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The Expression Dynamics of Key Immune-Related Genes in Response to *Mannheimia Haemolytica* in Sheep Alveolar Macrophages *In Vitro*

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ABSTRACT

Alveolar macrophages (AMs) respond to these infections as the first line of defense and trigger the lung's immune response. Knowing the expression dynamics of key immune-related genes in alveolar macrophages against *M. haemolytica* challenge will help deepen the understanding of disease immunopathogenesis. For this purpose, first time an *in vitro* obtained from bronchoalveolar lavage (BAL) fluid and treated with three doses (T1: 1800, T2: 2700, T3: 5400 CFU) of *M. haemolytica* inoculum, while keeping one untreated as a control. Then, total RNA was extracted, and cDNA was synthesized. The real-time quantification data indicated that the expression of *IL-1β, IL-6*, *IL-8*, *IL-10*, and *NF-κB* genes were significantly changed as compared to control. Our study revealed that the exposure to *M. haemolytica* stimulates the immune response in the sheep alveolar macrophages in a dose and time-dependent manner.

Koyun Alveoler Makrofajlarında *Mannheimia Haemolytica*'ya Yanıt Olarak Bağışıklık ile İlişkili Genlerin *İn Vitro* mRNA Ekspresyon Dinamikleri

MAKALE BİLGİSİ

Araştırma Makalesi

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ÖZ

Alveoler makrofajlar (AM) enfeksiyonlara ilk savunma hattı olarak yanıt verir ve akciğerin bağışıklık tepkisini tetikler. Alveoler makrofajlarda *Mannheimia haemolytica* mücadelesine karşı anahtar bağışıklık ile ilişkili genlerin ekspresyon dinamiklerini bilmek, hastalık immünopatogenezinin anlaşılmasını derinleştirmeye yardımcı olacaktır. Bu amaçla, ilk kez interlökin-1β, *IL-6*, *IL-8*, *IL-10* ve *NF-κB*'nin ekspresyon desenini doz ve zamana bağlı bir şekilde araştırmak

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Anahtar Kelimeler

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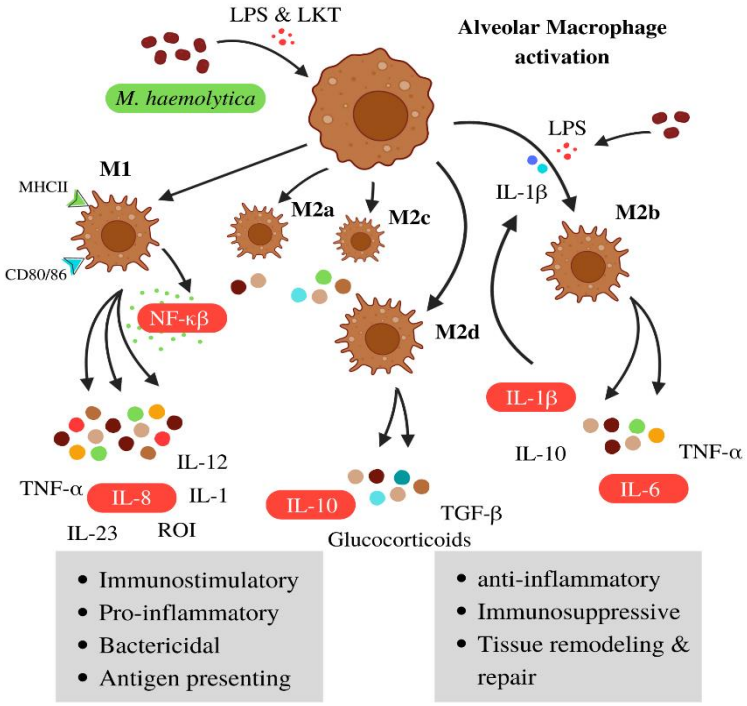
için *in vitro* deneysel bir enfeksiyon modeli kullanıldı. Bronkoalveoler lavajdan (BAL) sıvıdan toplam %95 saf alveoler makrofaj kültür plakaları elde edildi ve kontrol grubu ile *M. haemolytica* inokulumun üç dozu (T1: 1800, T2: 2700, T3: 5400 CFU) analiz edildi. Daha sonra toplam RNA ekstrakte edilerek ve cDNA sentezlendi. Gerçek zamanlı PCR verileri, *IL-1β*, *IL-6*, *IL-8*, *IL-10* ve *NF-κB* genlerinin ekspresyonunun, kontrole kıyasla önemli ölçüde değiştiğini gösterdi. Çalışmamız, *M. haemolytica*'ya maruz kalmanın, koyun alveoler makrofajlarındaki bağışıklık tepkisini dozda ve zamana bağlı bir şekilde uyardığını ortaya koymuştur.

Introduction

M. haemolytica is associated with ruminant respiratory diseases; along with climate and flock management conditions, it intensifies the pathogenesis in the lung (Singh et al., 2011). *M. haemolytica* is a common respiratory pathogen in both domestic and wild ruminants (Ackermann and Brogden, 2000; Ayalew et al., 2004). It causes severe fibrinous pleuropneumonia in sheep that is distinguished by fibrin merging and deposition, intra-alveolar hemorrhage, massive leukocyte infiltration in AMs of the lungs. Due of the intricate interactions between the infection source and valving host parameters, *M. haemolytica* infection considerably affects several economically significant features, such as condemnations in abattoirs, treatment cost reduction weight increase, and death. In a time- and dose-dependent manner, alveolar macrophages take a part in the creation of extracellular traps in response to *M. haemolytica* challenges (Aulik Nicole et al., 2012). *M. haemolytica*; a gram-negative bacterium is one of the major microbial stimulators of respiratory inflammation. Initial innate immune response is generated by the activation of macrophages (Picture 1), monocytes, and dendritic cells (DCs) to produce a wide variegation of cytokines including *TNF-α, IL-1*, and *IL-6.* These cytokines interact with diverse target cells and receptors to produce an inflammatory response (García-Alvarez et al., 2018). The defense mechanism against many microorganisms and different pathogens that cause various ailments, such as infections, pneumonia, hypersensitivity, and trauma, is inflammation, it is crucial to remember this. (Ackermann and Brogden, 2000). Due to the intricate interactions between the illness and valving host parameters, *M. haemolytica* infection has a considerable impact on a number of economically significant features in sheep, including condemnations in abattoirs, reduced treatment costs, weight increase, and mortality (García-Alvarez et al., 2018). It is significant to note that domestic animals are more likely than wild ruminants to contract this infection. On the other hand, more than $1 billion has been spent as a cost on this microorganism by the US livestock industry alone. This makes it one of the most significant bacterial pathogens of the complex of respiratory diseases in ruminants, including sheep (Ayalew et al., 2010).

M. haemolytica is a significant pathogen associated with respiratory illnesses in bighorn sheep (*Ovis canadensis*; BHS). It is one of the main reasons for the sharp decline in the bighorn sheep population worldwide, which was estimated to be two million in the 1800s and has since reduced to 70,000 (Ayalew et al., 2011). It is necessary to note that in the majority of experiments, *M. haemolytica* infection results in 100% mortality in bighorn sheep within two to three days of infection (Bowen et al., 2022). It is true that infections, particularly pneumonia brought on by bacteria of the species *Mannheimia*, *Bibersteinia*, and *Pasteurella*, have been

partially blamed for the significant and abrupt decline in the number of bighorn sheep in North America. According to several instances, bighorn sheep who come into touch with domestic sheep eventually succumb to pneumonia (Lawrence et al., 2010). Different diseases affecting the ruminants' respiratory system have a considerable detrimental influence on the global economy. *M. hemolytica* is Involved directly to cause pneumonic illnesses in both ovine and bovine (Ramírez Rico et al., 2017). Although the complex interactions between the environment, host, and pathogen as well as the mechanism of infection are still not fully understood, it has been suggested that the leukotoxin (LKT) produced by *M. haemolytica* is the predominant virulence factor in the case of respiratory diseases in ruminants (Oppermann et al., 2017). The most prevalent antigen-presenting cells in the alveolar spaces and the airways are alveolar macrophages (AMs). They have a crucial role in controlling inflammatory and immunological reactions in the lung. The AMs carry out several immunity-related responses, such as phagocytosis of particulate matter, cytokine and enzyme secretion, and microbial regulation. These cells serve a crucial role in maintaining and triggering local pulmonary immune responses because they are the first line of defense against many inhaled antigens, such as tiny particulate debris, allergens, and pathogenic pathogens (Guth et al., 2009). Macrophages originated in the typical multicellular organism to carry out phagocytic clearance of dying cells during development and adult life. These cells provide innate immunity through recruited cells made of monocytes and inhabitant tissue macrophages during inflammation, protecting multicellular host organisms (Martinez and Gordon, 2014).



Picture 1. Activation of alveolar macrophages and key immune-related genes in response to *Mannheimia haemolytica* stimulation.

Resim 1. *Mannheimia haemolytica* stimülasyonuna yanıt olarak alveolar makrofajların ve bağışıklıkla ilgili anahtar genlerin aktivasyonu.

In order to continuously defeat the host immune system in the upper respiratory tract, *M. haemolytica* colonizes the ruminant lungs due to a variety of predisposing factors, such as environmental or climatic conditions, adverse physical conditions, malnutrition, transportation, as well as prior infection or co-infections with other 3 pathogens (García-Alvarez et al., 2018). Determining the levels of mRNA expression against *M. haemolytica* in the alveolar macrophages was the purpose of this investigation. *NF-κB, IL-6, IL1, IL10,* and *IL-8* genes were among five selected inflammatory cytokine genes that showed a proportionate increase in gene expression following the *M. haemolytica* live bacteria challenge by PCR analysis. Although many samples use genes with cytokine properties to normalize the quantity of mRNAs, it is vital to remember that the expression of these cytokine genes can vary depending on the situation and in different cells or tissues. Therefore, choosing cytokine genes is crucial for gene expression investigations.

Materials and Methods

Preparation of bacterial inoculum

A field strain of *M. haemolytica* bacteria was isolated from an Akkaraman lamb lung, infected with chronic fibrinous pleuropneumonia. Samples were propagated in 6-well culture plates for 1-24 h with RPMI-1640 Medium (Phenol Red Free, Sigma-Aldrich, USA) modified and supplemented with L-glutamine, and incubated for 38ºC. Viability of bacteria was confirmed using optical density (OD) and effect of antibiotics on bacterial viability was also checked. Stock solution was prepared in the cell culture media and 0.05 MF turbidity was achieved at 0.008 OD to make a working solution containing 15,000,000/ml bacterial cells using Spectrophotometry.

Cell culture

Lungs were collected from Akkaraman lamb lungs after slaughtering and bronchoalveolar lavage (BAL) fluid was obtained by adding phosphate buffer saline (PBS) and shaking the lungs. BAL cells were collected after centrifugation and incubated for 4 hours in plates, after which the plates were washed to take out the floating cells. 95% purified alveolar macrophages were counted using trypan blue solution (Sigma-Aldrich, USA). 500 µl containing 800,000 cells were seeded in 6-well plates along with 1500 µl of RPMI-1640 to *M. haemolytica* challenge.

Stimulation of alveolar macrophages with M. haemolytica

Two batches of 6-well culture plates were made ready for two time periods (4 and 24 hours) and to treat them with three treatments (T1: 1800, T2: 2700, T3: 5400 CFU), while keeping one well untreated as a control. All plates were incubated overnight at 37 °C in 5% CO_2 and the viability of the cells were checked under the microscope (20X) before treating them. Cells were treated with three different doses of *M. haemolytica* as prescribed earlier and incubated at 37 °C in 5% CO_2 for 4 and 24 hours. After the treatment and end of each time, cells were washed thrice with 1X PBS and dissociated from the culture plates using 0.25% Trypsin-EDTA. A short incubation of 5 minutes was given to harvested cells at 37 °C. After incubation, the culture plates were placed on ice for 15-20 minutes to completely dissociate the

cells from the culture plates. Once the cells were properly harvested, they were processed for total RNA isolation, cDNAs synthesis, and gene expression analysis using real-time qPCR.

RNA extraction and cDNA preparation

Once all the AMs were dissociated from the culture plates, the function of trypsin-EDTA was stopped by adding 2 ml of warm culture media (Dulbecco's Modified Eagle Medium, Sigma Aldrich, UK). Cells were then carefully harvested from each well into 15 ml Falcon tubes and centrifuged at 700 × g for 5 minutes at room temperature. Then, was centrifuged and the supernatant was discarded, and 1 ml of warm PBS (1X) was added to each falcon tube and cells were mixed gently then transferred into 2 ml micro-centrifuge tubes. Finally, the mixed cells (AMs) were centrifuged at 700 × g for 5 minutes. Then, the PBS (1X) was removed carefully, and cells were stored immediately at -80 °C for RNA isolation. Total RNA was extracted from AMs cells by using a high pure RNA isolation kit (Roche Applied Science, Germany) according to the manufacturer's instructions with some modification adopted for cell samples. Immediately, after elution, the RNA was analyzed using the Biospec-Nano (Shimadzu Biotech, China) spectrophotometer to evaluate the quality and quantity of the isolated total RNA. The cDNA stands were constructed using Easy Script plus Reverse Transcriptase kit (Roche Applied Science, Germany) according to the manufacturer guidelines.

Quantitative real-time PCR (qRT-PCR)

For the quantification of *IL-10, IL-8, IL-6, IL-1β, NF-κB*, and *GAPDH* genes, cDNA strands were amplified by real-time qPCR using Cyber Green Super-mix with primer pair sets (designed using Primer3web, v4.1.0) listed in Table 1. According to the experimental design, 2 µl of cDNA from each sample was placed in each well of the 96 wells PCR. The negative controls (2 µl of water) were placed in the last row of the PCR microplate. PCR master mix was prepared with 10 µl of SYBR Green I master mix, 0.4 µl of forwarding primer, 0.4 µl of reverse primer and 7.2 µl of RNase-free water for each reaction. Each well received 18 µl of the master mix, for a total reaction volume of 20 µl. The amplification reaction was carried out as an initial pre-incubation at 50 ˚C for 2 minutes and one cycle at 95 ˚C for 10 minutes, followed by 45 cycles of amplification at 95 ˚C and 60 ˚C withholding for 15 and 60 seconds, respectively. Melting curve analysis was accomplished at 60 ˚C withholding for 15 seconds followed by 95 ˚C in continuous mode and 20 acquisitions (per ˚C) using LightCycler 480 instrument (Roche Applied Science, Germany). The background fluorescence and the crossing point Ct values are automatically calculated by the software associated with LightCycler.

Data analysis

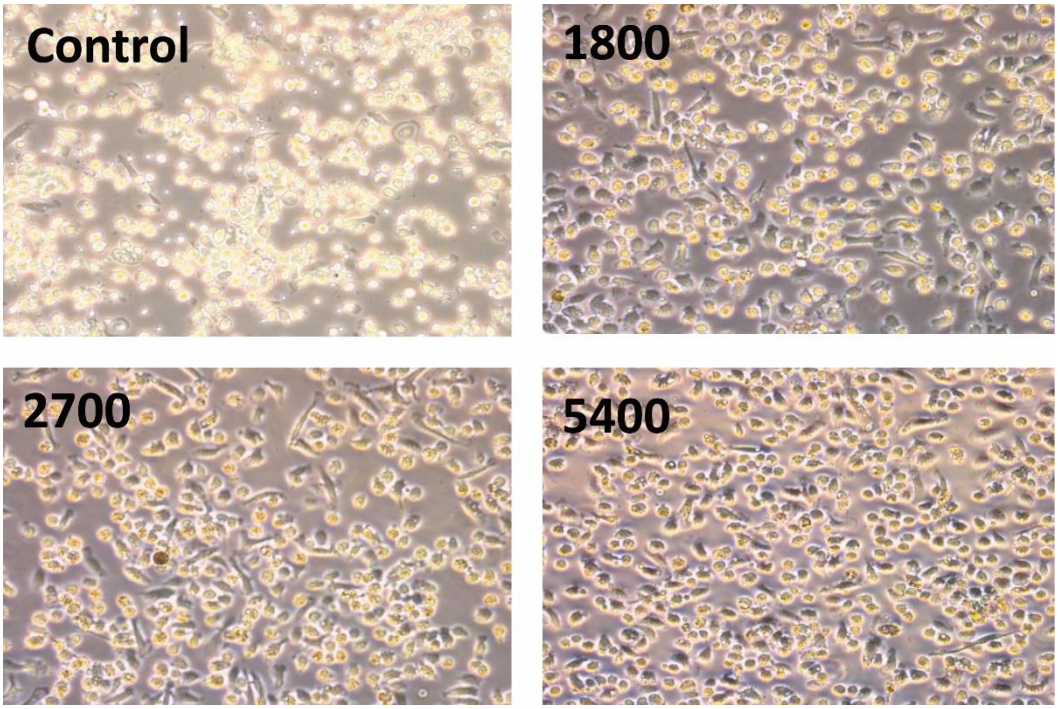
The levels of gene expression were analyzed using the equation $2^{-[\Delta CP\ sample - \Delta CP\ control]} = 2^{-\Delta\Delta CP}$ for each gene in Microsoft Office Excel 365, and further converted into fold change value (relative mRNA expression) as done earlier (Aksel and Akyüz, 2021). The significance of the relative expression between groups was determined by Student's t-test using the built-in data analysis tool in Microsoft Office Excel 365. The P-value ≤ 0.05 was considered as significant. The relative mRNA expressions were visualized in bar graphs using R programming in RStudio (version 1.4.1717) along with relative significance.

Results

Viability of alveolar macrophages (AMs)

Viability of AMs after 4 hours treatment

After 4 h of incubation, the alveolar macrophages were treated with different doses of *M. haemolytica* (T1=1800, T2=2700, and T3=5400 CFU) and incubated again according to the experimental design. The viability and growth of AMs in the control group on RPMI-1640 medium indicated that the nutrients present in the medium were sufficient to nourish the AMs under culture conditions. Culture plates' photos showed that most of the AMs survived and exhibited higher resistance against *M. haemolytica* in the T1 and T2 treatment groups. However, bacterial abundance altered the size and shape of the cells in T3 treatment. In addition, more dead cells were observed in the T3 treatment compared to other groups (Picture 2).

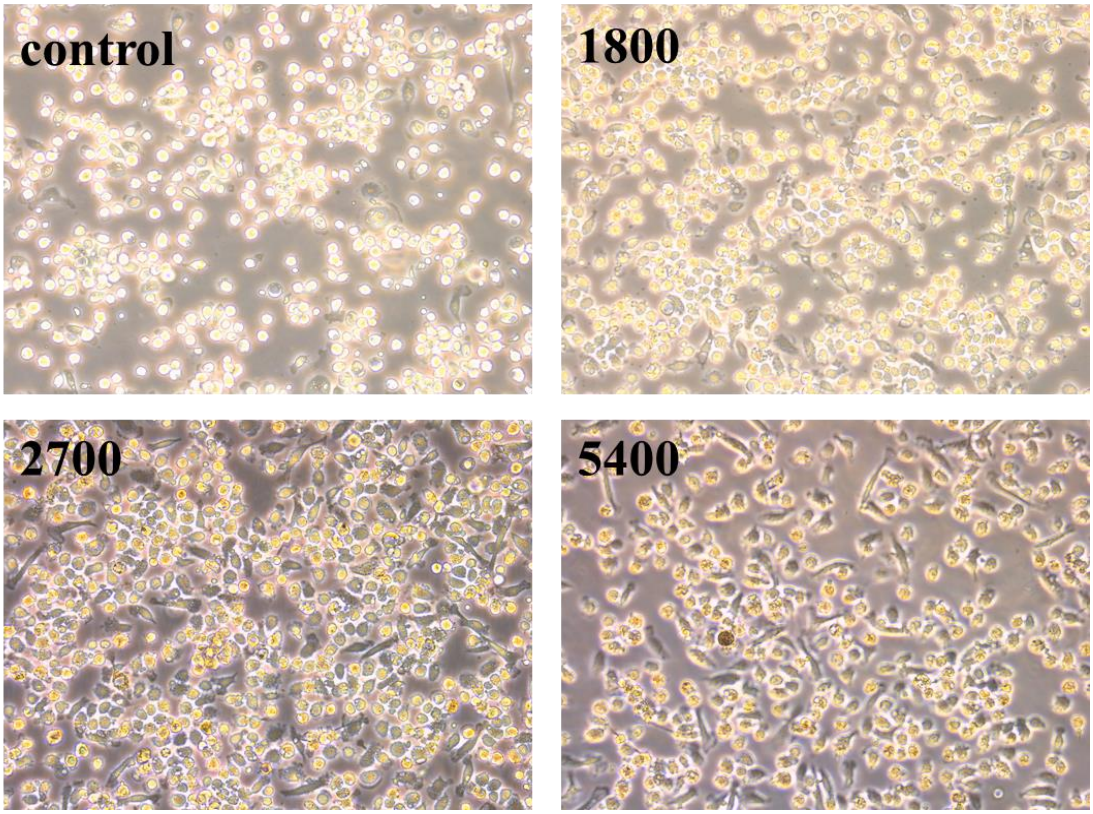


Picture 2. AM cells were cultured for 4-hour under different conditions and the pictures were captured using an inverted light microscope using 20X objective

Resim 2. AM hücreleri, farklı koşullar altında 4 saat süreyle kültürlendi ve resimler, 20X objektif kullanılarak ters ışık mikroskobu kullanılarak çekildi

Viability of AMs after 24 hours treatment

After 24 h incubation of AMs in RPMI 1640 medium, AMs were treated with three different doses of *M. haemolytica* bacteria, the same concentrations used at 4 h, and cultured according to the experimental design. We have seen that in the control treatment, the AM cells survived and were active and most of the cells were still alive after 24 h. The first dose of treatment T1 *M. haemolytica* showed that most of the cells survived and exhibited high resistance against *M. haemolytica*. The same trend was observed in plates of the last two treatments T2 & T3 of *M. haemolytica* (Picture 3).



Picture 3. Alveolar macrophage cells were grown in a 6-well culture plate. Cells were treated with live *M. haemolytica* bacteria for 24 h. At 24 h time point pictures were taken by a light microscope using 20X objective

Resim 3. Alveoler makrofaj hücreleri, 6 oyuklu bir kültür plakasında büyütüldü. Hücreler, 24 saat boyunca canlı M. haemolytica bakterisi ile muamele edildi. 24 saat zaman noktasında resimler, 20X objektif kullanılarak bir ışık mikroskobu ile çekilmiştir

Expression of key immune-related genes

The differential expression of *IL-1β, NF-κB, IL-6*, *IL-8*, and *IL-10* as a reference gene in alveolar macrophages were performed with three treated and one untreated control group at two-point time (4 h and 24 h).

The mRNA expression of NF-κB as master regulator for pro-inflammatory molecules

The role of NF-κB as a master regulator of proinflammatory signals was established based on several studies (Bannerman et al., 2002). *NF-κB* gene expression was triggered by lipopolysaccharide (LPS), IL-1 *β,* and some other molecules. *M. haemolytica* produces two major virulent toxins; LPS and LKT (McClenahan et al., 2008). In the current study, the higher relative expression of the *NF-κB* gene was observed in the AM cell when treated with *M. haemolytica* as compared to untreated control at the 4-hours' time point while there was a lower expression at the 24-hours' time point. Moreover, T3 treatment resulted in a significantly highest expression of *NF-κB* in the AMs, T1, also, triggered the higher expression of *NF-κB* gene. However, the expression of *NF-κB* was significantly lower in the T2 live bacteria treated cells as compared to T1 and T3 treatments at 4-hours' time point. The expression of *NF-κB* was significantly lower in the T1 live bacteria treated cells but it was comparatively higher than T2 and T3 treatment groups at 24 h time point. Furthermore, the expression of *NF-κB* was lowest in the T2 treatment group as shown in Figure 1.

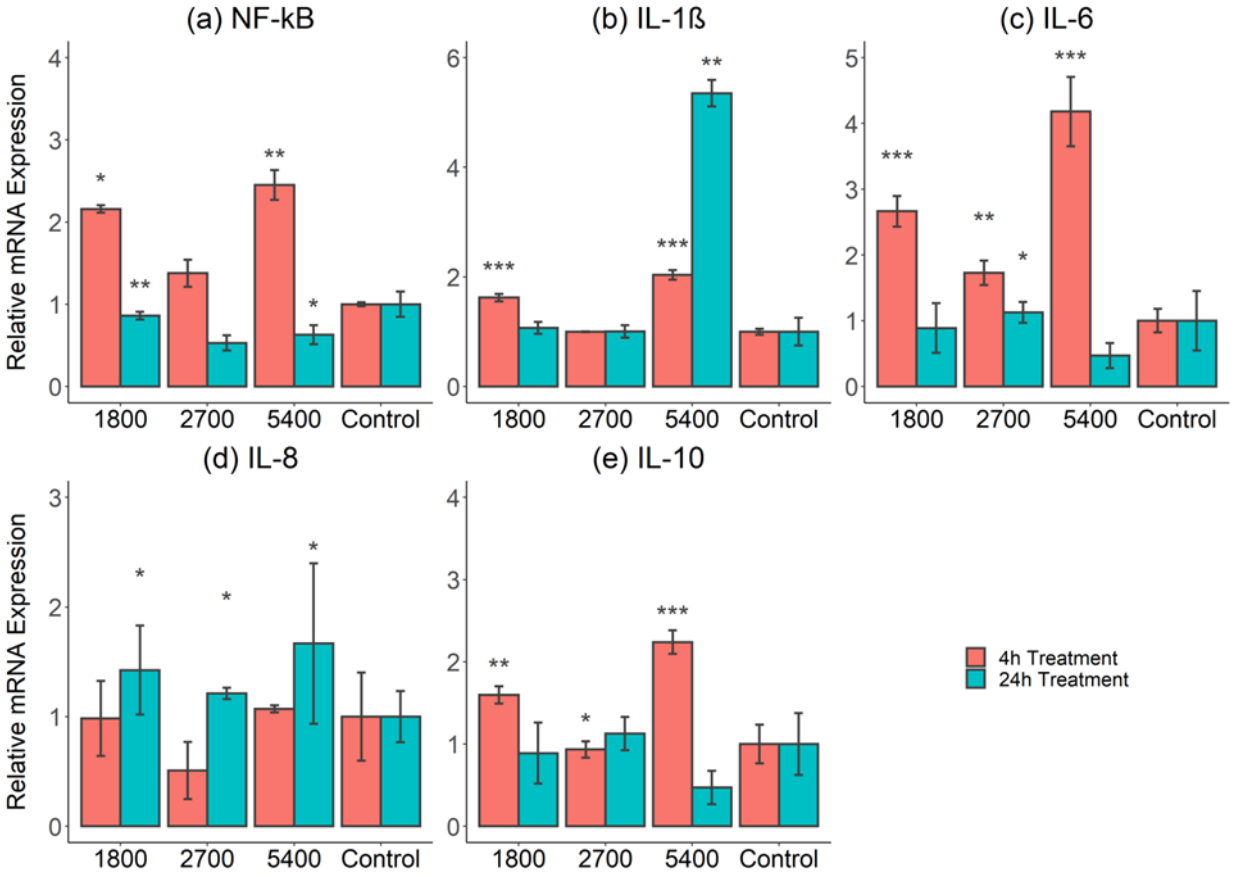


Figure 1. Relative mRNA Expression of key immune-related genes after 4h and 24h treatment points. **(a)** Relative mRNA expression of *NF-κB*. **(b)** Relative mRNA expression of *IL-1β*. **(c)** Relative mRNA expression of *IL-6*. **(d)** Relative mRNA expression of *IL-8*. **(e)** Relative mRNA expression *IL-10*.

Şekil 1. 4 saatlik ve 24 saatlik tedavi noktalarından sonra bağışıklıkla ilgili anahtar genlerin bağıl mRNA ifadesi. (a) NF-κB'nin nispi mRNA ifadesi. (b) IL-1β'nın nispi mRNA ifadesi. (c) IL-6'nın göreli mRNA ifadesi. (d) IL-8'in nispi mRNA ifadesi. (e) Bağıl mRNA ifadesi IL-10.

Error bars represent the means±SD of three biological replicates. Asterisk indicating the different level of significant differences between treatments groups compared with control, where *=P≥ 0.05, **=P≥ 0.01, ***= P≥ 0.001.

The mRNA expression of pro-inflammatory IL-1β gene

Alveolar macrophages are the primary cellular sites to produce *IL-1β* after lipopolysaccharide exposure (Blackwell et al., 2011). Stimulation of sheep alveolar macrophages with LPS producing *M. haemolytica* altered the expression of the *IL-1β* gene in all treated groups when compared with control at both time points (4 hours and 24 hours) of the study. Treatment with T3 dose of the bacteria revealed a significantly highest *IL-1β* expression in the AMs. Yet, the *IL-1β* expression noted lower in AMs at 4 hours treatment of T2 dose of the bacteria. However, treatment of AMs with T1, T2 doses for 24 hours and T1 treatment for 4 hours resulted in no significant alteration of the expression of *IL-1β* gene as compared to the control group. However, a significant increase in the *IL-1β* expression was observed with T1 treatment at 4 h time point, compared to control group (Figure 1).

The mRNA expression of pro-inflammatory IL-6 gene

IL-6 is a key signal in generating an over sustained and adaptive immune response during the transition of initial innate response to an infection (Naugler & Karin, 2008). The investigation of the *IL-6* gene expression on the stimulation of LPS producing *M. haemolytica* resulted in a higher expression at the 4 h treatment time point. Treatment with live T1 dose of the bacteria significantly increased the expression of *IL-6* gene. Surprisingly, *IL-6* gene expression was not significantly increased in AMs with the treatment of T2 bacterial dose. The highest *IL-6* gene expression was observed at 4 hours timepoint when the cells were treated with T3 bacterial dose. The *IL-6* gene expression, however, showed that the AM cells treated with T1 and T2 had a reduced expression compared to the control group. Moreover, T3 dose generated a significantly higher expression of *IL-6* gene in the AMs. After 24 hours of the treatment dose T2, the expression of *IL-6* was not increased, it was even lowered than the T1 and T3 doses (Figure 1).

The mRNA expression of pro-inflammatory IL-8 gene

According to the results of this study, the expression of the *IL-8* gene in the AM cells of all treatment groups was found to be lower than that of the control group. The expression of *IL-8* in the AMs was considerably higher after treatment with T3 live *M. haemolytica* bacteria than in the control group. At the 4 h time point, the expression of *IL-8* was dramatically reduced in the T2 live bacteria-treated cells, while the stimulation of AMs with all T1, T2, and T3 treatments of *M. haemolytica* resulted in a significantly higher expression of *IL-18* compared to control. Additionally, the T3 live bacteria treatment of AMs increased the expression of IL-8 in the AMs. However, at the 24 hours' time point, the T2 live bacteria treated cells' expression of *IL-8* did not increase significantly (Figure 1).

The mRNA expression of anti-inflammatory IL-10 as master regulator

The gene expression of *IL-10* was significantly increased in AMs stimulated with all T1, T2, and T3 treatments of *M. haemolytica* for 4 h of trials compared to the control. Additionally, 4 hours' time study using T3 *M. haemolytica* on AMs revealed increased *IL-10* gene expression. Moreover, compared to other treatments, the expression of T2 treatment was seen to be lower at the 4 hours' time point. As a result, when *M. haemolytica* was treated with T1, T3, and all the AMs, the gene expression of *IL-10* was significantly reduced compared to the control. Additionally, the 24 h study using T2 treatment on AMs showed increased *IL-10* gene expression. When cells were treated with T3 live bacteria, *IL-10* expression drastically decreased at the 24 h mark (Figure 1).

Discussion

Alveolar macrophages are the first line of defense against any inhaled antigenic particle and responded to them by the production of different cytokines and chemokines (Guth et al., 2009). In the current study, two master regulatory and three other genes showed changing in their expression data when treated with different doses for 4 h and 24 h time periods. *NF-κB*, *IL-1β* and *IL-6* genes expression were observed higher on all 4 hours treatments while *IL-10* gene expression was higher in only T1 and T3 treatments. The expression of *IL-1β* and *IL-8* was higher in all 24 h treatments while *IL-10* showed higher expression only with T3 dose at

the same period of treatment. *NF-κB*, and *IL-8* genes showed lowered expression at 24 h and 4 h treatments, respectively. IL-6 also showed lowed expression at 24 h for T1 and T3, but it was relatively high with T3 treatment (Figure 1). It is evident that the different treatments of the live *M. haemolytica* bacteria triggered the expression of these immune related genes in AMs of sheep.

The AMs are equipped with a wide variety of receptors for microbe-host interactive products, cytokines production by T helper cells, and lineage-determining growth factors. In live animals, the macrophages stimulated and activated in a dynamic response to perform their specialized functions in the form of M1 and M2 (Martinez and Gordon, 2014). *M. haemolytica* is used for the development of an in vitro lung pneumonia model as a major microbial stimulant. It triggers the first response system in many cells like macrophages, monocytes, and dendritic cells to synthesize the cytokines such as *IL-1, IL-6*, and *TNF-α*. The products play a vital role in the mediation of inflammatory interactions with different receptor-mediated cells (Rice et al., 2007).

As the signal regulator, *NF-κB* gene showed higher expression at 4 hours compared to 24 h period with all treatment, suggested that in the first 4 hours after stimulation the AM cell, CD4+ T and B cells were responding the antigens (Janeway et al., 2001) and after 24 h of stimulation the expression levels are decreased which is mean most of the AM cells went dead. The alveolar surface can face severe damage if the antigenic organisms continue to exist because of the strong inflammatory immunological reactions. Another signal regulator, *IL-10* gene was also exhibited high expression in both study time of the treatment, but it was highest in 4 h treatment which means gene expression of *IL-10* increased right after stimulation.

In a prior study, it was examined that the bovine alveolar macrophages (BAMs) during *M. haemolytica* infection, showed no significant difference in the *IL-1β*, *IL-6*, *IL-8*, *IL-10*, and *TNFa* gene expression at 24 h (Singh et al., 2011). This outcome was due to a longer period of stimulation, at which most innate immune response-related gene expression reaches a normal level. However, the current study found higher expression of *IL-10*, *IL-8*, *IL-6*, and *IL-1β* genes at 4 h. In another study, *M. haemolytica* treated cells showed upregulated expression response for *IL-6*, *IL-8*, and *IL-1* genes, 9, 10, and 20 times higher as compared to untreated control group, respectively (N'jai et al., 2013). This study's findings totally concur with our results in that there is a significant relationship between the expression of immune-related genes and exposure to *M. haemolytica* live bacteria. *M. haemolytica* stimulation significantly contributed to the pathogenesis of bovine lung pneumonia (BLM) with the selective activation of cytokine production by BAMs (Singh et al., 2012). The LKT-induced lysis of polymorphonuclear neutrophils (PMNs), which is a hallmark of *M. haemolytica* infection, is the main cause of lung damage and acute inflammation. The ineffective neutralization of the LKT released by *M. haemolytica* is caused by the low amounts of LKT-neutralizing antibodies at the pulmonary mucosal surface. The PMNs secrete high levels of IL-8 which directly affects the influx of more PMNs into the alveolar sites of lung. Based on our study and mentioned published data, we suggest the same pathogenesis model for Akkaraman sheep to explain the *M. haemolytica* infection, pathology and morbidity displayed by AMs. It is significant to mention that Akkaraman sheep have *M. haemolytica* in their nasopharynx as a commensal bacterium, same as other ruminants. Our finding clearly demonstrated that exposure to a substantially higher

number of *M. haemolytica* (T3 dose) led to a higher number of cell deaths when compared to the other two treatments (T1 and T2) at both time intervals. High cytokine responses can be induced by the high quantity of *M. haemolytica* per AM cell.

Protective immune response is always required for the removal of infectious organisms from the alveolar sites, in the absence of this response, inflammatory damage can lead to a much more severe pathological condition called Pneumoncystis pneumonia. Better understanding of different protective responses provide an opportunity for their manipulation, precise medication and preventive strategies for Pneumocystis infections. Proinflammatory cytokines including *IL-1, IL-6,* and *TNF* that are produced in excess during infections contribute to anaimal health damage (Chaudhry et al., 2013; Liu et al., 2016). The genetic code serves as a blueprint for the production of the proteins that give rise to a particular phenotype. Appropriately, the phenotype is less reliable measure to understand the immune responses as compared to gene expression (Klima et al., 2017). In this way, current study contributed reliable findings of the stimulated gene expression of key immune related genes with time and dose-dependent manner.

Conclusion

One of the most significant causes of financial losses in the livestock industry is pneumonia, which is caused by *M. haemolytica* and affects all ruminants, including sheep. It is crucial to note that *M. haemolytica* is a significant pathogen that causes pneumonia in sheep and has a considerable amount of potential to be used as an in vitro model for lung inflammation development. The AMs were obtained and purified in the lab from the lungs of a local Turkish breed known as Akkaraman. Our findings show that in 4 hours of treatments, most of the doses enhanced high gene expression of *IL-1β, IL-6, IL-8, IL-10, and NF-κB* in the treated groups compared to the control. In most treatments, 4 hours trials also revealed higher gene expression than 24 h tests, however *IL-10* and *IL-1* gene expression in 24 h treatments was higher in the 2700 and 5400 treatments. Additionally, *IL-8* showed increased gene expression in all treatments after 24 h compared to 4 h. Overall, the current research revealed that the enhanced lung pathology and prospect mortality of Akkaraman sheep are highly likely to be correlated with the bacterial manifestation and time until the animal survived. In addition, our research also revealed that the immune response in lung is mediated via AMs through the induction of different genes crucial to the innate immune response.

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