-phenol extraction protocol described by Farrell (2010). DNase application was performed as recommended by the manufacturer (DNase I recombinant-Sigma-Aldrich, Germany). For cDNA synthesis, Roche Transcriptor High Fidelity cDNA synthesis kit (Sigma-Aldrich, Germany) protocol was applied. At the end of the process, samples were stored at − 20 °C until use in the study.

Primer design

Primers of *TLR2*, *TLR4*, *MyD88*, *TRAF6*, *TNF-α*, *IL-1ß*, *IL-6*, *IL-10*, *NF-κß*, and *IFN-γ* genes as well as *GAPDH* and *β-actin* selected as the house-keeping, were designed using Primer 3 2020 (http://primer3.ut.ee/), ENSEMBL, and BLAST web programs to determine gene expression levels by qRT-PCR (Table 1).

Quantitative real time-polymerase chain reaction

For the qRT-PCR, Roche Fast Start Green Master PCR kit (Sigma-Aldrich, Germany) protocol, which is used for Roche Light Cycler Nano 1.0 device, was applied. The mixture completed to 10 μl with 0.2 μM forward (0.5 μl) and reverse primer (0.5 μl) belonging to each gene; cDNA (2 μl) and nuclease-free water (7 μl) were placed into sterile strips. Ten microliters of Fast Start Green Master was added to the mix, and it was loaded into the device.

The prepared mix was loaded into the device, and the qRT-PCR protocol was arranged to be at 95 °C for 10 min (5 °C/s ramp), touchdown from 95 °C (58–63) to 72 °C and 30 s 45 cycles at 95 °C. The melting temperature was adjusted from 65 °C to 95 °C to make it 0.1 °C/s. The products obtained

Table 1 Primers, PCR lengths, melting temperatures, and ensemble IDs of genes whose expression levels are to be determined

Genes	Primer sequence	AL (bp)	TM (°C)	Ensemble ID
TLR2	F: TCTCCCACTTCCGTCTCTTT R: TGATCTTCCGCAGCTTACAG	111	60	ENSOART00000016373.1
TLR4	F: AACTTCTCCAGGTTCCCAGA R: AGGGTTTCCCGTCAGTATCA	123	58	ENSOARG00000005792
MyD88	F: ATGGTGGTGGTTGTCTCTGA R: TGCTGGGGAACTCTTTCTTC	145	58	ENSOARG00000001031
TRAF6	F: TGGAAAGCAAGTACGAGTGC R: GGACATTTGTGACCTGCATC	127	63	ENSOARG00000019079
NF-κβ	F: ATCTGAGCATTGTGCGACTG R: AGCCAGCTGTCTTGTCCATT	159	58	ENSOARG00000000483
TNFα	F:TGGTTCAGACACTCAGGTCATC R: AGCGCTGATGTTGGCTACAA	77	60	ENSOARG00000008333
IL-1β	F: AGAGCAAAAATCCCTGGTG R: ACGAAGCTCATGCAGAACA	102	60	ENSOARG00000020866
IL-10	F: GAAGGACCAACTGAACAGCA R: CCTCCAGGTAAAACTGGATCA	107	60	ENSOARG00000006292
IFN-γ	F: GGAGGACTTCAAAAGGCTGA R: GGTTAGATTTTGGCGACAGG	110	60	ENSOARG00000001958
GAPDH	F: ACCCCTTCATTGACCTTCAC R: TTCCATTGATGACGAGCTTC	114	60	ENSOART00000006848
β-actin	F: TCCCTGGAGAAGAGCTACGA R: TAGAGGTCCTTGCGGATGTC	179	62	ENSOART00000003275

AL, amplicon length; *TM*, temperature of melting; *bp*, base pair

after qRT-PCR for each gene were viewed with 1% agarose gel electrophoresis.

Statistical analysis

The statistical significance between 24 h and day 7 time periods of the "RT" and "BP" of the experimental groups was determined by one-way ANOVA multiple comparison test. Also the statistical significance between experimental groups of the studied time periods was determined with the same test. Rectal temperature and blood pressure of the examined time periods are given in Fig. 1.

Relative gene expression data were compared with ΔΔCT method using Microsoft Excel 2007 software (Livak and Schmittgen 2001) for normalization by taking the Geometric mean of reference genes *GAPDH* and *β-actin* (Vandesompele et al. 2002). Statistical significance of expression differences between groups was determined by Kruskal-Wallis (nonparametric ANOVA) and Dunn multiple comparison test. Significance level was accepted as $P < 0.05$. Relative gene expression data for all genes are shown in Figs. 2, 3, 4, and 5 with means ± standard error.

Results

Mean body weight and age of all animals used in the study are presented in Table 2.

For RT parameters, a statistically significant difference was observed between LPS and LTA groups ($P < 0.05$) on day 7, and also statistical significance was observed between 24 h and day 7 time periods in LPS ($P < 0.01$) groups. For BP parameters, a statistically significant difference between control and LTA groups ($P < 0.05$) was determined at 24 h time period. The differences between control and LPS ($P < 0.001$) as well as LPS and LTA + LPS ($P < 0.05$) on day 7 were statistically significant. Also the statistical significance was observed between 24 h and day 7 in LPS ($P < 0.05$) groups (Fig. 1).

As a result of statistical analysis of qRT-PCR, *TLR2* [LPS ($P < 0.05$), LPS + LTA ($P < 0.01$)], *TLR4* [LPS + LTA ($P < 0.05$)], *TNF-α*, *IL-10* [LPS + LTA ($P < 0.05$)], and *IFN-γ* [LPS + LTA ($P < 0.05$)] genes increased in all groups compared to the control group at 24 h. Furthermore, the differences between the control and LPS + LTA ($P < 0.01$) groups of the *IL-1β* gene and the control and LTA ($P < 0.05$) groups in the *MyD88* gene were found to be statistically significant. In the same time period, according to statistical analysis out of the control group, differences between the groups *TLR4* [between LTA and LPS + LTA ($P < 0.05$)], *MyD88* [between LTA and LPS + LTA ($P < 0.01$)], *IL-1β* [between LTA and LPS + LTA ($P < 0.05$), LPS and LPS + LTA ($P < 0.05$)] and *IL-10* [LTA and LPS + LTA ($P < 0.05$)] were found statistically significant. Expressions of *TLR2*, *TLR4*, *TNF-α*, *IL-10*, and *IFN-γ* genes were decreased in all

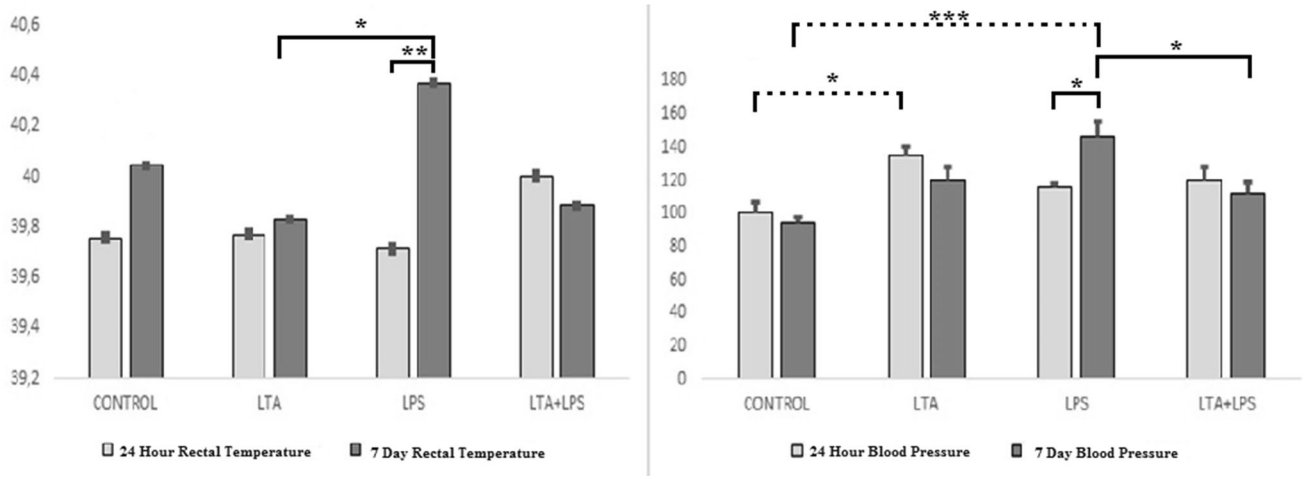

Fig. 1 Values of groups rectal temperature and blood pressure of 24 h and day 7. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

groups on day 7 compared to 24 h in two different time periods.

On day 7, compared to the control group, differences in *MyD88* [control and LTA ($P < 0.05$); control and LPS + LTA ($P < 0.05$)], in *IL-1β* [control and LPS ($P < 0.05$)], in *IL-10* [control and LTA ($P < 0.05$)] were found to be statistically significant. At the same time, differences in *TLR2* [between LTA and LPS + LTA ($P < 0.05$)], in *TLR4* [between LTA and LPS + LTA ($P < 0.05$)], in *IL-1β* [between LPS and LPS + LTA ($P < 0.05$)], and in *IL-10* [between LTA and LPS ($P < 0.01$); LTA and LPS + LTA ($P < 0.05$)] were statistically significant. The means ± SEMs and statistical significance values of expression levels of the genes examined at 24 h and on day 7 are shown in Figs. 2, 3, 4, and 5.

Discussion

In the early stages of infection, TLRs recognize invasive organisms such as bacteria, viruses, fungi, and parasites and provide a critical link between innate and adaptive immune systems (Crellin et al. 2005; Murphy 2014). Expression studies investigating the responses of TLRs in sheep PBMCs to bacterial PAMPs are very few. The periods examined in the study were at 24 h in which the early stimulated response was given and on day 7 in which immune cells were activated (Murphy 2014). According to the data obtained at 24 h time period, the mRNA expression of the *TLR2* and *TLR4* genes obtained from total PBMC as a result of intranasal administration of LPS, LTA, and LPS + LTA was performed to determine the increases in expression level in all groups compared to the control group. Similar to this result, in an in vitro study, as a result of two different doses of LPS in porcine PBMC *TLR2* and *TLR4* gene expression level has been reported to increase up to 48 h (Uddin et al. 2012). In another in vitro study, it was reported that the expression level of the same genes increased up to 48 h following the LPS stimulation in porcine alveolar macrophages (Islam et al. 2012). In this study, the expression levels of the *TLR2* and *TLR4* genes in the combined (LPS + LTA) group were found to be the highest compared to those of other groups where LPS and LTA were administered separately. Combination of both

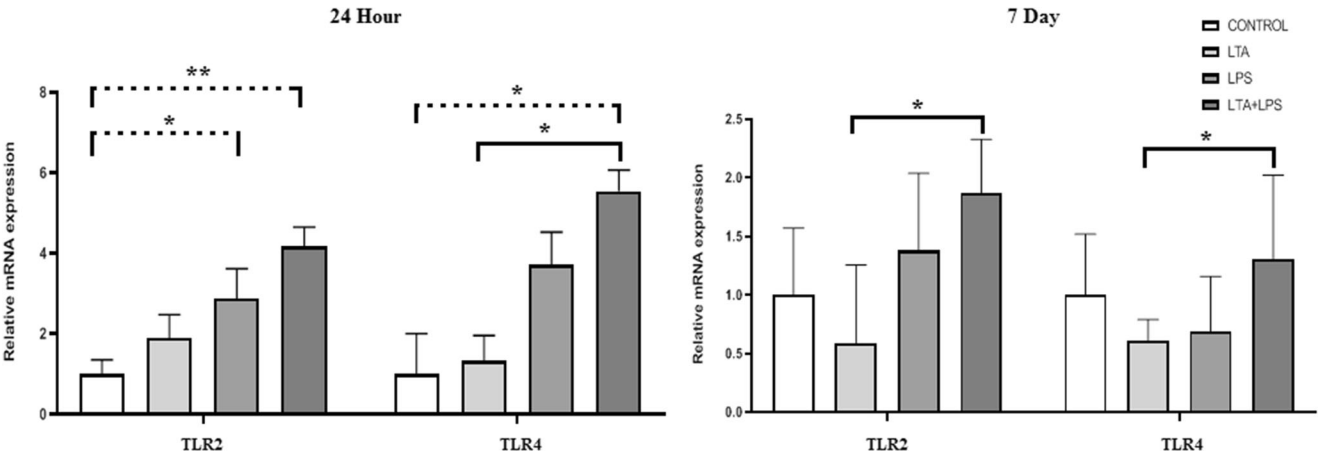

Fig. 2 Expression levels (mRNA expression level) of 24 h and day 7 of *TLR2* and *TLR4* genes examined in all groups. *$P < 0.05$; **$P < 0.01$

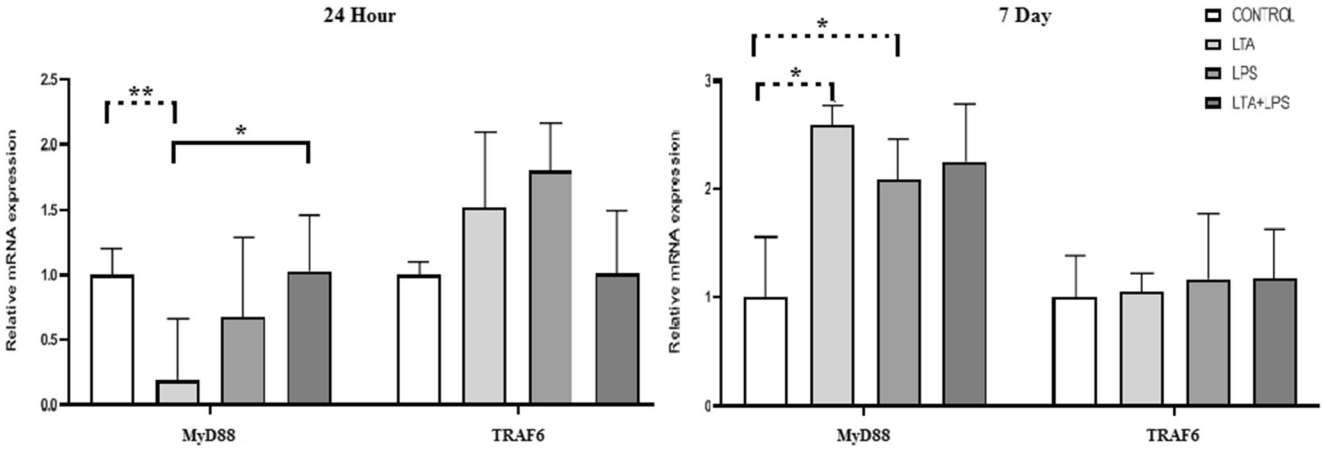

Fig. 3 Expression levels (mRNA expression level) of the 24 h and day 7 signaling *MyD88* and *TRAF6* genes examined in all groups. $*P < 0.05$; $**P < 0.01$

Gram-negative and Gram-positive agents has been observed especially in respiratory-related bacterial infections (Cinar et al. 2012), and the high expression levels of the *TLR2* and *TLR4* genes in the LPS + LTA group support this finding.

Studies have shown that TLR2 is a ligand associated with LTA (Echchannaoui et al. 2002; Takeuchi et al. 1999) and that the cellular receptor for LPS may also be a signal component (Kirschning et al. 1998). As a matter of fact, according to the data obtained at 24 h in our study, the expression of *TLR2* gene increased in LPS, LTA, and LPS + LTA groups compared to the control group. The TLR4 gene, which is reported to be directly linked to the applied LPS, was also observed to be increased at 24 h time period in all groups compared to the control group. After intraperitoneal administration of LPS and LTA in mice, the levels of *TLR2*, *TLR6*, and *TNFa* mRNA gene expression in lung tissue obtained at 4 h have been reported to increase significantly, especially after LPS administration, but LTA had no effect (Ehrentraut et al. 2011). In our study, the intranasal route of administration and application doses were found to be less effective in sheep PBMCs obtained at 24 h after the administration in the LTA group compared to the other groups but higher than the control group. Knapp et al. (2008) controlled pulmonary inflammation by *TLR4* either directly or indirectly, especially as a result of in vivo LTA administration; therefore, in our in vivo study, *TLR4* expression level may be expected to increase in the LTA group compared to the control group.

Studies conducted about TLRs focused on innate immune system cells, but it has been reported that they play important roles in adaptive immune cell function as well (Reynolds and Dong 2013). Lipopolysaccharide has been reported to affect differentiation in adaptive immune cells (McAleer and Vella 2008). It has been reported that some adaptive immune cells in pigs responded directly to proinflammatory bacterial products and that they express different TLRs, including *TLR4* (Caramalho et al. 2003). In particular, it has been reported that *TLR4* (Jin et al. 2012; Salem 2011) and *TLR2* have been functionally expressed in specific adaptive immune cells (Komai-Koma et al. 2004; Liu et al. 2006; Sobek et al. 2004). Therefore, in our study, it was thought that the increase in *TLR2* and *TLR4* expression levels detected in all groups at

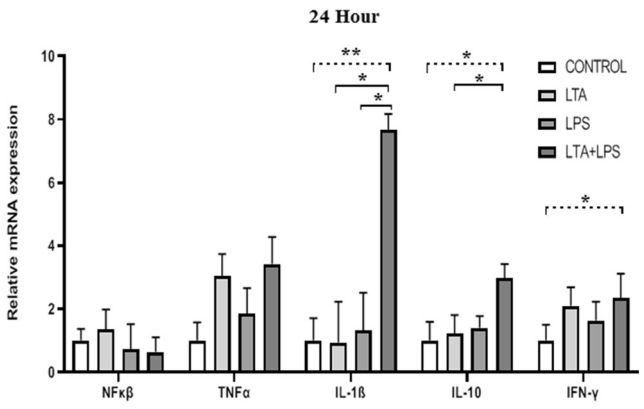

Fig. 4 24-h expression levels (mRNA expression level) of *NF-κβ*, *TNFα*, *IL-1β*, *IL-10*, *IFN-γ* genes examined in all groups. $*P < 0.05$; $**P < 0.01$

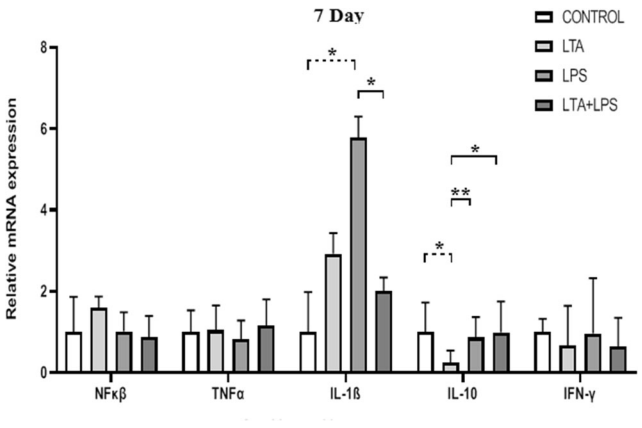

Fig. 5 Day 7 expression levels (mRNA expression level) of *NF-κβ*, *TNFα*, *IL-1β*, *IL-10*, *IFN-γ* genes examined in all groups. $*P < 0.05$; $**P < 0.01$

Table 2 Parameter values of groups average ± standard deviation (body weight, age)

Parameters	Control (*n*: 7)	LTA (*n*: 7)	LPS (*n*: 7)	LPS + LTA (*n*: 7)
Body weight	23.79 ± 2.68	30.94 ± 4.83	46.94 ± 1.47	24.45 ± 4.31
Age (month)	3	3	3	3

24 h may be due to the activation of the cells in the adaptive immune system.

Expression levels of *TLR2* and *TLR4* genes were decreased in all experimental groups on day 7 compared to the 24 h following LPS and LTA intranasal administration in lambs. Similarly, in the in vitro study conducted by Islam et al. (2012), it was reported that the expression level of *TLR2* and *TLR4* genes decreased after the 48 h following the LPS administration. In another study, it was reported that following the LPS administration in amniotic sac in sheep, the high level of expression of *TLR2* and *TLR4* genes on day 2 in fetal lung tissues decreased on day 7 (Hillman et al. 2008). If the immune system responses are not needed after completing the task of the system, the necessary mechanisms to put them into resting state are known to be activated (Murphy 2014). According to the data obtained from the study, the decrease in the expression level observed in all groups on day 7 compared to the 24 h suggested that the effect of the agent on the immune system was lost and that the immune responses entered the resting state.

Islam et al. (2012) reported that in porcine alveolar macrophage cells, the expression of the *MyD88* gene increased from 1 h to 72 h after LPS administration. In our study, at 24 h expression data of *MyD88* gene decreased in LPS and LTA groups compared to control and increased in all groups on day 7. The *MyD88* gene is an important link between innate and acquired immunity (Takeda and Akira 2004). It was thought that the increase in mRNA expression data compared to the control of the gene on day 7 may be due to regulation of the acquired immune responses. In addition, Loures et al. (2011) reported that *MyD88* is also effective in the acquired immune mechanism in mice exposed to the fungus of intratracheal agents. Seibert et al. (2010) reported that *MyD88* is particularly effective in protective acquired immunity. In our study, the increase seen in all groups belonging to this gene on day 7 was thought to be due to the acquired immune mechanism.

In this study conducted in vivo, it was determined that mRNA expression level of the gene encoding *TRAF6*, which is one of the adapter proteins, was increased in LPS and LTA groups at 24 h but this increase was not statistically significant. Furthermore, the gene expression data on day 7 decreased in all groups according to the 24 h expression data. Xue et al. (2015) reported that *TRAF6* gene increased at 24 h compared to control after *Mycoplasma ovipneumoniae* infection. In the study of Islam et al. (2012), *TRAF6* gene porcine alveolar macrophage cells after LPS showed the highest level of expression at 24 h and then it was found to be declined. According to the reported literature (Islam et al. 2012), the increase in expression data of this gene after 24 h LPS and LTA application was considered to be an expected result in our study.

In our study, we checked the expression of *NF-κβ* at 24 h and on day 7 after administration and we observed no significant differences in the expression of this gene in both time points. Enkhbaatar et al. (2015) have showed that the activity of *NF-κβ* gene is highest after 2–4 h of injection. Compared to their study, in our study measurements at 24 h and on day 7 of the *NF-κβ* gene were late, so it was considered that the timing of examination is not suitable for determining the expression level of this gene. PBMC cells in pigs have been reported to be decreased in the culture supernatant of TNFα after 48 h in vitro after LPS stimulation (Uddin et al. 2012). In a study, LPS and LTA were administered via intraperitoneal route in mice and LPS was reported to be more efficient in TNFα mRNA and protein expression over a period of 0–6 h compared to LTA administration (Ehrentraut et al. 2011). In our study, it was found that expression of this gene in PBMCs raised in all groups at 24 h compared to the control group, whereas LTA was expressed more than LPS and expression level was highest in the LPS + LTA group. Furthermore, it was observed that the expression of this gene diminished in all groups on day 7. Fernandez et al. (2013) found that LTA stimulates TNFα cytokine more than LPS at 24 h in rat alveolar macrophage culture. According to the data obtained from the study, it was thought that LPS and LTA activity in *TNFα* gene and protein may vary according to in vivo and in vitro applications. However, it is envisaged that the combined application of these agents rather than the separate applications will activate the highest level of expression.

Among the cytokines studied, IL-1β provides more leukocyte transfer to the site of infection by targeting the increase of natural and acquired immune system cells, especially stimulated by infectious agents (Murphy 2014). Ehrentraut et al. (2011) reported that the expression of this gene was higher in the LPS group than in the LTA group. In our study, the highest expression of this gene was observed in the combined group at 24 h. It has been reported that human PBMCs are expressed at different levels in macrophages and monocytes in which this gene is activated within 4 h after LPS stimulation (Netea et al. 2019). It was thought that the obtained expression difference may be due to the monocyte/macrophage ratio, which cannot be detected in PBMCs. In our study, the

expression levels determined in PBMCs on day 7 of this gene were increased by the treatments in all groups compared to the control. This suggests that acquired immune cells on day 7 may have increased expression levels of this gene. And also it was observed that the severity of fever on day 7 was directly proportional to the increase in the expression of the *IL-1β* gene in all groups.

In our study, it was observed that the IL-10 gene increased in all groups in the 24th hour and decreased in all groups in the 7th day. Previous study supports our findings (De Waal Malefyt et al. 1991).

Puech et al. (2015) reported that Concavalin A agent applied in sheep, goat, and bovine in vitro increased *IFN-γ* gene expression in all three species at 36 h expression. In our study, it was determined that in vivo bacterial agents in all groups increased the expression level of this gene at 24 h and lost its effectiveness on day 7. In a study conducted on human differentiated macrophages, Traore et al. (2012) have reported that LPS is more effective than LTA for inducing the expression of IL-1β, IFN-γ, TNFα, and IL-10 cytokines. In our study, it was observed that IL-1β, LPS in IFN-γ and TNFα cytokines were more effective in cytokines examined by mRNA expression analysis, and it had the highest effect in all genes in LPS + LTA groups.

As a result of intravenous administration of LPS in Dorset and Suffolk sheep, it was determined that the *TLR4* gene expression peaked at the 3 h in the plasma obtained from the Dorset breed and peaked at 6 h in the plasma obtained from the Suffolk breed, while decreased in both breeds at 9 h (Hadfield et al. 2018). Based on their study, Hadfield et al. (2018) reported that Suffolk breed lambs were more susceptible to disease than Dorset breed lambs according to their expression levels (Hadfield et al. 2018). Therefore, it was concluded that the responses of different breeds to the pathogen agent may be different and more studies are needed in the development of breed resistance against diseases (Hadfield et al. 2018). In genome sequence analysis of Djallonke and Sahelian sheep resistance to diseases and adaptation to the environment, it was reported that detection of heterozygous diminishing the regions related to these characters may be a sign for selection of economically important diseases (Yaro et al. 2019). In our study, it was thought that this study on Akkaraman sheep should be compared in different breeds to determine susceptibility or resistance to different diseases.

In our study, we found that LPS and LTA applied separately in this study at 24 h increased *TLR2*, *TLR4*, *TNF-α*, *IL-10*, and *IFN-γ* mRNA expression levels, but the expression level reached the highest level in the combined dose group. In addition, the decrease in the expression levels of these genes in all groups on day 7 compared to the 24 h after the application was considered an indicator of the transition of the immune system to resting state. As Gram-positive and Gram-negative agents co-exist in respiratory diseases (Cinar et al. 2012), in this study, where PAMPs of these microorganisms were examined, it was concluded that the expression level observed in the combined groups had more in vivo effect on *TLR2*, *TLR4*, *TNF-α*, *IL-10*, and *IFN-γ* genes in sheep PBMC cells at 24 h and on day 7 compared to the groups administered separately. This study, investigating the expression levels of TLR-pathway genes in PBMCs at *TLR-pathway* genes in PBMCs at 24 h and on day 7 following the treatments are thought to act as a guide for further similar studies on bacterial respiratory diseases, especially in sheep. More specific studies are needed examining the levels of expression responses of the genes to the agents, route of administration, and doses in sheep breeds. Lambs raised on farms should be controlled clinically at every stage from birth to the time of application for the studies that would be planned in the future. Studies to be planned with lambs in younger age ranges can enable the researchers to investigate the mechanism of natural immune system in more detail.

The specific cell originating from the expression differences detected in the study can be examined in more detail in peripheral blood mononuclear cells. It can contribute to planned in vivo bacterial vaccination studies in sheep in terms of dose determination. The response mechanism of the immune system can be elucidated with sequence analyses, in which larger data are processed in shorter time intervals.

Conclusion

This in vivo study may contribute to understanding the host-pathogen relationship in PBMCs at 24 h and on day 7. Particularly, during the 24 h period, sensitivity/resistance comparisons can be made by measuring the immune system responses of different sheep breeds to the applied antigenic molecules at the level of gene expression. Thus, the immune response of different breeds to bacterial agents taken naturally can be measured especially on *TLR2* and *TLR4* genes.

Funding This work has been supported by Erciyes University Scientific Research Projects Coordination Unit under grant number [TDK-2017-7343].

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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