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Investigation on the Expression Profile of Immune-Related Genes in Response to Lipopolysaccharide and Lipoteichoic Acid in Alveolar Macrophages of Akkaraman and Romanov Lambs

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ABSTRACT

The alveolar macrophages (AMs) are frontier of defense against foreign materials that initiate immune response in lungs. Knowledge of the expression dynamics of major immune-related genes in the alveolar macrophages in response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) challenge can help to understand disease mechanism involved in several respiratory diseases. The aim of this study was to investigate the mRNA expression of selected immune-related genes in response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) challenge in sheep alveolar macrophages *in vivo*. Results revealed that Romanov lambs exhibited higher mRNA expression of *TLR2*, *TLR4*, *NF-κβ*, *TNFα*, *IL-1β*, *IL-6*, *IL-8*, and *IL-10* genes as compared to Akkaraman lambs along with the control of all treatments. Moreover, the expression of *TLR2*, *TLR4*, *NF-κβ*, *TNFα*, *IL-1β*, *IL-6*, *IL-8*, and *IL-10* genes was higher in combine treatment of LPS and LTA as compared to separate treatments of LPS and LTA in both breeds. The results showed that the mRNA expression of immune-related genes was significantly increased in the sheep AMs in response to LPS and LTA treatment whereas a synergistic effect was observed in LPS+LTS treatment. Also, breed comparison showed that the native Akkaraman was more resistant to disease compared to exotic Romanov.

Akkaraman ve Romanov Kuzularının Alveolar Makrofajlarında Lipopolisakkarit ve Lipoteikoik Asite Yanıtta İmmün İlişkili Genlerin Ekspresyon Profilinin Araştırılması

MAKALE BİLGİSİ

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Koyun
TLRs yolu

*** Sorumlu Yazar**

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Alveolar makrofajlar (AM'lar), akciğerlerde bağışıklık tepkisini başlatan yabancı maddelere karşı savunma bariyeridir. Lipopolisakkarit (LPS) ve lipoteikoik asit (LTA) tehdidine yanıt olarak alveolar makrofajlardaki majör immün bağlantılı genlerin ekspresyon dinamiklerinin bilinmesi, çeşitli solunum yolu hastalıklarında yer alan hastalık mekanizmasının anlaşılmasına yardımcı olabilir. Bu çalışmanın amacı, koyun alveolar makrofajlarında lipopolisakkarit (LPS) ve lipoteikoik asit (LTA) stimülasyonuna yanıt olarak seçilen bağışıklıkla ilgili genlerin mRNA ekspresyonunu *in vivo* araştırmaktır. Sonuçlar, Romanov kuzularının *TLR2*, *TLR4*, *NF-κβ*, *TNFa*, *IL-1β*, *IL-6*, *IL-8* ve *IL-10* genlerinin mRNA ekspresyonunu Akkaraman kuzularına kıyasla kontrol grubuna göre daha yüksek saptandığını ortaya koymuştur. Ayrıca, her iki ırkta LPS ve LTA'nın kombine uygulanmasında *TLR2*, *TLR4*, *NF-κβ*, *TNFa*, *IL-1β*, *IL*-6, *IL-8* ve *IL-10* genlerinin ekspresyonu, LPS ve LTA'nın ayrı uygulamalarına kıyasla daha yüksektir cinste. Çalışmanın sonuçları, LPS ve LTA uygulamalarına yanıt olarak koyun AM'larında bağışıklıkla ilgili genlerin mRNA ekspresyonunun önemli ölçüde arttığını, LPS+LTS uygulamasında ise sinerjik bir etkinin gözlemlendiğini gösterdi. Ayrıca, ırk karşılaştırması, yerli Akkaraman'ın Romanov'a kıyasla hastalığa karşı daha dirençli olduğunu gösterdi.

Introduction

Respiratory infections contribute to significant financial losses, health management problems (Lacasta et al., 2019) and have a direct impact on health and productivity of sheep flocks (Nettle et al., 2010). Several studies depicted the consequences of respiratory illnesses and diseases involved 6.5% financial loss of lamb cost (Luzon, 1999), 78% cause of mortality (Gonzalez, 2015) and 22-34% condemnations in abattoirs (McRae et al., 2016; Goodwin et al., 2004; Gonzalez, 2015). In response to respiratory antigen-related stimulation or inflammatory stimulus, innate immune response of the lungs acts quickly and mediates its effects on the foreign material (Rock & Hogan, 2011; Hoebe et al., 2004). Alveolar macrophages are the most abundant pulmonary cells and the first line of the defense in this innate immune response (Hu & Christman, 2019) by playing a central role in the pathogen recognition that enters the lung (Daly et al., 2009; Twigg, 2004; Medzhitov, 2007). In this response mechanism AMs recognize the foreign particles signals through Toll-like receptors (TLRs), intracellular pattern-recognition receptors, interleukin-1 receptor (IL-1R), cytokines, growth factors and other mediators released in the lung environment and AMs phenotypically and functionally modified to acquire anti & pro-inflammatory, anti & pro-fibrotic, pro-resolving, pro-asthmatic and tissue regenerating properties (Mosser & Edwards, 2008; Duffield et al., 2013).

In the same pattern, when macrophages are exposed to LPS, they can produce an array of cytokines including *IL-6*, *IL-1*, and *TNF-α* which later work as an internal mediator of inflammation through the interaction between receptor and ligand of a variety of target cells (Dempsey et al., 2003). By contrast, LTA can bind with the target cells following both nonspecific and specific pathways (Pinilla-Vera et al., 2016). Toll-like reporters (TLRs) are considered as the prime components in the immune system against various microorganism-

mediated infections, the improper response of TLRs can result in chronic and acute inflammatory diseases as well as a variety of autoimmune diseases (Islam et al., 2013;Yoshimura et al., 1999; Perkins & Vogel, 2015). In addition, Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) are the important outer membrane components of Gram-negative and Gram-positive bacteria, respectively; and they are the causal factors for the respiratory disease in sheep (Uebelhoer et al., 1995; Su et al., 2006; Sriskandan & Cohen, 1999). Despite the importance of AMs in host defense, there is limited knowledge of the factors that are crucial for their effector's functions. It has been reported that different cytokines can be produced by porcine AMs after initial stimulation with LPS (Islam et al., 2012a). Although some investigations have been published recently on the effects of LPS and other pathogen-associated molecular patterns (PAMPs) in porcine immune cells (Cinar et al., 2012; Raymond & Wilkie, 2005), yet the response of AM cells against LPS and LTA challenge in sheep has not been studied extensively.

In this study, we aimed to investigate the expression of some immune related genes associated with immune response against respiratory infections, for understanding of immune status and immunogenomic landscape of sheep breeds in detail. Ultimately, it could enhance the effectiveness of sheep selection on the basis of resistance to respiratory infections by testing different kinds of breeds and compare them with the native breed through the investigation of expression profile of some immune-related genes. Thus, this study may provide an insight to enhance the productivity of sheep flocks by limiting the lung diseases.

Materials and methods

Preparation of Reagents

Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Sigma-Aldrich, St. Louis, MO, USA) were used to treat the experimental animals for 24 hours. Briefly, 20 µg of LPS was administrated for each kg of live weight of the animal. On the other hand, 50 µg of LTA was used per kg of an animal. For the control group, sterilized PBS (1X) 500 µl per animal was administrated in the form of an intranasal aerosol.

Preparation of Animals

The whole procedure was carried out in strict accordance with the protocol approved by the Animal Ethics Committee of Erciyes University (Permit No: 17/009 on 15/02/2017). Twenty-four healthy lambs of two different breeds (Akkaraman: n=12 and Romanov: n=12) were used in this study. The experimental procedures were approved by the Animal Welfare Committee of Erciyes University. The age of the experimental animals was about four months with approximately equal body weight (BW); and animals were evenly divided into four groups (LPS, LTA, LPS+LTA, and Control). The weather conditions and dietary supplements were equal for all animals in this experiment. Animals were fed *ad libitum*. After the treatment, the rectal temperature and heartbeats were measured Table 2. All lambs from the two different breeds were euthanized after 24 h of the treatment period in a licensed slaughterhouse in Kayseri province. The animal trial experiments were performed at the Agricultural Farm Research Site at Erciyes University, Kayseri province of Turkey.

Isolation of Alveolar Macrophages (AMs)

Lungs were collected and transported on ice to the laboratory within 2 h following slaughter. The bronchoalveolar lavage (BAL) fluid was obtained by adding cold and sterilized PBS (1X) into the lung. The lungs were palpate and shaken gently for 2 to 3 mins. The BAL fluids were collected in a sterilized 50 ml falcon tube and filtered through sterile gauze. The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) by centrifugation at 4 °C for 10 min at 400 × g. Following this, 500 µl of RBC Lysis buffer (1X) was added to the BAL cells and mixed gently by pipetting and incubated at room temperature for 30 s for complete removal of erythrocyte contamination. Isotonicity was restored by adding 10 ml of complete RPMI-1640 media. The cell mixture was centrifuged in 700 × g for 5 min and resuspend the cells by adding culture media (RPMI-1640 medium with L-glutamine and sodium bicarbonate, R8758) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (100 µg/ml). A total of 1.6×10^6 viable cells/ml were dispensed into each well of the 6-wells cells culture dish (Corning, NY, USA) and incubated in an incubator at 37.0 °C and 5% CO_2 in a humidified environment for 4 h to allow the alveolar macrophages to adhere to the bottom of the wells (Yang et al., 2016; Gao et al., 2010).

Harvesting the Alveolar Macrophages (AMs)

After 4 h, the culture plates were washed twice to remove floating cells and 750 µl of 0.25% Trypsin-EDTA was added to each well of the culture plate and incubated in the incubator (37.0 °C and 5% CO_2) for 5 min. Afterward, the plates were taken out from the incubator and immediately put on the ice for 20 min and the plates were periodically shaken and checked under a microscope to ensure the complete detachment of AM cells. Once all the AMs were disassociated from the culture plate, 2 ml of warm culture media was added to each well to stop the activity of the Trypsin-EDTA and centrifuged at 700 × g for 5 mins at room temperature to collect the AMs in the form of pellets and stored immediately at -80 °C freezer for later use (Hussell & Bell, 2014; Zhou et al., 2010).

RNA Extraction and cDNA Synthesis

The total RNA was extracted from AMs (n = 3) by using high pure RNA isolation kit (Roche Life Science, Penzberg, Germany) according to the manufacturer's instructions with some modification. The concentrations and purity of total RNA were checked by a BioSpec-Nano Spectrophotometer (Shimadzu Biotech, Japan). The total RNA was then reversed transcribed to cDNA using a commercially available cDNA synthesis kit (Applied Biological Materials, Vancouver, Canada) according to the manufacturer's protocol. The resulting cDNA was diluted with RNase free H_2O (two floods: approximately 40 µl) to have a suitable concentration of cDNA and stored at -20 °C freezer for later use.

Quantitative Real-Time PCR (qRT-PCR)

The Real-Time PCR reaction was performed in a Roche LightCycler 480 quantitative Real-Time PCR (qRT-PCR) instrument (Roche Life Science, Penzberg, Germany). The primers were designed using online software Primer 3 (Koressaar & Remm, 2007) and the primer sequences are presented in Table 1. *GAPDH* and *ACTB* were used as endogenous control. Two

housekeeping genes (*GAPDH* and *ACTB*) were used as normalizer based on the information described in Cinar et al. (2012). Relative expressions of each mRNA were analyzed using a comparative ct ($2^{\Delta\Delta ct}$) method (Sohel et al., 2017).

Table 1. List of primers that were used in this study for qRT- PCR experiment
Tablo 1. qRT-PCR deneyi için bu çalışmada kullanılan primerlerin listesi

Genes	**Accession number**	**Primer sequences (5' -> 3')**	**Annealing temp.(°C)**
GAPDH	NM_001034034.2	F-GAAGCTCGTCATCAATGGAAA	58
		R-CCACTTGATGTTGGCAGGAT	56
ACTB	171120-1-76	F-ATTCCATCATGAAGTGTGACG	53
	171120-1-77	R-TGATCCTCAAGAAGGATGGAA	55
IL-1β	180221-1-27	F-CAGCCGTGCAGTCAGTAAAA	56
	180221-1-28	R-GAAGCTCATGCAGAACACCA	55
IL-6	171120-1-84	F-TGACGAGTGTGAAAACAGCAA	55
	171120-1-85	R-CTGATTGAACCCAGATTGGAA	53
IL-8	180221-1-29	F-CTATCAACCACCCTCCTCCA	54
	180221-1-30	R-CCATTTGGGCTGAAAACAGT	53
IL-10	180221-1-31	F-TTTAAGGGTTACCTGGGTTGC	53
	180221-1-32	R-AGTTCACGTGCTCCTTGATGT	56
TNFα	171120-1-88	F-TGATGCTGATTTGGTGACCGA	55
	171120-1-89	R-CACTTTATTTCTCGCCACTGA	54
NF-$_k$B	171120-1-86	F-GCCATTGTCTTCAAAACTCCA	53
	171120-1-87	R-GTTTTGGTTCGCTAGTTTCCA	54
TLR2	171120-1-90	F-GCAATTCACCGATGACAGTTT	54
	171120-1-91	R-GTAAAATCGCCAACTCCATCA	53
TLR4	171120-1-94	F-TGGATTTTCAGCATTCCACTC	53
	171120-1-95	R-ACAATCCGGATGTTGGTGTAA	53

* bp: base pair**.**

Statistical analysis

The qRT-PCR data were displayed as the mean of triplicates data ± standard deviation for each cycle threshold (Ct) value. Microsoft Excel (365, student version) 2016 was used for the statistical analysis and graph preparation. The significance of difference among three

treatment groups was determined by Student's t-test. A p-value of ≤ 0.05, is considered significant. A clustering heat map analysis for all treatment and control groups was done by using fold change values and visualized with R Software (Wickham, 2009).

Results

Post-treatment phenotypic changes in the experimental animals

We have observed slight phenotypic changes in the animals after 24 h exposure to LPS, LTA or LPS + LTA. The remarkable changes were sneezing, lack of movement, redness of the eyes, dripping tears from the eye and the tendency of the animal to stagnate near sources of food and drink. No significant differences were observed in the rectal temperature, heartbeats, and live weight of the animals after the treatments Table 2.

Table 2.The weight, rectal temperatures, heartbeats and the weight of the Lambs were measured after 24 h of exposed to LPS, LTA and with the combination of LPS + LTA treatments
Tablo 2. LPS, LTA ve LPS + LTA tedavilerinin kombinasyonu ile 24 saat sonra kuzuların ağırlıkları, rektal sıcaklıkları, kalp atışları ve ağırlıkları

No	Breeds	Treatments	Weight/ kg	Doses/µl/kg	Temp after treatment ºC	Heartbeats after treatment/min
1	Akkaraman	Control	32.6	500	39.5	112
2	Akkaraman	Control	37	500	39.7	114
3	Akkaraman	Control	30.4	500	40	96
4	Akkaraman	LPS	51	210	40.5	100
5	Akkaraman	LPS	45.8	190	40.1	118
6	Akkaraman	LPS	46.5	190	40.2	112
7	Akkaraman	LTA	42.6	430	39.3	136
8	Akkaraman	LTA	42.3	420	39.5	148
9	Akkaraman	LTA	40.7	410	39.7	140
10	Akkaraman	LPS+ LTA	51.5	120 + 515	39.9	114
11	Akkaraman	LPS+ LTA	55.4	220 + 560	39.7	134
12	Akkaraman	LPS+ LTA	61.6	250 + 620	40	136
13	Romanov	Control	15.1	500	39.4	94
14	Romanov	Control	15.5	500	40.1	126
15	Romanov	Control	25.4	500	39.2	110
16	Romanov	LPS	24.5	100	39.8	102
17	Romanov	LPS	20.3	90	40.8	112
18	Romanov	LPS	16.7	60	39.9	100
19	Romanov	LTA	32.2	322	40	118
20	Romanov	LTA	19.9	200	39.9	112
21	Romanov	LTA	20.4	204	40.4	140
22	Romanov	LPS+LTA	32.5	130 + 370	39.9	112
23	Romanov	LPS+LTA	20.3	90 + 200	40	114
24	Romanov	LPS+LTA	17.9	80 + 180	39.4	136

Expression levels of candidate reference genes

Mean Cycle threshold (Ct) values of 7 candidate reference genes ranges from -4.83 for *IL-1* to 9.89 for *IL-10* gene in 4 hours treatment of respectively while -2.26 for *IL-1* to for 11.16 for *IL-8* gene in 24 hours treatment of combine LPS+LTA and LTA respectively. The expression level

of *TNF-α* had the largest variation among all treated samples with the average Ct SD value of 0.885 in 4 hours treatments followed by *IL-1* and *IL-10* with average Ct SD value of 0.705 and 0.5, respectively. While the treatments of 24 hours, highest variation observed in *IL-8*, *TNF-α* and *IL-1* with average CT SD values of 1.01, 0.71 and 0.61, respectively. All mean Ct values with their SD values are represented in Fig. 1.

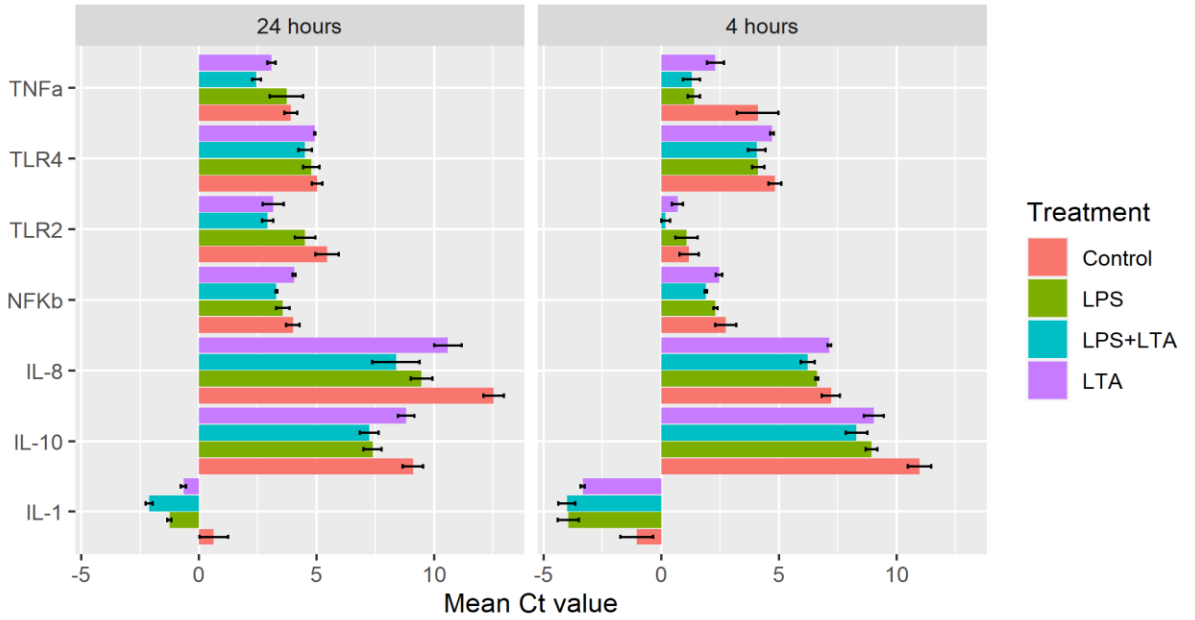
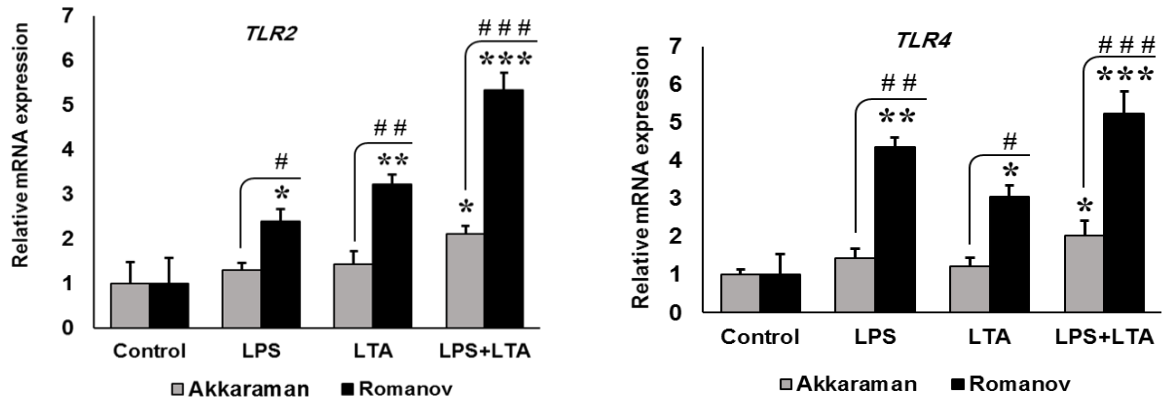


Figure 1. Graphical presentation of mean cycle threshold (Ct) values of candidate reference genes in AMs under treatment of LPS, LTA and LPS+LTA and their control with Ct SD values (4 hours and 24 hours groups)

Şekil 1. LPS, LTA ve LPS+LTA tedavisi altındaki AM'lerde aday referans genlerin ortalama döngü eşiği (Ct) değerlerinin grafiksel sunumu ve bunların Ct SD değerleri (4 saat ve 24 saat grupları) ile kontrolü

Gene expression of TLR2 and TLR4

In the case of Akkaraman lambs, LPS and LTA treatments did not significantly increase the gene expression of *TLR2* and *TLR4* compared to control, while, LPS+LTA treatment showed a significant increase compared to control. However, Romanov lambs showed a significant increase in the expression of *TLR2* and *TLR4* in all treatment groups compared to control Figure. 2. Interestingly, significantly higher expression of *TLR2* and *TLR4* was observed in the AMs of Romanov lamb compared to those of Akkaraman lambs in all the treatment groups Figure 2ab.



(a) **(b)**

Figure 2ab. The mRNA expression differences of *TLR2* and *TLR4* in Alveolar Macrophages cells between Akkaraman and Romanov lambs exposed to LPS, LTA and a combination of LPS + LTA treatment for 24 h time point

Şekil 2ab. 24 saatlik zaman noktası için LPS, LTA ve LPS + LTA tedavisinin bir kombinasyonuna maruz kalan Akkaraman ve Romanov kuzuları arasındaki Alveolar Makrofaj hücrelerinde TLR2 ve TLR4'ün mRNA ekspresyon farklılıkları

Asterisks (*) indicate the significant mRNA expression levels differences between Akkaraman and Romanov breeds compared with control in response to LPS, LTA and with both LPS + LTA treatments (*P<0.05)

Gene expression of IL-1β, IL-6, IL-10, and IL-8

Although there was no significant increase in the expression of *IL-1β* and *IL-6* in the AMs of Akkaraman lamb treated with LPS and LTA, the expression was significantly increased in LPS+LTA treatment compared tho those of control Figure 2ab. On the other hand, the expression of *IL-10* and *IL-8* was significantly increased in the AMs of Akkaraman lamb treated with LPS and LPS+LTA compared to control, while no increase was observed in LTA treatment Figure. 3c,d. However, the expression of *IL-1β*, *IL-6*, *IL-10*, and *IL-8* was increased with a various significance level in the AMs of Romanov lamb in all treatment groups compared to those of control Figure. 2a-d. In addition, it is important to note that the highest expression of all these genes was observed in the AMs of Romanov lambs treated with LPS+LTA. Nevertheless, the lowest level of *IL-1β*, *IL-6*, *IL-10*, and *IL-8* mRNA expression increase was observed in AMs of Akkaraman lambs treated with LTA. The expression of these genes was significantly higher in all treatment groups in the AMs of Romanov compared to those of Akkaraman lamb Figure 3a-d.

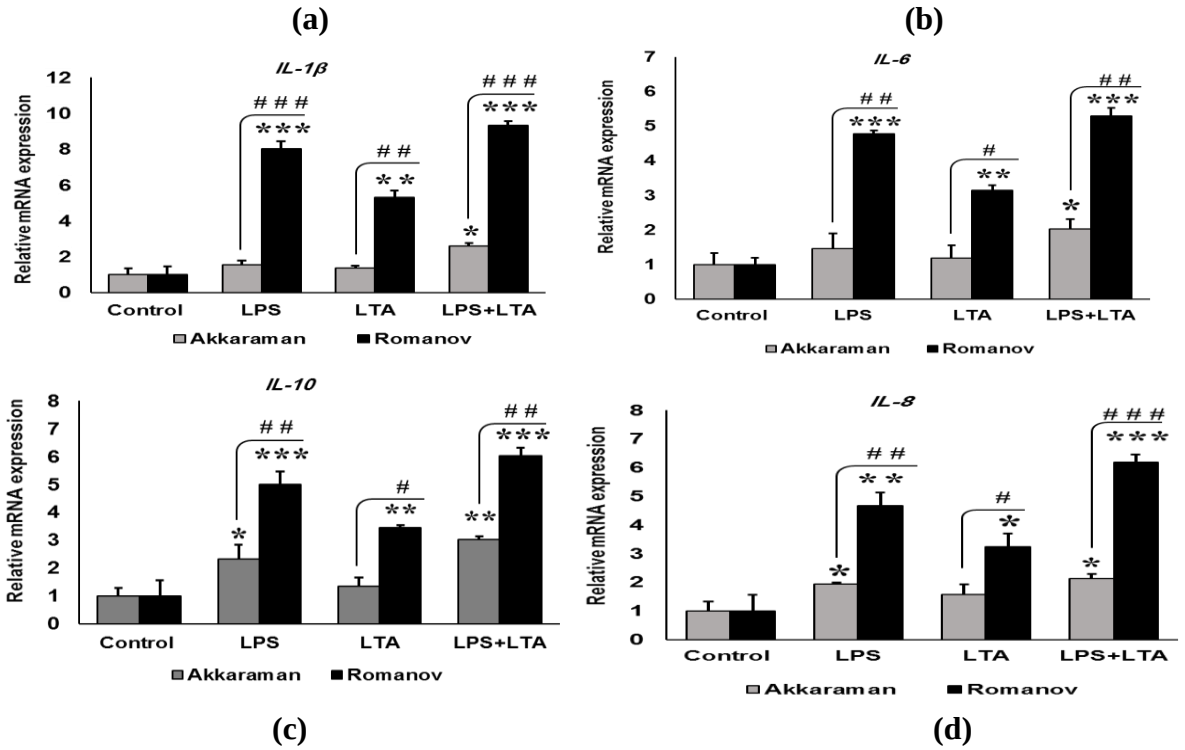


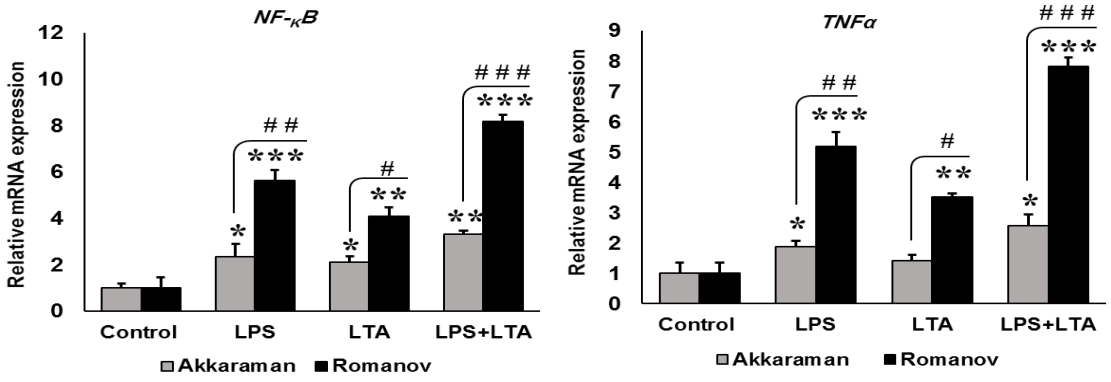
Figure 3a-d. The differential mRNA expression levels of *IL-1*β, *IL-6*, *IL-10* and *IL-8* in Alveolar Macrophages cells of Akkaraman and Romanov lambs treated with LPS or LTA or a combination of LPS + LTA after 24 h of treatment

Şekil 3a-d. 24 saatlik tedaviden sonra LPS veya LTA veya LPS + LTA kombinasyonu ile tedavi edilen Akkaraman ve Romanov kuzularının Alveolar Makrofaj hücrelerinde IL-1β, IL-6, IL-10 ve IL-8'in diferansiyel mRNA ekspresyon seviyeleri

(#) refer to the mRNA expression levels differences between the two different sheep breeds (Akkaraman and Romanov) in response to LPS, LTA, and with the combination of LPS + LTA (*P<0.05)

Gene expression of NF-κβ and TNFα

Almost similar expression patter *NF-κβ* and *TNFα* was observed in the AMs of both breeds. The expression of *NF-κβ* and *TNFα* was significantly increased in the AMs of both Akkaraman and Romanov lambs in all treatment groups compared to those of control except in the AMs of Akkaraman treated with LTA Figure. 4a,b. The highest level of expression was observed in the AMs of Romanov lamb when they were treated with LPS+LTA, while the lowest expression was observed in the AMs of Akkaraman lambs when they were treated with LTA Figure. 4a, b. In addition, compared to Akkaraman lambs, the AMs of Romanov lamb expressed a significantly higher level of *NF-κβ* and *TNFα* in all treatments Figure 4a,b.



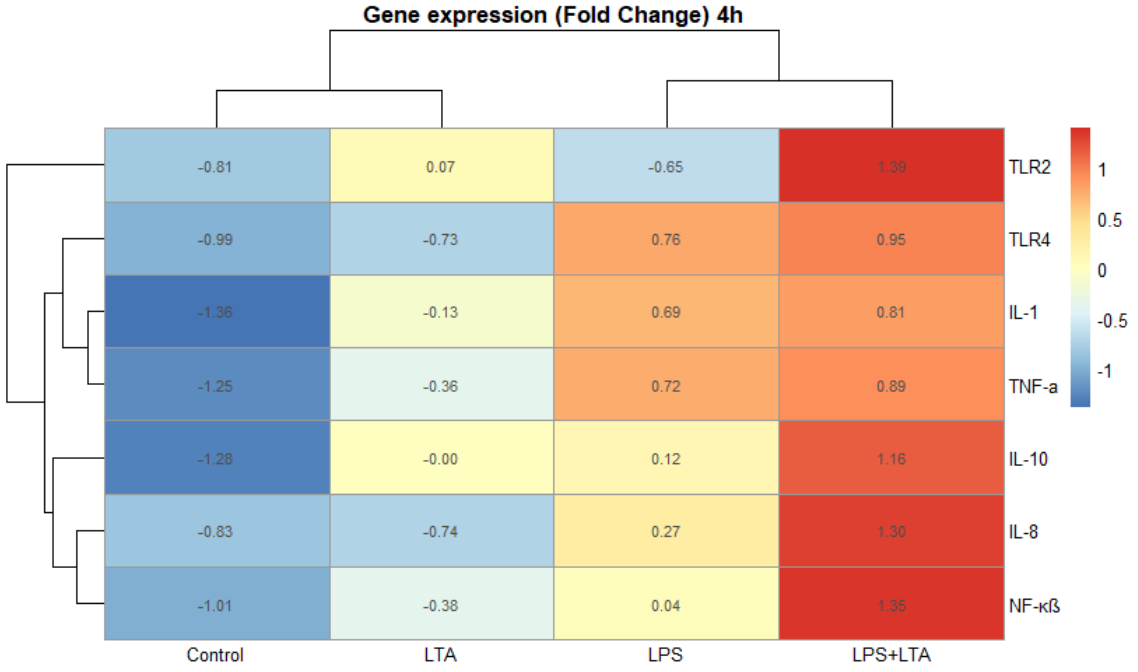
(a) **(b)**

Figure 4ab. The mRNA expression level of *NF-κB* (a) and *TNFα* (b) mRNA in Akkaraman and Romanov lambs in response to LPS, LTA and a combination of LPS + LTA treatments after 24 h time point

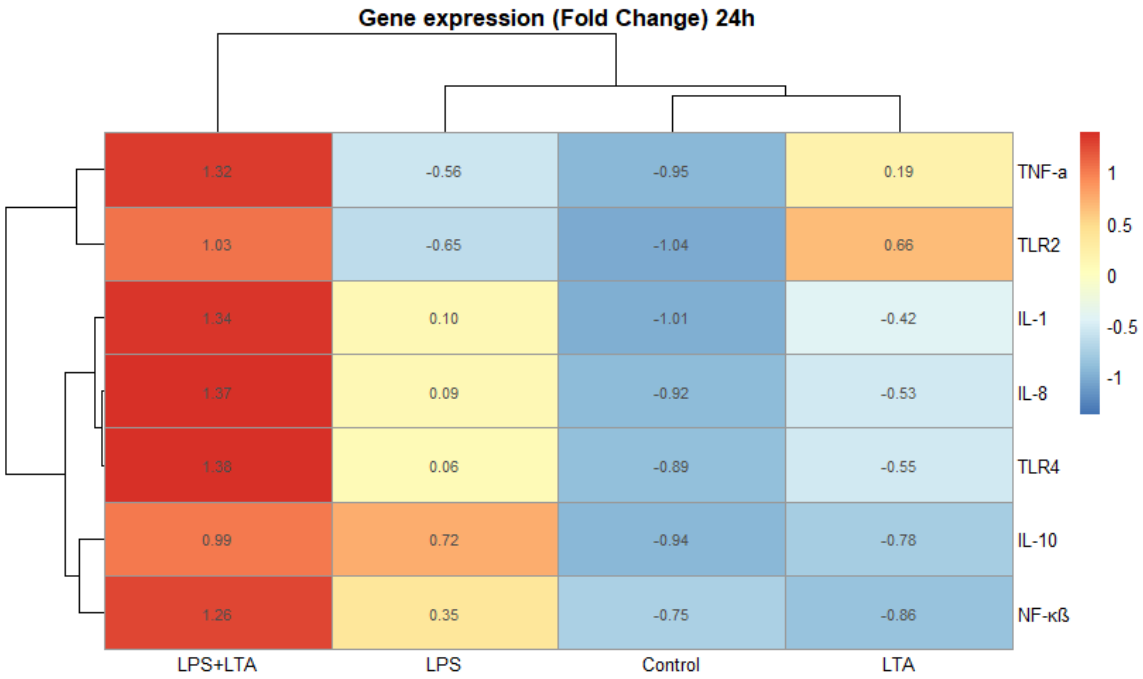
Şekil 4ab. 24 saatlik zaman noktasından sonra LPS, LTA ve LPS + LTA tedavilerinin bir kombinasyonuna yanıt olarak Akkaraman ve Romanov kuzularında NF-κB (a) ve TNFa (b) mRNA'nın mRNA ekspresyon seviyesi

Discussion

The immune response is intimately regulated in lungs because the organ is regularly exposed to environmental pollutants and respiratory microorganisms, this demands an excessive response to prevent tissue damage. That inflammatory response starts from the recognition of pathogens by Toll-like Receptors (TLRs), interleukin-1 receptor (IL-1R) and cytokines like mediators in the alveolar compartment that lead to elimination of pathogens and protection of lung tissue (Akira et al., 2006; Murphy & Weaver, 2016). Therefore, it was highly important to investigate the gene expression regulation in alveolar macrophages during the immune stimulation through pathogens and stimulants of various origin to understand the innate and adaptive immune responses (Wang et al., 2001). We were interested to challenge aveolar macrophages with LPS or LTA that are important proinflammatory component of gram-positive bacteria and gram-negative bacteria respectivly, and a combine stimulation of LPS+LTA. So, this was actually non-pathogenic but, antigentic gram-positive or gram-negative and mixed stimulation in sheep *in vivo*. Results demonstrated the upregulation of expression in response to LPS or LTA and combine triggering of LPS and LTA as shown in Fig.5a,b.



(a)



(b)

Figure 5ab. The expression levels of genes are presented using fold-change values in Akkaraman and Romanov lambs in response to LPS, LTA and a combination of LPS + LTA treatments after 4 hours (a) and 24 hours (b). Fold-change values and the color scale are shown at the left of each heat map.

Şekil 5ab. Genlerin ekspresyon seviyeleri, 4 saat (a) ve 24 saat (b) sonra LPS, LTA ve LPS + LTA tedavilerinin bir kombinasyonuna yanıt olarak Akkaraman ve Romanov kuzularında kat değişim değerleri kullanılarak sunulmaktadır. Katlama değişim değerleri ve renk skalası, her bir ısı haritasının solunda gösterilir.

Studies have described that the *TLR2* induce cell specific inflammatory cytokine production in macrophages (Yoshimura et al., 1999). So, higher the expression of *TLR2*, resulted in more the production of inflammatory cytokines.

Thus, the up regulated expression of *TLR2* in the AMs of Romanov in comparison to Akkaraman lamb possibly suggesting that with the same degree of infection, Romanov lamb could suffer more as they will produce more inflammatory cytokines. *NF-κβ* pathway is responsible for the activation of immune cells through the receptors of *TLR2* and *TLR4* (Perkins & Vogel, 2005) in response to LTA of gram-positive bacteria and LPS of gram-negative bacteria (Hussell & Bell, 2014). Moreover, the overexpression of TLR4 resulted in an increase in the level of expression of *TNF-α*, *IL-6*, and *IL-8* (Gao et al., 2010). The same argument is given by many other studies that the activation of NF-κβ resulted in the higher expression of pro-inflammatory cytokines, chemokines, antimicrobial peptides (Zhou et al., 2010), *iNOS* and *COX2* (Donaldson et al., 2005; Barbalat et al., 2009) that are involve in adaptive and innate immune response and inflammation. So, higher the expression of TLR2 and TLR4 in Romanov lambs as compared to Akkaraman lambs in all treatments suggests that Akkaraman breed is more resistant to both gram-negative and gram-positive in context of NF-κβ pathway activation. Similar gene expression was shown in peripheral blood mononuclear cells (PBMC) in response to LPS, LTA and LPS+LTA treatments in Akkaraman sheep (Aksel & Akyuz, 2021).

The interaction between inflammatory and immune cells is primarily mediated by proteins known as interleukins (ILs). In the current study, we have studied gene expression of four important ILs such as *IL-1β*, *IL-6*, *IL-8*, and *IL-10* because they are engaged with the respiratory immune response in AMs. *IL-1β* plays an important role in the defense mechanism cascade against a wide variety of bacterial infections. It is also involved in familial auto-inflammatory syndromes induced pathogenesis, and the blocking of *IL-1β* in systemic diseases reduce *IL-6* levels (Wang et al., 2000; Schwandner et al., 1999). Though it has been shown *in vivo* that the phagocytosed of bacteria is suppressed by *IL-10*, and neutralization of endogenous *IL-10* led to enhanced survival in murine models of *Mycobacterium avium* infections, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae*. Moreover, several cytokines including *IL-l*β and *TNF-α* can induce the secretion and expression of *IL-8* in astrocytes (Thompson et al., 1991; Cohen, 1997). In the case of Akkaraman lambs, the significant increase in the expression of ILs was observed mostly in the LPS+LTA groups. In contrast, in the case of Romanov lambs, a significant increase in the expression of ILs was observed in all the treatment groups. In addition, higher expression of ILs was observed in all treatment groups of Romanov lambs compared to Akkaraman lambs. Recent studies reported that *IL1β* is a highly inflammatory cytokine response by mediating its production and by stimulating the synthesis of other cytokines such as *IL-6*, *IL-8*, and tumor necrosis factor *TNF-α* and any reduction of its production or activities are more likely to have an impact on clinical medicine (Hoshino et al., 1999; Zhang et al., 2019). Our findings are agreed with a previous study in which authors reported an increase in the expression of *IL-6* in response to Gram-negative bacterial sepsis (Surh et al., 2001). A study showed that the phagocytosis of *Escherichia coli* is suppressed by *IL-10* and attenuated the activity of microbicidal neutrophil toward internalized bacteria, which is correlated with the reduction of the expression of complement receptor type 3 (CR3); and

they further showed that the major function of *IL-10* is to limit and finally terminate the response of inflammation (Thorley et al., 2007). Many studies indicated the involvement of *IL-8* in most acute and chronic inflammatory diseases and several acute infections can alter the hemodynamics and the clotting and fibrinolytic systems in ways that can precipitate ischemic events (Ishida et al., 1994; Micera et al., 2009).

The transient expression of *TNF-α* helped to prevent over inflammatory reaction. It has been shown that *TNF-α* is a major pro-inflammatory cytokine produced by mammals during infection with Gram-negative bacteria. This cytokine is generated mainly by macrophages that reside in many tissues and trigger an array of innate immune responses upon encounter of invading pathogens (Choussat et al., 2000). In the current study, we found that the expression of *TNFα* in the AMs of both Romanov and Akkaraman lambs was significantly increased in LPS and LPS+LTA treatments compared to control. The higher expression of *TNFα* in LPS treated AMs maybe because Gram-negative bacteria induce the production of pro-inflammatory cytokines like *TNFα*. (Hack et al., 1989) have reported that *TNF-α* dominated acute and excessive release of proinflammatory cytokines can be triggered by the LPS.

In conclusion, the higher mRNA expression of certain immune related genes; *TLR2, TLR4, NF-κB, TNF-α, IL-1β, IL-6, IL-10*, and *IL-8* in AMs of Romanov in response to LPS or LTA or LPS+LTA challenge possibly indicating that this breed is susceptible to a wide variety of respiratory infection induced by Gram-positive and Gram-negative bacteria which could be characterized by the production of a higher level of other inflammatory cytokines. Moreover, the lower expression of these genes in AMs of the Akkaraman in response to different treatments indicating that this sheep is stronger and has well developed adaptive and innate immune response which can easily handle the infection induced by Gram-positive and Gram-negative bacteria-induced infection. These findings can help in selection criteria of respiratory disease resistant breeds for protection and production improvement of sheep flock.

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Conflicts of interest

The authors declare that they have no competing interests.

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