REPUBLIC OF TURKEY
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL SCIENCE AND TECHNOLOGIES

INVESTIGATING THE EXPRESSION OF IMMUNE GENES IN RESPONSE TO LIPOPOLYSACCHARIDE (LPS) AND LIPOTEICHOIC ACID (LTA) STIMULATION IN SHEEP LUNGS

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Ph.D. Thesis

June 2019
KAYSERI

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SCIENTIFIC ETHICS SUITABILITY

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Ahmed Qasim NAJI

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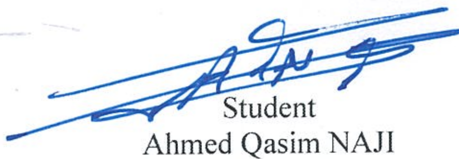

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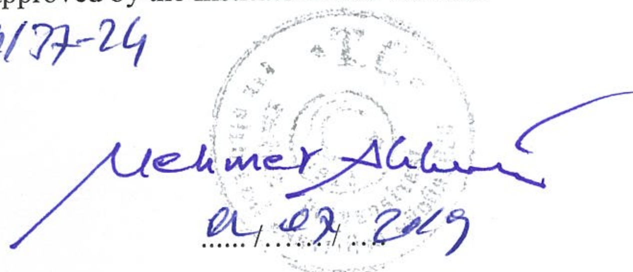

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INVESTIGATING THE EXPRESSION OF IMMUNE GENES IN RESPONSE TO LIPOPOLYSACCHARIDE (LPS) AND LIPOTEICHOIC ACID (LTA) STIMULATION IN SHEEP LUNGS

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ABSTRACT

Sheep is one of the most economically domestic important animals that provide meat, milk, and wool products in every corner of the world. This study has been done in two steps which are *in vitro* and *in vitro* studies. For *in vitro* study, alveolar macrophages (AMs) were isolated from the lamb lung and incubated for 4 h and 24 h with 10µg/ml of LPS, 10µg/ml LTA and with the combination of 10µg/ml LPS + 10µg/ml LTA and control. Results showed that the exposed AMs for 4 h showed higher mRNA expression level of *TLR2*, *TLR4*, *NF-κB*, *TNFα*, *IL-1β*, *IL-6*, *IL-10,* and *IL-8* compared with control in all treatments than 24 h experiment. Moreover, there were significant differences in the mRNA expression of all the above genes between 4 h and 24 h experiments in response to LPS, LTA and with the combination of LPS+LTA treatments. On the other hand, *in vivo* study has been performed on twenty-four healthy lambs from two different sheep breeds (Akkaraman and Romanov); and they were exposed to 20µg/kg LPS, 50µg/kg LTA and with the combination of 20µg/kg LPS+50µg/kg LTA at same time and left for 24 h. Animals in each breed category were divided into four treatment groups (LPS, LTA, LPS+LTA and Control). Animals were euthanized after 24 h of the treatments, the lungs were collected, and AMs were isolated and purified. Then, the total RNA extracted, cDNA synthesis and mRNA expression has been done by real time PCR to check the expression levels for the same above genes compared with control within the breed and between the two different breeds. Results showed that Romanov lambs present high mRNA expression of *TLR2*, *TLR4*, *NF-κB*, *TNFα*, *IL-1β*, *IL-6*, *IL-8* and *IL-10* compared with control in all treatments than Akkaraman lambs. Overall, obtained results could enhance the effectiveness of selection for sheep against respiratory diseases.

Keywords: Lung inflammation, *In vitro*, *In vivo*, Immunity, Cytokine, Alveolar macrophages, Lipopolysaccharide (LPS), Lipoteichoic acid (LTA)

KOYUN AKCİĞERLERİNDE LİPOPOLİSAKKARİT (LPS) VE LİPOTEİKOİK ASİT (LTA) STİMÜLASYONLARI İLE OLUŞAN İMMUN YANITIN GEN İFADELERİNİN ARAŞTIRILMASI

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ÖZET

Koyun, dünyanın her köşesinde et, süt ve yün ürünleri sağlayan, ekonomik açıdan en önemli evcil hayvanlardan biridir. Bu çalışma *in vitro* ve *in vitro* olmak üzere iki aşamada gerçekleştirildi. *In vitro* çalışma için, alveolar makrofajlar (AM'ler) koyun akciğerlerinden izole edildikten sonra saflaştırıldı ve 4 saat ve 24 saat süreyle 10µg/ml LPS, 10µg/ml LTA ve 10µg/ml LPS + 10µg/ml LTA ve kontrol kombinasyonu ile inkübe edildi. Dördüncü saatte alınan örneklerde *TLR2*, *TLR4*, *NF-κB*, *TNFα*, *IL-1β*, *IL-6*, *IL-10*, ve *IL-8* genlerinin ekspresyonlarının 24. saateki örneklere göre daha fazla olduğu bulunmuştur. Ayrıca, *NF-κB, TNFa, IL-1β*, *IL-6*, *IL-8* ve *IL-10*'un mRNA ifadelerinde LPS, LTA ve LPS + LTA kombinasyonuna verilen immune yanıtta 4. ve 24. saatler arasında önemli istatistiki yanıt olduğu bulunmuştur. *In vivo* çalışma iki farklı koyun ırkından (Akkaraman ve Romanov) toplamda 24 sağlıklı kuzu üzerinde gerçekleştirilmiş ve bu kuzular 20µg/kg LPS, 50µg/ml LTA ve 20µg/kg LPS+ 50µg/ml LTA kombinasyonuna 24 saat boyunca maruz bırakılmışlardır. Her iki ırktaki hayvanlar 4 farklı uygulama grubuna (kontrol, LPS, LTA ve LPS+LTA kombinasyonu) bölünmüşlerdir. Hayvanlar 24. saatte ötanazi edilmiş, akciğerleri alınmış ve alveolar makrofajlar izole edilmiş ve saflaştırılmıştır. Daha sonra, total RNA izolasyonu, cDNA sentezi ve Real-Time PCR ile aynı genler için ekspresyon seviyeleri ölçülmüş ve kontrol grubu ve birbirleri arasındaki gen ekspresyonları karşılaştırılmıştır. Romanov kuzularda Akkaramanlar'a göre *TLR2*, *TLR4*, *NF-κB*, *TNFα*, *IL-1β*, *IL-6*, *IL-8,* ve *IL-10* genlerinin ekspresyonlarının daha yüksek olduğu görülmüştür. Genel olarak, elde edilen sonuçlar koyunlarda solunum yolu hastalıklarına karşı seçimin etkinliğini artıracaktır.

Anahtar Kelimeler: Akciğer inflamasyonu, *In vitro*, *In vivo*, Bağışıklık, Sitokin, Alveolar makrofajlar, Lipopolisakkarit (LPS), Lipoteikoik asit (LTA)

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LIST OF ABBREVIATIONS

AMs	: Alveolar Macrophages
AP	: Activating Protein
ARDS	: Acute Respiratory Distress Syndrome
ATF	: Activating Transcription factor
B cells	: Bone Marrow Cells
BAL	: Bronchoalveolar Lavages
BALF	: Bronchoalveolar Lavage Fluid
BW	: Body Weight
CD4	: Cluster of Differentiation 4
DAMPs	: Danger Associated Molecular Patterns
ddH_2O	: Double-distilled water
DMEM	: Dulbecco’s Modified Eagle’s Medium-High Glucose
EDTA	: Ethylenediaminetetraacetic Acid
FBS	: Fetal Bovine Serum
GAPDH	: Glyceraldehyde-3-Phosphate Dehydrogenase
GHG	: Greenhouse Gas
IL-10	: Interleukin-10
IL-1β	: Interleukin- 1
IL-6	: Interleukin- 6
IL-8	: Interleukin- 8
INF	: Interferon
IPCC	: Intergovernmental Panel on Climate Change
LPS	: Lipopolysaccharide
LTA	: Lipoteichoic acid
MAPK	: Mitogen-Activated Protein Kinases
NF-$_K$B	: Nuclear Factor Kappa B
NK	: Natural Killer Cells
NO	: Nitric Oxide
PAMPs	: Pathogen-Associated Molecular Patterns
PBS	: Phosphate Buffer Saline
PRRs	: Pattern Recognition Receptors

RPM1640	: Roswell Park Memorial Institute 1640 Medium
T cells	: Thymus Cells
TLRs	: Toll-Like Receptors
TNFα	: Tumor Necrosis Factor Alpha
Union	: Antibiotic Bacteria and Fungus

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INTRODUCTION

Sheep are one of the main and the most important economic and domestic animal's species in the world that use for meat, milk, and wool products [1,2]. For long decades, the traditional selection of animals including sheep was depended on phenotype and production of animals [3,4]. It has recently been shown that for the planning an efficient breeding program, the knowledge of animals' phenotypic parameters and genetic markers are essential [5,6]. It is crucial to incorporate the traits related to the production robustness in the breeding program for sustainable and stable genetic changes in relation to the environmental sensitivity [7,8,9].

In fact, historical intensive selection for milk production in dairy cows has succeeded in increasing milk yield but has done so to the detriment of functional traits such as animal health and reproductive performance [10,11,12]. Among all the diseases in sheep, the respiratory diseases the most infectious, serious, and important disease causing severe economic loss in the industry through a direct impact on the animal's health and productivity [13]. Acute respiratory diseases syndrome (ARDS) due to long-term pneumonia, inhalation injuries, burns and sepsis is considered to be a most common clinical entity that causes the majority of mortality and morbidity in farmed and domestic sheep [14,15]. Sheep are frequently used as an animal model to study lung pathology and physiological responses because of its similarities to human. The progenitor cells in the ovine alveolar and bronchioalveolar tissues could be a new tool for studying new curative targets or for the regeneration of lungs which can further be used lung repair and embryogenesis [16,17,18].

There are two major defense mechanisms (namely adaptive and innate immune system) in host animals against external stimuli including different antigenic stimulation, viral, and bacterial infection. In response to antigen-related stimulation and inflammatory stimulus, innate immune response acts quickly and mediates its effects on the foreign body [19,20,21]. Alveolar macrophages are one of the most important types of phagocytes which performs major functions, not only the host defensive responses

against foreign antigens but also in maintaining lung homeostasis and orchestrate the mechanisms to repair the tissue injuries [22,23,24]. In addition, there are a few pieces of evidence exists that explain the genetic roles in either cell-mediated immune response or disease resistance in lamb's respiratory tract which warrant a further robust study to characterize the molecular mechanisms of alveolar macrophages mediated immune responses in sheep [25,26]. Both external and internal factors are responsible for the overproduction of bacterial infection [27,28]. 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 [29,30].

In fact, 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. By contrast, LTA can bind with the target cells following both nonspecific and specific pathway [31,32,33]. TLRs are considered as the prime components in the immune system against various micro-organism mediated infections, the improper response of TLRs can result in chronic and acute inflammatory diseases as well as a variety of autoimmune diseases [34,35,36,37]. Indeed, the *TLR4*, *TLR2* is associated with the identification of different microbial PAMPs mostly from microorganisms like virus, fungi, bacteria, and parasites [38,39,40,41]. These studies provide an insight that to enhance the productivity of domestic animals including sheep through avoiding the lung diseases, it is essential to understand the underlying molecular mechanism of the economically important lung diseases caused by both Gram-positive and Gram-negative bacteria.

More recently, disease free healthy animals are the premium choice of farmers in terms of economic point of view. Most of the exotic small ruminants, particularly foreign lamb breeds in Turkey, suffer from respiratory diseases due to the different environmental conditions and lower adaptive capacity of the animal itself. In fact, the understanding of respiratory diseases in the molecular and genetic level become the research priority in many research laboratories in the world. Thus, in this study, we aimed to identify underlying immunogenetic mechanisms associated with resistance to respiratory diseases that could enhance the effectiveness of selection for sheep against

respiratory diseases. Therefore, this study has been done in two steps which are *in vitro* and *in vivo* studies, to find out the level of mRNA expression when alveolar macrophages cells were exposed to LPS, LTA and with the combination of LPS + LTA treatments in different time points 4 h and 24 h for *in vitro* study and for 24 h for *in vivo* study. We hypothesized that it's possible to use the genetics of innate immunity as the main assistant in sheep selection against respiratory diseases.

The primary aims of the current study were to investigate the immune responses; particularly innate immunity, in the healthy lambs of two different breeds, namely Akkaraman (Turkish native breed) and Romanov (imported exotic breed) in response to LPS, LTA and the combination of LPS+LTA for 24 h as a time point study. In addition, the expression of candidate genes in the TLR pathways will be determined according to experimental design. Finally, we are going to determine the differences in terms of the immunity responses by carrying out the relevant work between these two breeds.

CHAPTER 1

LITERATURE REVIEW

1.1. A Brief History of the Domestication of Sheep

Sheep is one of the earliest domesticated animals in human history and the domestication of sheep started long back which dated back to 11000 to 9000 BC. The domestication of sheep started with the wild mouflon in the old Mesopotamia. There are several pieces of evidence which support the idea that the sheep farming was also started at the same time in Iranian statuary and radiocarbon examination of the sheep fossil indicating that it is one of the first animals that human had tamed to fulfill their needs of protein (meat and milk) as well as for the skins to protect them from extreme environment. Sheep that provide fine wool was developed in Iran during the 6000 BC and the culture, livelihood, and the trades was heavily influenced by the sheep wool. Eventually, these fine wool sheep breeds are imported to Europe and Africa through trading [42].

1.2. The Regional Distribution of Sheep

Although sheep are thought to be domesticated in the ancient Mesopotamia, eventually it was spread throughout the world via trading or colonization. Before the domestication of sheep, they used to depend on foraging as their main source of food. In that scenario, they primarily grazed the lower pastures to fulfill their nutritional needs. In addition to grazing lower pastures, the also browsed small trees with young leaf and the young shoots of bushes. However, in under the domestic farmed environment, sheep are also depending on the food supply from its owner. Due to its small size and non-aggressive behavior, the early agricultural society easily tamed them to mitigate their needs for food and clothes. Probably the domestication of sheep started through catching a young one and instead of killing it instantly, the early humans brought it back to their shelter and confined it for a while to grow bigger. Although the imprinting memory of a

captured young animal is important for domestication, a number of other factors including behavioral and environmental factors made it easier to domesticate the adult sheep as well. [43–45].

It is a well-accepted notion that the sheep are first domesticated in the south-west part of Asia because the root of agriculture was based on this area. After that, the domesticated sheep were moved with their keepers throughout Africa, Asia, and Europe and in recent centuries to the New Zealand, Australia and most part of the North and South America. Although the total number of sheep are declining in recent days, totaling almost 1000 million, the sheep have spread throughout the world because of its adaptability under a wide range of environmental condition. It is important to note that there is a considerable variation in the genetic makeup of the sheep within the world population. This is mainly due to the selection process attributed by both human and nature in order to develop animals that are well adapted to a certain climatic condition. For example, African sheep breeds need to be adapted to the climate which is tropical in nature and extremely hot in summer. Because of these adaptation strategies, sheep in the tropical region don’t have wool, instead, they have hair to reduce the impact of hot weather. In addition, to wool modification, the fats are not accumulated in the subcutaneous region instead accumulated in the tail [46,47].

Table 1.1. By analyzing the mitochondrial DNA control region sequence, the origin and distribution of domestic and wild sheep are traced. Quoted from [45]

Name[a]	**Breed**[b]	**Specimen numbers**	**Region / Country**
Ovis canadinesis canadinesis	Unknown	1	Rocky Mounties / Canada
Ovis musimon	Unknown	2	Hessen / Germany
Ovis vignei bocharienis	Unknown	2	Southeast / Turkmenistan
Ovis vignei arkal	Unknown	1	Ust-Urt / Kazakhstan
Ovis ammon ammon	Unknown	1	Altai / Mongolia
Ovis ammon darwini	Unknown	1	Gobi-Altai / Mongolia
Ovis ammon nigrimontuna	Unknown	2	Kara-Tau / Kazakhstan
Ovis ammon collium	Unknown	1	Kaaganda / Kazakhstan
Ovis aries	Edilbey	1	Alma-Ata / Kazakhstan
Ovis aries	Astrachan	1	Tschimkent / Kazakhstan
Ovis aries	Gizarr	1	Tadjikistan
Ovis aries	Awassi	1	Syria
Ovis aries	Kivircik	1	Aegean / Turkey
Ovis aries	Daglic	1	The western region of Turkey
Ovis aries	Akkaraman	1	Central Anatolia / Turkey

a. Nomenclature is according to Vorontsov et al. (1972).
b. n.a., not applicable.

On the other hand, in temperate cold weather sheep developed course wool to protect themselves from extreme cold. In addition, they are able to store a substantial amount of energy in the form of fat in their internal organs as well as in the tail to use in the dry and cold season when the food is scarce. Furthermore, sheep in the temperate region including Europe have developed a cover of thick subcutaneous fat layer and an incredible amount of internal fat deposition in order to avoid cold shock [48,49].

1.3. The Economic Importance of the Sheep Industry

Sheep industry, along with other domestic animal industry, is the most important constituents of the world economy. A variety of products including cosmetics like hand lotion, clothing like a woolen blanket and leather products, and a protein source like lamb chops are the contribution of the sheep industry. We cannot even think the world without sheep and sheep products. In this capacity, this industry promoted the establishment of other input industries such as sheep health supply, sheep feed, meat processing, leather processing, and wool processing industries. Therefore, it is easy to understand the importance and effects of the sheep industry and other related industries in the whole economic system [50,51].

Despite there have been many changes occurred in the husbandry practice in the sheep industry as well as the genetic, productive, and reproductive performance of sheep since the first domestication, it provides a decent way of livelihood for many people and has significant contribution in the economic system in the industry and agricultural sector. The population of the world is increasing with an exponential rate and it has been estimated that by the end of 2050, we need to face the challenge of producing more than 60% of the agricultural output using the same or even fewer resources. Considering this fact, it is also estimated that the demand for meat and meat products will also increase substantially to mitigate the increasing demand for quality protein. In this scenario, the sheep industry is one of the most important industries that could play a substantial role in providing quality meat for the increasing demand [52,53]. It has been estimated that the production of world meat will grow almost 20% at the end of 2021 through primarily by increasing the productivity of beef, pig, and poultry. In this regard, the sheep industry should play a significant role. However, the current standpoint of the industry may not be able to fulfill the demand because increasing the number of sheep

simply does not work because of the competition for natural resources. Therefore, we have to enhance the productive and reproductive performance of the sheep through the genetic and marker-assisted selection process.

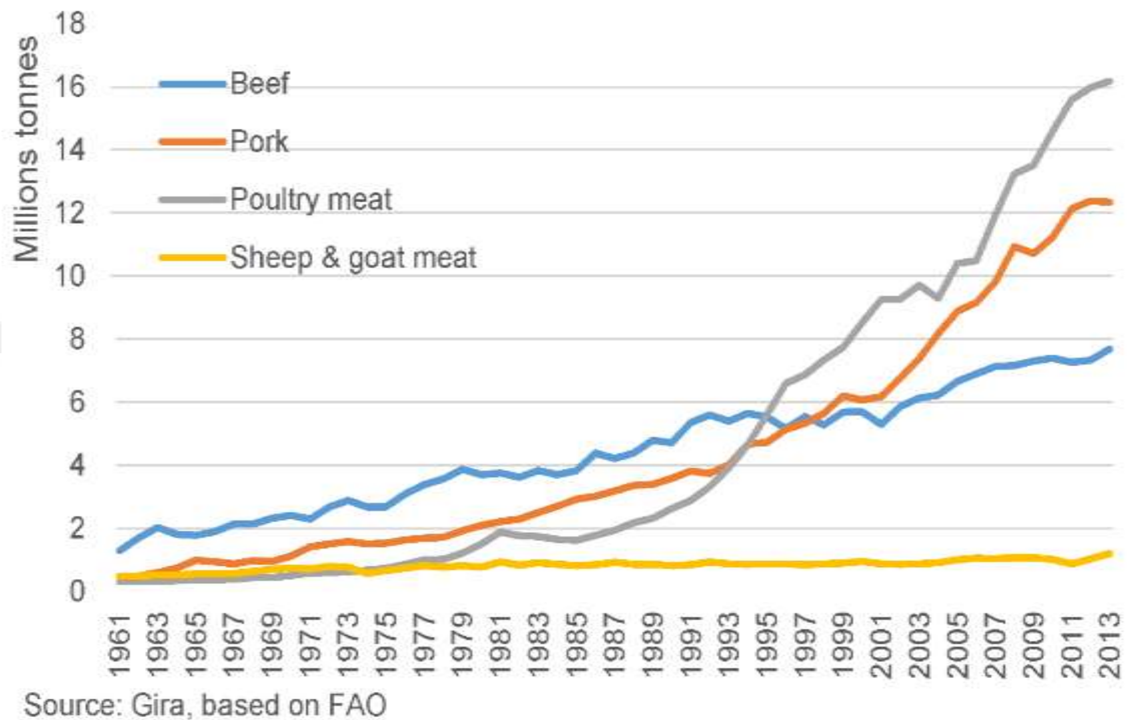

Figure 1.1. World meat trade volumes from 1961 till 2013. Quoted from [54]

In fact, it is true that, besides the demand for meat in the USA or EU (the traditional importing markets), the demand will also be increasing in the developing Asian countries including Qatar, Turkey, India, United Arab Emirates, Jordan, Saudi Arabia, and China. To fulfill the demand in the USA, EU, and Asia, it is evident that the sheep industry will face several challenges. Among these, there are two main challenges that should be solved in order to compete with other industries. The first challenge is to solve value and consistency, contemplating product differentiation, and increase production and efficiency. Secondly, the adaptation of technological changes. However, it would not be easy to compete with other meat industries like beef, pig, and poultry because of the type and size and the total amount of capital invested [54,55] see Table 1. 2. In the current situation, the sheep industry and its supporting industries have a significant economic contribution to the world economy.

Table 1.2. Meat production globally (,000 tons) and presented by the region. Quoted from [46]

Region	Cattle meat	Buffalo meat	Sheepmeat	Goat meat	Chicken meat	Pig meat	Total ruminant meat	Total meat
Africa	4601	270	1205	1073	3276	820	7149	11245
Latin America	16527	0	305	126	15737	5695	16958	38390
North America	12707	0	103	0.001	16869	11303	12810	40982
Soviet Union	3866	0	535	46	2118	2650	4447	9225
Eastern Europe	930	0.2	117	20	1868	4089	1067	7024
Western Europe	7935	8	1299	132	7827	18717	9374	35918
India	1334	1501	234	527	1900	497	3596	5993
China	5357	345	1800	1704	9964	46622	9207	65793
Asia	2786	1025	387	806	7052	8074	5004	20130
AUS-New Zealand	2814	0	1138	23	917	441	3974	5332
Middle East	720	22	766	233	2912	20	1740	4672
World	59576	3172	7888	4691	70451	98927	75327	244705

1.4. The Importance and Challenges of Sheep Husbandry

It is now an established fact that sheep can adapt with a wide range of production system under different climatic condition. In addition, another advantage of sheep rearing is that the feed requirement of sheep is less when compared to cattle farming. Due to bigger body size cattle require a large quantity of feed. Higher adaptability in a wide variety of environments and lower feed requirements make it easier to integrate smaller ruminant like sheep to the different farming system [56]. It is true that the grazing area for the large ruminants is decreasing day by day due to the increasing number of population and their housing which makes it difficult to continue large ruminant farming. Because of the increasing pressure on the land and other resources due to the exponential growth of the population, the arable land allocation for agriculture and the animal rearing will decrease in the near future. Therefore, it would not be wise to depend only on big ruminants for high-quality protein. Instead, the focus should be made on the small ruminant like sheep as it requires small grazing land and can survive with poor nutrition [57]. In addition, the sheep population can grow rapidly within a short period of time as the generation interval is short. These characteristics allow the farmer to sell the surplus animals to get financial support for other agricultural enterprises they involved with [58]. Due to their high growth and economic sustainability, sheep and sheep farming sometimes considered as the “village bank”. Although sheep and other small ruminants comprise only the 7% share total capital

investment in the mixed production system (crop-livestock), it accounts more than 40% cash return and on an average 19% of the value of livestock-derived foods [59,60].

Despite sheep industry played a significant role in the world livestock trade and its involvement is increasing day by day, very little attention has been paid to improve the production system and increase the reproductive and productive ability. This, in turn, takes the sheep industry to the backward position which is one of the major challenges in the sheep industry. Therefore, it is important to give emphasis on the housing system, integration of modern technologies in reproduction and production, and finally the use of genetic information to produce the superior animals [61].

The most significant challenge faced by the sheep farmers today is the producing animals that contribute less to global warming. Recently, it has been shown that the ruminants including sheep contributing to global warming by emitting methane gas as a result of the microbial activity in the rumen. The impact of ruminants on the climate change or global warming has been assessed by the climate change scientists that indicating the fact that ruminants are one of the major contributors for the increase of greenhouse gas [62]. It has been estimated that livestock agriculture, particularly ruminant industry, contributes around 8–10.8% of the total global greenhouse gas (GHG) emissions which can be as high as 18% depending on the lifecycle analysis. Almost all continents including South and North America, Latin America and Europe contributes to the methane emission through ruminant rearing, however, the significant contribution comes from Asian countries. Among all the ruminants, cattle dominate the methane emission [46,63]. Although sheep contribute to the methane emission, the contribution is far less than that of cattle. In this regard, sheep is a better choice than other ruminants. Scientists throughout the world are trying to develop breeds or variety of sheep that are producing far less methane in their lifecycle. If they succeed in producing such an animal model, the sheep industry will mitigate the challenge and will take place of other ruminants.

Table 1.3. Major livestock-mediated greenhouse gases emitter regions (million tonnes of CO_2-equiv./yr.). Quoted from [46]

Region	Enteric fermentation (CH4)	Manure (CH4)	Manure (N_2O)	Soils (N_2O)
Latin America	460	17	11	394
Africa	280	14	6	361
China	259	22	69	536
India	218	23	0	58
Asia	175	24	35	192
Western Europe	160	69	26	257
North America	136	43	22	300
Soviet Union	97	12	28	76
AUS-New Zealand	88	3	1	32
Eastern Europe	28	7	12	43
Middle East	27.3	1.6	0.7	50.7
World	1929	235	211	2299

1.5. Economically Important Disease in Sheep

The disease is one of the most important causes of economic losses in the livestock industry as well as in the sheep industry. Controlling livestock diseases now receiving immense interest not only from the livestock operators, whose primary objective is to cut the cost of farming and make it profitable but also from the policymakers who are interested to enhance the animal health and make the livestock farming more sustainable. Like other ruminant animals, sheep may suffer from a wide variety of diseases where the key component of the pathophysiological condition is the inflammation. Among all the diseases, sheep are highly suffering from different diseases caused by an increased level of inflammation. Foot and limb lesions are economically important inflammatory diseases that include dermatitis, interdigital and digital dermatitis, foot-rot, mastitis, and pneumonia which are commonly associated with sheep scab. In fact, the disease can cause economic loss in domestic farms in many ways. The most important causes of economic loss due to diseases include diagnosis of the disease, preventive measures, treatments, loss of productive and reproductive performance, and so on. Therefore, a small decrease in the disease prevalence and reduction of severity may have larger economic gain in the sheep industry through cutting the cost of production and increasing the productivity [64,65].

Besides the inflammatory diseases, there is another disease like Urinary Calculi, Stomach and Nodular Worms, Enterotoxemia or Overeating Disease, Scours, Scrappy,

Ketosis or Pregnancy Disease, and White Muscle Disease or Stiff Lamb Disease are also common in sheep that need to be taken care of in order to have a profitable sheep farming. Therefore, it is important both for the sheep industry owners or the sheep farmers that they need to check their animals on a daily basis for any abnormalities or any change in the health of the animals as well as the loss of productivity. The farmers should follow the saying “prevention is always better than cure!”. Indeed, to take the preventive measures, it takes less cost input. In turn, these small preventive measures could save some large amount of money by preventing the occurrence of diseases on the farm. The most common and effective preventive measures are a vaccination against common diseases, balanced nutritional input, animal hygiene, personal hygiene, and finally the good management.

Through researching the prevalence of the economically important diseases in the area, sheep farmers can know what measures should be taken to prevent and treat the animals. There are several diseases common in sheep including gut diseases, mastitis, abscesses, bluetongue, respiratory diseases, heartwater, and arthritis [66]. Among these diseases, respiratory diseases may cause a huge economic loss in the production system through decreasing productivity and increasing the mortality of the animals.

1.5.1. Respiratory Diseases

Sheep lung is a delicate organ that is continuously exposed to external air-borne pathogens. A schematic diagram of sheep lung is presented in Figure 1.2. It has been estimated that the respiratory system of an adult sheep may come across on an average 7-8 liters of air in each minute. Therefore, it is logical to think that the air inhaled by sheep can influence the prevalence of respiratory disease in them. Indeed, the air quality where the sheep are raised has a major impact on the health of the domestic or farmed sheep, particularly respiratory health. Despite the fact that the sheep’s respiratory tract is adapted to a wide range of environment and quite resistant to a variety of pathogens or contaminants, they can suffer from respiratory diseases if the defense mechanism is disrupted or the immunity is dysfunctional due to the suboptimal environment or the animals are exposed environmental stress. Under such conditions, i.e. suboptimal health condition, reduced immunity, and environmental stress, air derived contaminant components could serve as the prime causes of respiratory disease or induce the

expression of the pathogenesis of disease that previously existed or pulmonary diseases [67]. It is, therefore, crystal clear that previously existed disease conditions or if the lungs are exposed to disease and the health is compromised then a moderate or low level of air contamination could cause severe lung damage through the production inflammatory cells [68].

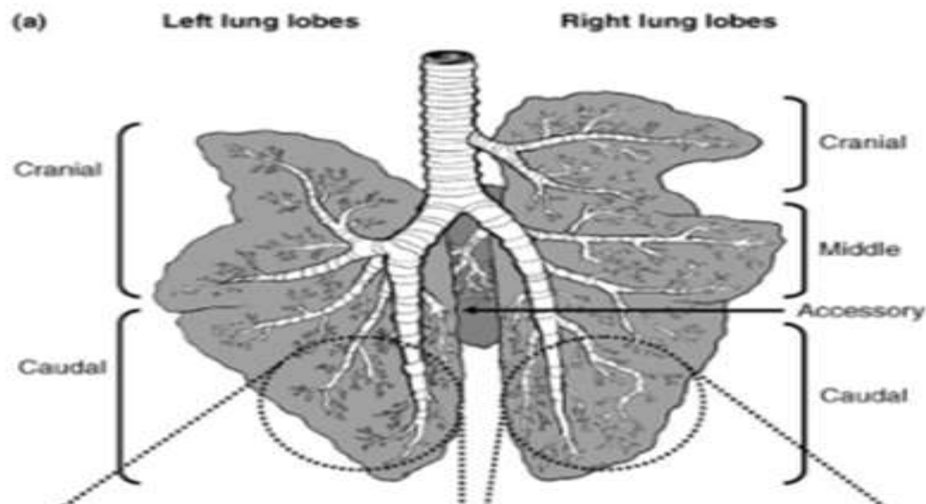

Figure 1.2. The anatomy of sheep lung diagram and was Quoted from https://www.studyblue.com/notes/note/n/resp-path-3-lung-dz-pleura/deck/ 4452952

The consequence of lung disease or respiratory disease is huge and have a direct influence on the economic outcome of a commercial farm. Respiratory diseases characterized by vulnerability to infection, loss of appetite, lower body weight gain, higher mortality and at the end of the day a considerable level of economic loss for the production system. Besides, the commercial losses, these diseases also have great concerns with animal welfare. It has been shown that the small ruminants including sheep can be suffered highly from respiratory diseases that have direct or indirect implications on the animal's health as well as the production system [69,70]. A combination of viral or bacterial infection and the suboptimal environmental condition often resulted in severe respiratory diseases in sheep [71].

Irrespective of the causative agents of the infection (whether virus or bacteria), the infectious diseases in the respiratory system of the sheep is contributing up to 5.6 % of the occurrence of the total disease in the of small ruminants. The infection-induce respiratory diseases can be categorized into two major groups such as upper respiratory

tract diseases which include sinusitis (causative agent- the parasitic larvae, enzootic nasal tumors, gaseous irritation, and nasal foreign bodies) and lower respiratory tract disease that include primarily pneumonia. Most of the time the infection in both upper and lower respiratory tract is caused by the infectious agents including fungal, viral, or bacterial origin. However, we must not overlook the role of environment originated pollutants such as mechanical induction of respiratory distress, toxicants, and pollutants which may have a significant role in the respiratory disease progression. The respiratory disease condition can be acute, chronic, and or progressive in nature based on the etiological factors, environmental, and physiological factors [72,73].

It should be noted that virus infection alone cannot create a situation of acute respiratory diseases. For instance, pneumonia caused by *Mannheimia haemolytica* bacteria causes severe damage and economic losses when the lambs exposed to other secondary infection caused by other pathogens resulting in an outbreak of pneumonia. In addition to secondary infection, housing conditions such as temperature fluctuations, ammonia levels, humidity, and stocking density or drastic alteration of the weather including severe wind or rain and other managemental practices like repeated transportation and handling induce stress, moving sheep from poor grazing to silage dominated food, and finally mixing sheep from multiple sources can have marked effects on the outbreak of *Mannheimia haemolytica* [74,75]. Indeed, respiratory diseases in sheep caused by *Mannheimia haemolytica* could result in unwanted mortality or causing severe suffering to the animals that reduce the productivity of the animals. It has been shown that these symptoms are highly correlated with the *Mannheimia haemolytica* induced pasteurellosis. Although the role of mycoplasma, particularly Mycoplasma ovipneumoniae, is not taken into consideration sometimes, the combination of mycoplasma and *Mannheimia haemolytica* may have severe impacts on the health of sheep of all age-group, particularly in newborns [72,76].

It is true that several pathogenic microorganisms are involved in disease progression in the respiratory tract. However, previous studies have shown that the relation of respiratory diseases progression and the effect of the altered environment can never be overlooked. These environmental factors irritate the respiratory tract producing stress in the microenvironment causing a decline in the immune status of the small ruminants and thereby assisting bacterial, viral, and parasitic infections in breaking down the tissue

defense barrier. As well as, the environmental pollutants cause acute or chronic reactions as they deposit on the alveolar surface, which is characterized by inflammation or fibrosis and the exhibition of transitory or persistent tissue manifestation. The disease development can be portrayed as three sets of two-way communications among pathogen, environment, and host but the interactions are highly variable. Moreover, the environmental scenario is never static; new compounds are introduced daily making a precise evaluation of the disease burden almost impossible [69,77].

1.6. *In Vivo* and *In Vitro* Studies to Understand the Underlying Mechanisms of Disease

1.6.1. *In Vitro*

The word *in vitro* stands for something which is in the glass. Therefore, the word *in vitro* experiments are the experiments that are performed outside the general biological context with biological molecules, cells, or microorganisms. Experiments conducted based on the small part of the organisms which has been separated from their normal biological environment allows a more convenient or detailed analysis than conducting the experiment using whole organism [78,79]. In order to monitor the clinical status of the patients or for the diagnosis of a disease using tissues, cells, or blood samples *in vitro* experiments are generally used to have the precise information about the disease. It is true that *in vitro* studies-derived results are not always trustable and could give false negative/positive results which may not predict the scenario of the whole organisms. Besides, in the organism in a biological surrounding, a particular mechanism or a pathway can be influenced by hundreds of molecules which are quite impossible to mimic in the *in vitro* condition outside the animal. Nevertheless, the use of *in vitro* experiments for the induction of different disease models such as respiratory tract disease or disease related to digestive system or other diseases in the lab condition allows researcher the opportunities to identify the best suitable way to solve the problem regarding a particular disease in addition to find the suitable [80].

Although *in vitro* experiments have many advantages, there are some disadvantages too. The first disadvantage is that it could be quite challenging to mimic the results from *in*

vitro experiments to the biology of an intact organism. The researchers doing *in vitro* experiments must keep an eye not to over-interrupt their results which may lead to a false conclusion about system biology as well as the whole organism. For instance, a new viral drug developed by the scientists in order to treat a viral pathogenic infection (HIV-1) may find that the function of a candidate drug is particularly useful to avoid the replication of the virus in *in vitro* experiments typically in cell culture [81], however, in the organism setting it may not work as it is working in the *in vitro* conditions.

1.6.2. *In Vivo*

The type of experiments where the effects of different biological or synthetic stimuli were examined in the whole live organisms is known as *in vivo* studies. It is important to note that the *in vitro* (performed within the glass) are performed generally outside the animal body, i.e., in Petri dishes, cell culture plates, and test tubes in a laboratory setting such as an incubator. On the other hand, the *in vitro* studies generally involve the living organisms to test the effects of a specific drug or the altered environmental conditions directly on the animal. Typical example of *in vivo* investigations include but not limited to: the development of antibiotics, effects of availability of nutrients on the performance of the animals, induction of disease in the laboratory animals to understand the disease etiology, to understand the pathogenicity of particular disease caused by bacterial infection through the use of bacterial toxins which is purified according to the need, and antiviral drugs. Although *in vitro* studies give the preliminary idea of the effects of some stimuli outside the body of the animal, it is always important to conduct *in vivo* experiments to confirm the effects of stimulus to confirm the biology of the disease. Therefore, testing and clinical trial of the animal is considered better to observe the overall effects of an experiment on a living subject [82,83]. It is inevitable to verify the effects of drugs in *in vivo* model during drug discovery because sometime the *in vitro* experiment could identify some candidate molecules for a specific disease or disorder which might be irrelevant in the *in vivo* condition. For instance, one molecule can be effective in the *in vitro* setting, this molecule may not reach the site of action in the *in vivo* condition due to the rapid catabolism [84,85].

It is important to note that different branches of biology and medical science define *in vitro* and *in vivo* studies in a different way. For instance, in nutritional studies or in drug

delivery or drug discovery studies *in vivo* studies involve the whole organism. However, brunches like microbiology, *in vivo* experiments often involve live isolated tissue instead of the whole animal or organisms. Experimentation involves a small part of an organ or tissues from the organ often termed as *ex vivo* experiments. It is important to note the when the tissues are disrupted to individual cells and tested or analyzed, it is known as *in vitro* [86]. Although the aim of a study can be different in their objectives i.e. to have information or gain knowledge of system biology or to develop or discover a new drug, the properties and the nature of a chemical cannot be independently considered. For instance, a chemical tool that can disturb cell function is one thing; compound that binds to the recombinant proteins are another, and agents which are considered as pharmacological may resist the defense mechanism of the living organism and disturb the physiology are yet another. It is as simple as discovering something in *in vitro* which also performs the same in the *in vivo* condition, drug discovery could be as simple as the manufacturing of the drug. [87]. Both *in vitro* and *in vivo* studies a variety of agents are used to identify the effects of microorganisms including Gram-positive and Gram-negative bacteria.

1.7. Gram-Positive and Negative Bacteria

Depending on the surface composition and the staining characteristics of the outer cellular wall, all bacteria are divided into two main categories namely Gram-negative and Gram-positive bacteria. Particular components in the cell wall which is unique such as *Lipoteichoic acid* (LTA) and *Lipopolysaccharide* (LPS) are able to induce the stimulation of cells to belong to immunity and are considered as PAMPs which can be recognized by TLR family [88]**.**

1.7.1. LPS and LTA Structure and their Mode of Action

Lipopolysaccharide (LPS) is a component found in the outer layer of Gram-negative bacteria which is also recognized as bacteria originated endotoxins and lipoglycans. LPS are large molecules and the molecular structure indicating that these molecules are composed of a polysaccharide and lipid and a covalent bond joins the inner and outer core. The outer membrane of the Gram-negative bacteria is largely composed of LPS which is not only protecting the bacterial membrane from a wide variety of chemical

shock and attack but also contributing substantially to maintain the integrity of the organism [89,90]. In addition to physical and protective support, LPS has been found to be involved in increasing the negative charge of the membrane of cells which also maintain the overall structural integrity [91]. Because of its binding ability to different cell membrane receptors including MD2/TLR4/CD14 receptor complex, LPS are able to mediate a robust induction of immune response in the animal by working as prototypical endotoxins. Although a wide range of cells can be stimulated by LPS exposure, it is particularly the immune cells especially B cells, macrophages, dendritic cells, and monocytes are quickly responded to the exposure which is characterized by secretion of a wide variety of chemical compounds including eicosanoids, nitric oxide, and different pro-inflammatory cytokines [92].

Due to its exogenous pyrogenic nature, LPS can induce the oxidative stress response in various types of cells that have TLRs and as part of the cellular stress response, the cells are producing excessive level of superoxide which is the major category of reactive oxygen species [93,94]. By the use of the *TLR4* receptor on the cell surface of macrophages, they can recognize the LPS mediated infection [95]. Immediately after recognition of the LPS, the ligand binds with the *TLR4* which ultimately promote the activation of the Myd88 dependent pathway that culminates with the activation of *NF-κB* and AP-1 transcription factors that recognize specific sequence motifs in the nucleus [96]. Other toll-like receptors such as *TLR9*, *TLR3*, and *TLR2* also share the same pathway to mediate the immune response in different immune cells. In addition, activation of *TLR3/4* may also lead to the activation of *Myd88*-independent pathway culminating with the activation of interferon response factors 3 and 7 (*IRF3/7*) transcription factors that recognize the canonical interferon-response element (ISRE) motif [97].

On the other hand, the major component of the outer cell wall of the Gram-positive bacteria is *Lipoteichoic acid* (LTA). LTA is a large compound which is composed of lipid and other materials which is the characteristic protein structure of the Gram-positive bacterial cell wall. In addition to the lipid structure, LTA also contains thick peptidoglycan which can be measured up to 80 nanometer that is present in the inner layer of the cell membrane. Depending on the length of glycerol phosphate and ribitol chains, the functional structure of LTA can be varying within different Gram-positive

bacterial species. By the help of a diacylglycerol, LTA binds with the cell membrane. Sometimes it also performs as the regulator of wall enzyme which is autolytic in nature. Furthermore, LPS may exhibit antigenic characteristics because of its ability to stimulate a highly specific immune response [98].

LTA can exert its effect on the target cells by both specific and non-specific bindings. For instance, through a non-specific binding with the target cells and membrane phospholipids. Alternatively, LTA can bind with the target cells through a specific binding through CD14 and Toll-like receptors. In either case, it induces the production of a wide range of cytokines and chemokines. It has been shown that the binding of *TLR-2* induces the expression of a central transcription factor *NF-κB* and depending on the physiological condition can increase the expression of both anti-apoptotic and pro-apoptotic genes [99]. Subsequently, the activation of mitogen-activated protein kinases (MAPK) takes place along with phosphoinositide 3-kinase activation as a result of the activation of *NF-κB* and other genes related to apoptosis. It has been shown by examining the molecular structure of LTA that it has a robust hydrophobic bond of the whole bacteria [100]. An overview of the LTA and LPS structure is presented in Figure 1.3.

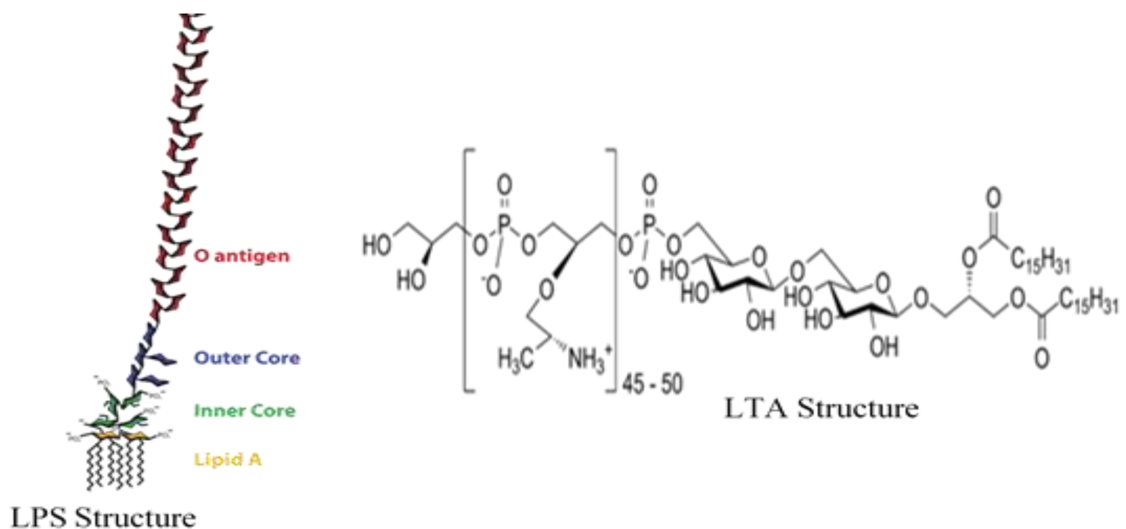

Figure 1.3. The overview of LPS and LTA structure. Quoted from [101,102]

1.8. The Response of the Immune System Against Pathogens

When a foreign protein compound entered into the body, the body reacted immediately against it to kill or destroy the foreign particles. This process is called the immune response and the ability to kill the foreign substances is known as immunity. The response in the physiological level due to the immune system activation through the foreign body or antigens could lead to two type of effects on the body. The beneficial immunity is killing the pathogenic microorganisms and the detrimental autoimmunity against the antigens produced by the animal and causes allergies and graft rejection. Both the macrophages and B and T lymphocytes are immanently involved in the immune response of a cell [103,104]. Lymphokines which is produced by T cells can induce the activities of other host cells. On the other hand, antibodies or other immunoglobulins are produced from B cells which can later react with antigens or foreign protein bodies. It is true that the property in terms of the immune response may transfer from sensitized animals to unsensitized animals through circulating cells or micro-molecules in the serum. The immune response is normally active against protein substances which is non-self to the organism and is highly specific to recognize the antigens [105].

1.8.1. Innate Immunity and Involved Cells

As we discussed before that the defense system of the host organism against any pathogens is divided into two categories such as adaptive and innate immunity. Innate immunity is the first line defense system in an animal’s body, and it is considered as the omnipresent arm of the immunity. It is important to note that innate immunity is unspecific in nature and immediately activated upon the recognition of any danger. Innate immunity includes different immunity effector cells known to be involved in recognizing and eradicating the foreign particles that invade the body, skin and mucosa which are considered as physical barrier, the complement system, the chemical protection system of different antimicrobial substances secreted on epithelial [106,107]. It is necessary to activate innate immunity in order to activate the adaptive immune system. Adaptive immunity generally starts slowly compared to innate immunity and the important thing is that the adaptive immunity can recognize a certain pathogen by keeping an immunological memory. A crucial bridge between adaptive and innate

immune system are the antigen presenting cells including dendritic cells and macrophages [20,108,109].

1.8.1.1. Bronchoalveolar Lavage (BAL) Fluid Functions and its Dominant Cells

The notion of bronchoalveolar fluid is new, in fact, it is an old idea. Bronchoalveolar fluid from the lungs is also known as bronchoalveolar lavage (BAL). In the case of tuberculosis patients, a technique called bronchial lavage is performed during rigid bronchoscopy in order to collect the samples for further analysis. BAL fluid contains many cells related to immunity and other bioactive molecules. Therefore, it is extremely important to understand the biology of BAL fluid and it's content (especially the dominant cells) in order to have substantial knowledge about the innate immune system in the lung. In addition to immune-related information, the BAL fluid also provides detail information about other cells and non-cellular constituents which are present in the lower respiratory tract. Because of its characteristics cellular and noncellular contents, BAL fluid is considered a new window to understanding the pathophysiological condition in the lung. Any deviation of the content in the BAL fluid and cells may represent the changes in the physiological condition to a pathological condition in the lung [110].

The analysis of BAL fluid cytology provides a unique insight into the pathophysiological condition of a patient. Therefore, collecting and analyzing BAL fluid become popular to understand the health and/or disease condition of the lung which is later known as the “BAL procedure” and applied as a research tool. In a clinical setting, it is used to a preliminary diagnostic tool to determine the non-infectious and infectious initial lung disorders [23]. Because of minimal invasive nature, easy, tailored, and safe in practice, collecting BAL fluid for the analysis to predict pathophysiological condition has many advantages compared to biopsy procedures. Nowadays, analyzing BAL fluid has become one of the prime choices of the diagnostic tool in pulmonary disease. In this regard, it is important to standardize the interpretation method of the cytology of BAL fluid to improve its diagnostic potential. This is particularly true for the diagnosis of immunological, infiltrative, infectious, and non-infectious lung disease [111]. The term cytology stands for the analysis of the cells and their contents. Therefore, the cytology of BAL fluid referred to the method that analyzes

the cellular components in the BAL fluid. Analyzing the BAL fluid reveals that the most available and prominent cells in the alveolar space are the inflammatory cells which are composed of neutrophils, lymphocytes, and macrophages [112].

Among all the immune cells in the BAL fluid, the most common and abundant cells are the alveolar macrophages. This cellular type represents more than 80% of the cellular population in a normal healthy subject. On the other hand, lymphocyte represents approximately 10% while the neutrophils represent less than 5% of the cells retrieved from the BAL fluid. In the lung of smokers, the concentration of neutrophils is higher than normal totaling more than 5-10% of the total BAL fluid cells. However, in normal health non-smoker individual's BAL fluid the neutrophils are rarely seen. Because of these characteristics, the BAL fluid is considered as an accepted method for the diagnosis of infection or deviation in lung physiology [113]. In addition, infections caused by bacteria and unusual bacterial infections such as Legionella can also be diagnosed by the BAL fluid cytology analysis [114]. In addition, cytology of BAL fluid has also been adopted in many clinical and hospitals to detect bacterial loads and routine tests to diagnose bacterial infections [115]. Therefore, it appears that the cytology of BAL fluid is very effective to diagnose the disease or any deviation in the lung which is non-invasive in nature and easy to perform. A brief description of different cellular components of BAL fluid is given below.

1.8.1.1.1. Alveolar Macrophages Cells

Alveolar Macrophage (AM), is the major type of BAL fluid cells, can be found near the pneumocyte in the pulmonary alveolus which is separated from the wall. In general, the activity of the AM is higher than any other immune cells because it is present in the place between the outside world and the body. The respiratory tract is a delicate organ and needs to be kept clean for its proper functioning. To keep the respiratory tract clean and functioning, AMs remove foreign particles including all types microorganisms and dust particles both upper and lower respiratory surfaces [116]. It is true that the respiratory tract comes in contact with the toxic substances and pathogens and affected severely. The air we breathe is not always clean and it may contain some substances which can be considered as pathogens by the body. In fact, in the modern day due to the industrial revolution, the air becomes so polluted which was never before. Therefore,

not only human but also domestic animals are suffering from air-born disease including pneumonia and other lung diseases. The respiratory tree of the lung is covered through a line of ciliated epithelial cells and it is composed of bronchioles, trachea, and larynx. Because of respiratory activity, the respiratory tree continuously exposed to materials which are toxic and harmful [77]. When these harmful and toxic substances break the superficial barriers, the body's immune system responds in an orchestrated manner in order to defend the body [117]. There are at least three types of cells present in the alveolar microenvironment that is as follows:-

a. Cells that are forming the alveolar wall structure known as Squamous Alveolar representing the **Type I** pneumocyte.

b. Great alveolar cells are known as **Type II** pneumocyte which is responsible to secrete the pulmonary surfactant and increase the gas exchange by lowering the surface tension of water.

c. The third and most important type of cells are the **Macrophages** which are responsible for the destruction of and foreign materials the enters into the body.

Due to its phagocytic nature, AMs may play a crucial part in the maintenance of host defense system, organ homeostasis, generate an immune response against foreign substances, and finally the tissue remodeling [118]**.** The pulmonary concentration of the AMs is one of the important deciding factors in lung immunity because it plays a critical role in the maintenance of local immunological homeostasis [119]. By expressing the surface receptor molecules, AMS are interacting with other cells and release several kinds of secretory products. In addition, AMs are found to be involved in many physiological actions such as necrosis and apoptosis. In case of necrosis and apoptosis AMs, accumulated near the site and exerts its effects through phagocytosis. To identify the PAMPs present on the pathogenic microorganism’s cell surface, the innate immune system adopts pattern recognition receptors (PRR) to facilitate the recognition of the pathogen and combat against infection. Indeed, the AMs are reacted almost instantly after the recognition of the external foreign body and play a central role in the innate immune system in the alveolar microenvironment and acted as a front-line defense mechanism against toxins and pathogens. That’s why the research focused on

AM biology and physiology has got the research priority nowadays in order to understand the disease biology in lungs and the innate immune defense mechanisms against environmental toxins and other bacterial pathogens [120].

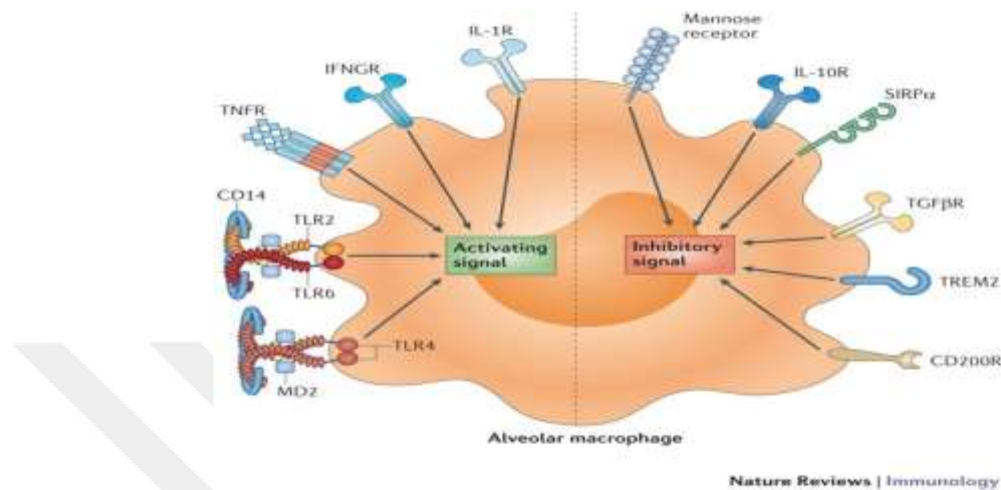

Figure 1.4. Typical pictorial presentation of alveolar macrophages along with its cell surface receptors. Quoted from [24]

1.8.1.1.2. Lymphocyte Cells

In the vertebrate's immune system, a lymphocyte the white blood cell subtype and it induces the activation of B cells (in the case of humoral, adaptive immunity driven by antibody), T cells (in the case of cellular, cytotoxic adaptive immunity) and natural killer cells (which function in cytotoxic and cell-mediated innate immunity). Because these cells are primarily found in the lymphatic organ, they are deliberately called lymphocytes. There are three major types of lymphocytes that can be found in the AMs including natural killer cells, B-cells, and T-cells. Lymphocytes are characterized by its large nucleus and can be identified under a microscope using this criterion [121].

Bursa or bone marrow-derived B-cells and thymus-derived T-cells are the major cellular constituent of is the adaptive immune system in sheep lung. Although both the B and T-cells are an integral part of the immune system, it is important to note that the B-cells are primarily involved in humoral immune response (immunity related to antibodies) whereas T-cells are involved in cell-mediated immunity. In the process of antigen presentation, both the B-cells and T-cells are involved in the recognition of specific 'foreign/non-self' antigens [122,123]. Upon the recognition of foreign pathogen, the

immune cells exert a specific response that is programmed or aimed to destroy or eliminate both the pathogen-infected cells and pathogen itself. In response to pathogens, B-cells are producing a huge quantity of antibodies that can neutralize or kill foreign components such as virus or bacteria [124]. There are two types of T-cells in the AMs such as T helper cells and cytotoxic T cells. It is interesting to note that in response to pathogenic infection, T helper cells are producing various cytokines that directing immune response, whereas cytotoxic T cells are involved in producing powerful toxic enzymes enwrapped by granules which initiate the death of the pathogen or pathogen-infected cells [125].

In case of an innate immune system, the natural killer (NK) cells are one of the most important components which are primarily involved in the identification and defending the host cells from both virally infected cells and tumor cells. By recognizing the changes is the surface molecule known as major histocompatibility complex (MHC) class I, NK cells can discriminate tumor cells from normal cells and infected cells from noninfected cells. NK cells are activated in response to a family of cytokines called interferons. Once the NK cells are activated, they are able to release granules containing cytotoxic materials to destroy the deviated cells. Due to the fact that NK cells don't require any previous activation to kill cells that are missing MHC class I, they are called natural killer cells [126].

1.8.1.1.3. Neutrophil Cells

Neutrophils are the major types of white blood cells in most of the mammals. These are the most abundant types of granulocytes (40% to 70%) in white blood cells. Although neutrophils played an essential role in the innate immune system of an animal, the function greatly varies depending on the species [127]. There are generally two types of neutrophils such as neutrophil- cagers and neutrophil killers. Both cells are generated from bone marrow-derived stem cells. Because these cells have the ability to enter parts of tissues (where other molecules/cells are unable to do so), they are extremely mobile, highly motile, and lived a short life [128,129].

The name neutrophil derives from staining characteristics on hematoxylin and eosin (H&E) histological or cytological preparations. Whereas basophilic white blood cells

stain dark blue and eosinophilic white blood cells stain bright red, neutrophils stain a neutral pink [130]. Normally, neutrophils contain a nucleus divided into 2–5 lobes. Neutrophils are a type of phagocyte and are normally found in the bloodstream. During the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, environmental exposure, and some cancers, neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation [131].

They migrate through the blood vessels, then through interstitial tissue, following chemical signals such as Interleukin-8 (*IL-8*), C5a, Leukotriene B4 and H2O2 in a process called chemotaxis. They are the predominant cells in pus, accounting for its whitish/ yellowish appearance [132,133]. Neutrophils are recruited to the site of injury within minutes following trauma and are the hallmark of acute inflammation; however, due to some pathogens being indigestible, they can be unable to resolve certain infections without the assistance of other types of immune cells [134].

1.8.1.2. Genetics of Innate Immunity

The existence of specific receptors in the outer surface of the immune cells help them to recognize any foreign bodies in most biological systems. Multicellular organisms are different from the microorganisms in a variety of ways that have been implemented to recognize and subsequent elimination of foreign pathogens. In this scenario, these characteristics are defined as microbial patterns and the molecules that detect them are known as pattern-recognition receptors. The prime examples of microbial patterns are phosphoglycan of parasites, peptidoglycan and lipopolysaccharide (LPS) of bacteria, and β-1,3-glucan of fungi [135]. It is true that with a limited number of pattern recognition receptors (PRRs) which is encoded in the germline innate immune system can identify microorganisms-associated pathogens. All PPRs has few characteristics which are common in nature such as, firstly, microbial components can recognize that is defined as PAMPs (we have discussed previously pathogen-associated molecular patterns). The recognition pattern is extremely important for microorganism survival and is, hence, very difficult to change by the microorganisms. Secondly, Second, PRRs can be expressed consistently and detect the pathogenic organism irrespective of the stage of lifecycle of the host. Thirdly, PRRs are encoded in the germline encoded, independent of immunologic memory, can be expressed in all cells. It is important to

note that various PRRs are reacting with specific PAMPs to exert distinct expression pattern of some genes that are able to modulate the activation of a specific signaling pathway, and finally induce the distinct antipathogenic responses [136].

Interleukin-1 receptor, also known as *IL-1R*, is the first mammalian protein that was shown to have similarities with the Toll. The first mammalian protein that was shown to have homology to Toll was the interleukin-1 receptor (*IL- 1R*). The *IL-1R*-associated kinase (IRAK) is signaled and stimulated by this Toll receptor which further stimulates the activation of *NF-κB* translocation. It works like a similar way that REL family members are activated by Toll family members. Despite the fact that *IL-1R* is one of the key regulators and play a central role in the innate immune system, it is not performing as the key sensor of infections. Instead of *IL-1R*, macrophages-derived *IL-1* can sense the infection at an early stage [137,138].

1.8.1.3. Pathogens Recognition by Immunity

Because of continuous breathing, the lung is constantly exposed to the external toxic materials and various pathogenic microorganisms as well. In the lung microenvironment, to protect the delicate lung tissues from the external invaders, macrophages are employed to induce the innate immune response via the phagocytic process or through the production of a variety of molecules line nitric oxide or cytokines [139]. Phospholipids and the proteins are the main components of the lung surface which aligned through a complex mixture. This line surface is sometimes also defined as a pulmonary surfactant. It is true that the innate immune response has the central role in distinguishing the non-self and self-compounds in order to respond against danger properly [140]. For instance, multicellular organisms including human have cells that contain a variety of receptors which are used for the surveillance of the intracellular and extracellular environment to detect the foreign proteins. These receptors are known as PRRs and the substances they recognize are defined as either danger-associated molecular patterns (DAMPs) alternatively known as pathogen-associated molecular patterns (PAMPs). A variety of classes of PRRs are defined such as 2-like receptors (*ALRs*), absent in melanoma (AIM), nucleotide-binding oligomerization domain (*NOD*)-like receptors (*NLRs*), retinoic acid-inducible gene (*RIG*)-I-like receptors, C-type lectin receptors (*CLRs*), and most importantly TLRs [141].

1.8.1.3.1. Toll-Like Receptors Pathways

Microbes from extracellular space can be detected by Toll-Like Receptors (TLRs). They are the transmembrane proteins encoded in germline which may also recognize structures from the endosomal pathway as well [33]. TLRs, are the first line defense warrior against external pathogens and other microbes and play an inevitable part in the innate immune system [5,142]. The ligand binding domain consists of leucine-rich repeats (LRRs) and the cytoplasmic Toll IL-1 receptor (TIR) domain is responsible for signaling. There are 10 – 12 type I integral membrane receptor paralogs present in TLR family that are associated with the inflammatory response as well as the pathogen-associated molecular signature [143]. While cell surface receptors are recognizing a wide range of bacterial cell wall components, the receptors are localized and they can identify the structure of nucleic acids in the endosomal components or intracellular space [38].

When TLR dimerization occurs due to the ligand binding, it initiates the TLR signaling and subsequently, the adaptor proteins are recruited by the dimerized TIR-domains. There are known five adaptor proteins which are associated with TLRs. These are myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor protein inducing IFN-β (TRIF), inhibitory sterile and HEAT/ Armadillo motif-containing protein (SARM), TRIF-related adaptor molecule (TRAM), and MyD88 adaptor-like protein (MAL) [144].

Differential downstream events are activated due to the bonding of differential adaptor protein with different TLRs. For instance, TLRs recruiting *MyD88* can induce a variety of inflammatory cytokines. On the other hand, TLRs like *TLR3* and *TLR4*, are inducing both inflammatory cytokine responses and type *I IFN*. For proper function and signaling of the TLRs, depicted should signal the adaptor and downstream mediators. Therefore, they are considered as the key TLR molecules. Molecules like *IRAK1/4*, *MyD88*, and *TLR7/9* are central to the immune pathway and are being investigated thoroughly in order to find the potential of therapeutic targets [145] see Figure 1. 4.

1.8.1.3.1.1. Toll-Like Receptor 2 (TLR2)

Toll-like receptor 2 (TLR2) can be found in the external surface membrane which is basically a membrane protein and able to identify the exogenous substances and mediate a proper signal to the immune system of the cells [146]. In fact, TLR2 is a protein structure which is the product of the translation of the *TLR2* gene. *TLR2* is one of the vital members of the innate immune system and plays an important role in the recognition of the pathogen and subsequent activation of the immune system [147]. PAMPs are present on the surface of the infectious agents which can be recognized by *TLR2* in order to develop effective immunity by producing necessary cytokines. Abundant level of *TLR2* can be found in leukocytes of peripheral blood and mediates a response through the stimulation of *NF-κB* in response to yeast and Gram-positive bacteria [37]**.**

1.8.1.3.1.2. Toll-Like Receptor 4 (*TLR4*)

Toll-like receptor 4, also known as TLR4, is also a surface molecule and transmembrane protein which is encoded by a gene called *TLR4*. One of the vital members of the toll-like receptor family is *TLR4* it belongs to the PRR family [148,149]. The activation of TLR4 resulted in the activation of *NF-ₖβ* intracellular signaling pathway and stimulate the production of a variety of inflammatory cytokine which subsequently activates the innate immune system [150,151]. In addition, the well-known function of TLR4 is *lipopolysaccharide* (LPS) recognizing. LPS is a surface protein complex found in the Gram-negative bacteria (e.g. Neisseria spp.). It can also detect heat shock protein, beta-defensins, a wide range of endogenous proteins including low-density lipoprotein (LDL), polysaccharide, and several viral proteins [152]. TIR-domains, which contain adaptor molecules, are recruited by the *TLR4* receptors upon the LPS recognition. *TLR4* cluster family and these adaptors are closely associated through the homophilic interactions between the domains of TIR [153].

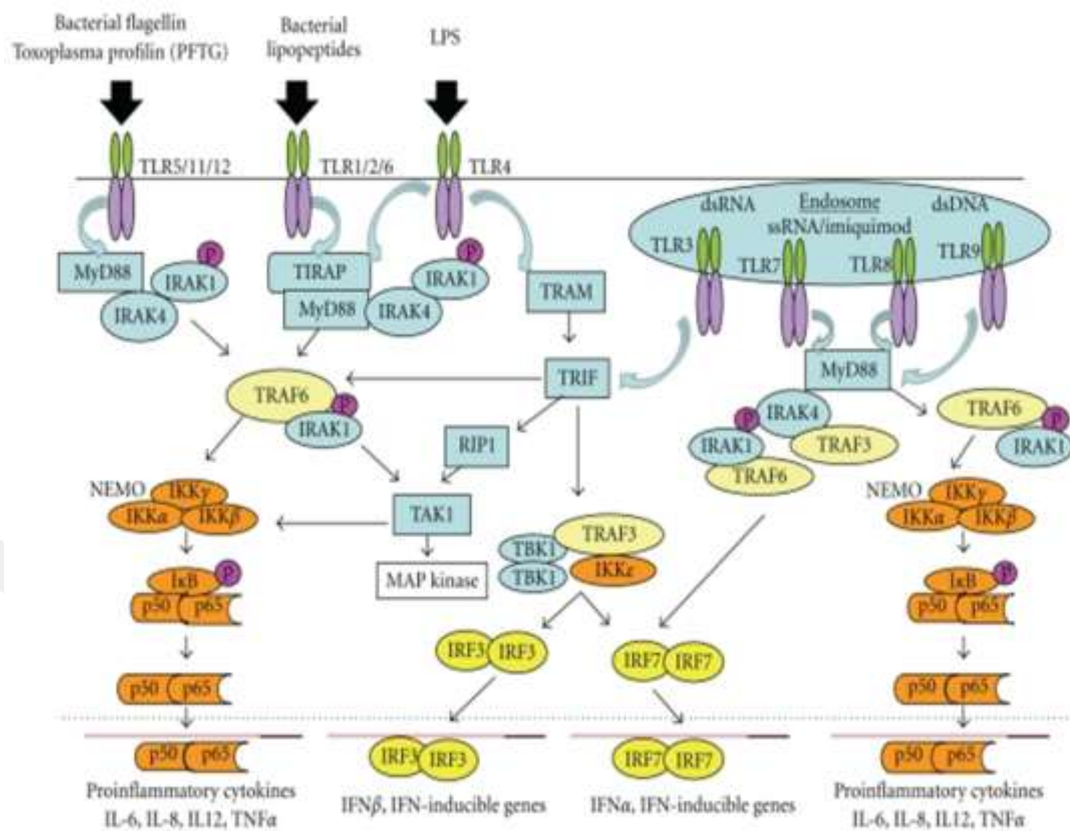

Figure 1.5. TLRs signaling pathway Quoted from [154]

1.8.1.4. Cytokines

Cytokines play an important role in cell communication which is found to be a pleiotropic signaling molecule (~5–20 kDa). They can mediate a wide variety of functions related to innate immunity. In addition, these molecules are equally involved in immune cell activation, antigen presentation, and cell trafficking. It is important to note that cytokines may act in both paracrine and autocrine fashion through recognizing the receptors neighboring or in the same cell, respectively [155]. In addition, systemic effects can be mediated by the cytokines occasionally. Cytokines can be secreted by many different cell types and express the receptors of cytokines on the surface of the cells. These cells include immune cells like mast cells, T lymphocytes, B lymphocytes, and macrophages as well as various stromal cells, fibroblasts, endothelial cells [156].

It is important to note that cytokines include the expression of interferons (*IFNs*), Tumour Necrosis Factor (*TNF*), adipokines, growth factors, chemokines, and interleukins (*ILs*). There are two major features that can define cytokines. The first one

is the redundancy, representing that various cytokines showed similar types of function, and second feature is pleiotropy, representing that one cytokine may exert several functions and if two stimulus signals making a solid response of cytokine than two different stimulators, it is assumed that there is a synergistic response of cytokines [157]. Often one signal can reduce the cytokine response stimulated by other cytokines through an antagonistic manner. *IL-12* is such type of proinflammatory molecule that has a heterodimeric domain containing comprises p35 and p40 subunits. Furthermore, signaling cascade of *IL-12* receptor is composed of *IL-12Rβ1* and *IL-12Rβ2* subunits [158]. Stimulation of *IL-12* signaling activates the tyrosine kinase 2 (*TYK2*) and Janus-activated kinase 2 (*JAK2*). The activation of these molecules further phosphorylates and involved in transduction of transcription factors signal and activator of transcription 5 *STAT5*, *STAT4*, *STAT3*, and *STAT1*. In addition, dendritic cell-derived *IL-12* in association with the macrophages synergistically enhance the naïve T cells differentiation to T helper 1 (Th1) cells that connecting the adaptive and innate immunity [159]. It is important to note that many studies indicated that in response to TLR stimulation through various TLRs supposed to exert a synergistic production of *IL-12* [160]. This synergistic effect is particularly important to control the production of proinflammatory cytokines [161,162].

1.8.1.5. Chemokines Cells

Chemokines are signaling proteins secreted by cells which are a family of small cytokines. The name chemokines originated from their capability to produce toxins with neighboring cells. Based on their structural characters, proteins of the chemokine family are classified. However, in this regard, the ability to attract cells do not take under consideration [163]. The chemokine molecules are generally small that ranging a molecular mass of 8-10 kiloDalton (kDa). It is worth to note that different cytokines share around 20-50% similarities within them. That means that they have shared amino acid sequence as well as gene homology. Many of the cytokines hold amino acid sequence that is conserved which is crucial in order to create their corresponding tertiary or 3-dimensional structure. There are main four classed of chemokines: such as XC, CX3C, CC, and CXC. By interacting with a complex protein called G protein-linked transmembrane receptor which is commonly known as chemokine receptors, these proteins mediate their biological effects [163–165].

In one hand, chemokines like *CXCL10*, *CCL11*, *CCL5*, *CCL4*, *CCL3*, *CCL2*, and *CXCL8* (*IL-8*) are well known for their pro-inflammatory properties. They might be activated for the recruitment of immune cells such as *IL-1*. In addition, these cytokines are actively participating in mediate the inflammatory response by recruiting the cells related to inflammation at the inflammation site. On the other hand, other chemokines are best-known as homeostatic because they participate in controlling cell migration at the time of tissue development and maintenance [166,167].

1.8.1.6. Signaling Pathways that are Controlling the Expression of Cytokine Genes

The innate immune system activation is highly complex and tightly controlled. Any deviation in the immune response or improper immune activation may have harmful effects on the host and no protection against pathogens. The production of cytokine is primarily maintained at the level of transcription through a wide variety of transcription regulators and hence, the production and/or the expression of different cytokines. The IRF, mitogen-activated protein kinase (MAPK)-pathways, and the nuclear factor of **κ** light polypeptide gene enhancer in B-cells (*NF-κB*) are the best-known signaling cascades that regulate and promote the expression of cytokine genes [153].

The activation of *MyD88* adaptor-dependent pathway through all TLRs but *TLR3* resulted in an interaction with *IL-1* receptor-associated kinase 4 (*IRAK4*), *IRAK2* and *IRAK1* to form a macromolecular complex in which the protein *IRAK4* is involved in phosphorylating *IRAK2* and *IRAK1* [168,169]. Upon the activation of *IRAK*s, they signaled the activation of *TNF* receptor-associated factor 6 (*TRAF6*) and initiate a bonding with TAK1-binding protein 3 (TAB3), TAB2 and TAB1 which results in the recruitment and activation of transforming growth factor- β- activated kinase 1 (*TAK1*) [170]. Inhibitor of *NF-κB* kinase β (IKK β) can be phosphorylated by the *TAK1* that subsequently involved in the phosphorylation of inhibitor of *NF-$_{k}$B α* (*I $_{K}$Bα*) [35]. It is important to note that the proteasomal degradation and polyubiquitination *K48* of *I $_{K}$Bα* resulted in the increased expression of the *NF-κB* components. The modifications in the expression of *NF-κB* components signals to the nuclear translocation in order to activate genes the bear *NF-κB* binding sites. In addition, *TAK1* is also involved in the activation of *MAPK* kinase 1/2 (*MKK1/2*), *MKK3/6*, and *MKK4/7*, which can further initiate the

activation of extracellular signal-regulated kinase *ERK2* and *ERK1*, p38 *MAPK*, and Jun N-terminal kinase (*JNK*) [148].

It is important to note that the cascades of *MAPK* signaling is involved in the activation of activating protein (AP)-1 transcription factor. The AP1 primarily consists of dimers which are formed through the activation of transcription factor (*ATF*) subunits. On the other hand, *NF-*$_k$*B* activation and AP-1 activation resulted in the production of cytokines including *TNF-α, IL-6,* and *IL-12*. Indeed, to activate a *MyD88*-independent pathway, TRIF adaptor protein is exclusively used by *TLR3* and *TLR4* [171]. It is well-known that *NF-κB* pathways can be activated by TRIF through the stimulation of *TRAF6/RIP1* or *TRAF6* that leads the stimulation and activation of the *MyD88*-dependent pathway and its downstream kinases as well. In addition, the TRIF pathway equally involved in the *IRF3* transcription factor activation, which is considered to an important step in the induction of *IFN* gene expression [149,172,173]. It is noteworthy that the phosphorylation of *IRF3* resulted in the formation of dimers that subsequently translocated in the cell's nucleus in order to form bonding with *IFN*-stimulated response element (ISRE) sites in *IFN* gene promoters [174].

By interacting with CARD domains, the cytoplasmic RLRs is able to signal the mitochondrial antiviral-signaling protein (MAVS) adaptor protein, which is commonly known as IPS-1, VISA or Cardif [175–177]. In a homeostatic condition, to block the initiation of signaling pathways, the RIG-I domains of CARD are folded over one another [178]. Once it bins with the corresponding RNA ligands, RIG-I undergoes a conformational change through an ATP-dependent mechanism which creates the availability of CARD domains for signaling. Following this step, dephosphorylation of CARDs and ubiquitinylate of CTD take place [179,180]. The addition of ubiquitin to the CARDs in RIG-I via the tripartite motif-containing protein 25 (TRIM25) ubiquitin ligase resulted in the MAVS binding in the mitochondrial-associated membrane and oligomerization of RIG-I (MAM) [181,182].

As a result, the activation of *TBK1* and *IKKε* take place that resulted in the *NF-κB* and *IRF3* activation. Immediately after the activation of *NF-κB* and *IRF3* translocated into the nucleus in order to provide the opportunity to the production and higher expression of pro-inflammatory cytokine and *IFNs* genes. The regulation of *RIG-I* was extensively

studied at many levels of regulation have been described by different research groups [183–185]. The regulation includes microRNA-mediated negative feedback regulation of signaling pathway components [186], *MAVS* mRNA [187,188], and posttranscriptional mechanisms including alternative splicing of *RIG-I* [189]. It is important to note that similar type of signals can be mediated by *MDA5* via *TBK1*/*IKKε* and *MAVS* [190].

1.8.1.6.1. Nuclear Factor Kappa B (*NF-κB*)

Nuclear factor kappa-light-chain-enhancer of activated B cells commonly known as *NF-κB*. It is a complex protein structure which is involved in the production of cytokines and survival of cells, as well as transcription control of DNA [150,191]. This protein complex is present in nearly all types of animal cells and particularly, involved in mediating the cellular response against external stimuli including bacterial or viral antigens, oxidized LDL, ultraviolet irradiation, heavy metals, free radicals, and cytokines, [151,192]. It is important to note that by regulating the response of the immune system against infection *NF-κB* plays an inevitable role in the defense mechanism of an organism. In addition, it has been shown that the improper activation and/or regulation of *NF-κB* is closely related with improper immune development, poor response against viral or bacterial infection, septic shock, inflammatory and autoimmune diseases, and cancer. Furthermore, the process of memory and synaptic plasticity may also be influenced by *NF-κB* [193,194]. Moreover, being a major factor of transcriptional regulation, *NF-κB* may regulate the expression of genes that are important not only for the adaptive immune response but also for the innate immunity [195,196].

Because of its nature that belongs to the "rapid-acting" primary transcription factors, *NF-κB* has an important role in regulating cellular responses. "Rapid-acting" group of transcription factors are those which might present in an inactive form in the outer cell surface and importantly, to become activated, these rapid-acting transcription factors do not need to synthesize new proteins. Members of this transcription factor family include nuclear hormone receptors, STATs, and c-Jun [197]. These transcription factors give liberty and the ability to *NF-κB* to perform a fast response against harmful cellular stimuli. Although the inducers of NF-κB activity are known but their characteristics are

highly variable. These inducers are ionizing radiation, cocaine, isoproterenol, bacterial lipopolysaccharides (LPS), interleukin 1-beta (*IL-1β*), tumor necrosis factor alpha (*TNFα*), and reactive oxygen species (ROS) [198].

The central activator of the *NF-κB* is RANK, which is basically *TNFR* in characteristics. Many factors are involved in regulation of the activation of *NF-κB* including through osteoprotegerin (OPG). OPG is a decoy receptor homolog for the *RANK* ligand (*RANKL*), which particularly inhibits the expression of *RANK* through forming a bond with *RANKL* [199]. Therefore, it is considered as the most complex activation procedure in the innate immune system. Stimulation of a wide range of receptor present in the cell surface and several products of bacterial origin leads to the activation of *NF-κB* and induce a rapid gene expression change. Different studies have shown that the surface molecule *TLR4* is an important receptor of Gram-negative bacteria associated LPS components. In addition, it is important to note that the members of TLR family are the key regulators of both adaptive and innate immune responses [200,201].

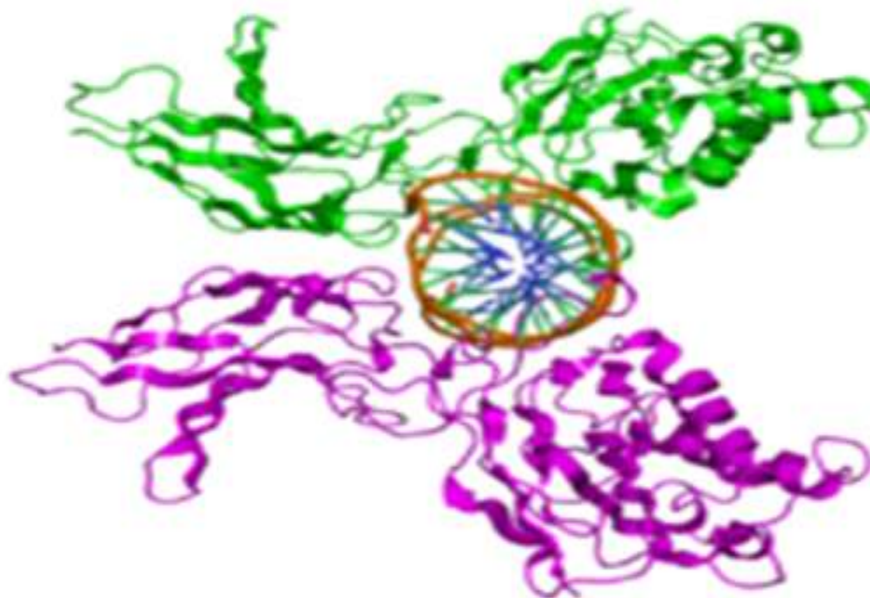

Figure 1.6. A homodimer bound to DNA (brown) and a homodimer of the *NF-κB1* protein (magenta and green) of the crystallographic structure (PDB: 1SVC) from the top view. Quoted from [202]

1.8.1.7. Using Gene Expression for Genetic Analysis Traits

The basic principle of sequence-specific transcription factors (TFs) binding to DNA and thereby activating or repressing gene expression is also conserved in eukaryotes and many of the sequence motifs have already been identified. However, an extra layer of complexity is that, in contrast to prokaryotes, eukaryotic DNA is located in the nucleus and 15 tightly packed around the nucleosomes. This adds two additional levels of regulation. First, since protein synthesis happens in the cytoplasm, the localization of TFs can be regulated as well. For example, the *NF-κB* complex is normally sequestered to the cytoplasm and is only localized to the nucleus after the repressor proteins have been degraded. Secondly, because nucleosomes have a much stronger affinity for DNA than single TFs do, a single instance of a TF motif is usually not sufficient for a TF to bind [203].

Since gene expression is regulated by TFs, to understand cell type-specific gene expression we first need to understand the principles of cell type-specific TF binding. Genome-wide profiling of TF binding has led to three key observations: (1) different factors in the same cell type often bind to the same locations, (2) the same factor in different cell types can often have different binding sites and (3) the same biological processes (such as self-renewal) can be regulated by distinct set of regulatory elements in different cell types [204–206].

In addition to total gene expression level, RNA-Seq data can also provide information about the relative expression of the gene from the maternal and paternal chromosomes. This is possible when an individual is heterozygous at sites within the gene body, making it possible to count the number of RNA-seq reads that come from each allele. The allele-specific expression has been shown to increase the power to detect gene expression quantitative trait loci (eQTLs) [207].

Therefore, eQTLs can be used to interpret GWAS associations, so, if the same genetic variant is associated both with expression level of gene A and increased risk of disease B then this can provide a mechanistic hypothesis that the expression level of gene A influences the risk of disease B. However as highlighted above, eQTLs are extremely common and because of strong LD between variants there is often a large number of

variants that are significantly associated with either gene expression level and/or disease risk. As a result, it is easy to get random overlaps between eQTLs and GWAS hits where the two associations are driven by different causal variants [208].

1.8.1.7.1. Tumour Necrosis Factor Alpha (*TNFα*)

The systemic inflammation is mediated by a cell signaling protein known as tumor necrosis factor alpha (*TNFα*). This protein is cytokine in nature and responsible for making up the acute phase reaction. Although activated macrophages are the primary sources of *TNFα*, it can be produced by a wide variety of cells including neurons, eosinophils, mast cells, neutrophils, NK cells, and CD4+ lymphocytes, [209]. The regulation of immune cells is the fundamental role of *TNFα*. The activation of *TNFα* is associated with cachexia, apoptotic cell death, inflammation, and induction of fever as it is an endogenous pyrogen. *TNFα* can inhibit tumor propagation and replication of virus and sepsis response through *IL6* & *IL1* producing cells. A variety of disease including inflammatory bowel disease, psoriasis, major depression, cancer, and Alzheimer's disease have been shown to be associated with the differential regulation of *TNFα* production [210–212]. Despite the controversies, the expression level of *TNF* has been currently linked to IBD and depression. In order to stimulate the immune system under the INN, the recombinant *TNFα* is sometime [213].

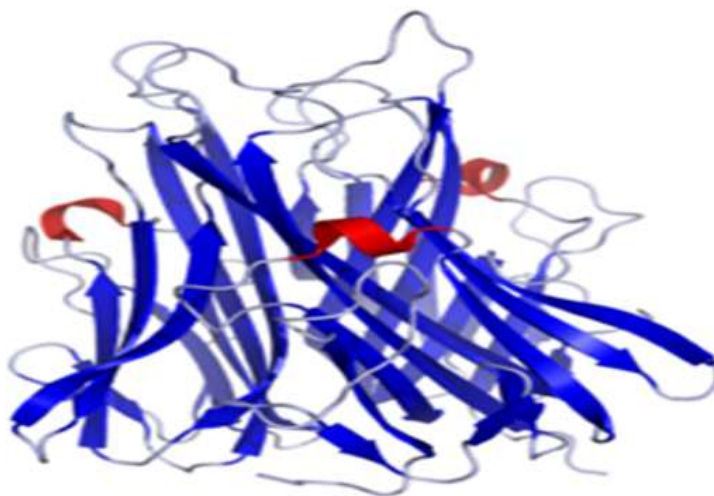

Figure 1.7. Crystal structure of *TNFα* published in the Protein Data Bank (PDB: 1TNF) Quoted from [214]

1.8.1.7.2. Interleukin 1 β (*IL-1β*)

Interleukin 1 (*IL-1*) is best-known for its leukocytic pyrogenic properties. In addition, it is also known as lymphocyte activating factor, mononuclear cell factor, leukocytic endogenous mediator, and other names [215,216]. The activated mononuclear phagocytes are the primary producers of *IL-1* and the primary job is to maintain the innate immunity-related inflammatory responses in hosts. By contrast to *IL-1α*, *IL-1β* is synthesized only after proper stimulation by the ligands as a precursor protein form. The expression of *IL-1β* is often induced by the activation of *NF-κB* transcription factor once innate immune cells are exposed to pathogens. This pathway is activated when the dendritic cells and macrophages are exposed to lipopolysaccharide (LPS) and may bind to *TLR4* and acts as PAMPs [217,218]**.**

Stimulation of innate immune cells by RIG-like receptors (RLRs) or TLRs can induce the synthesis of *IL-1β* precursor (and *IL-18*). However, in order to have access for binding with the *IL-1* receptor, it is necessary to cleave the *IL-1β* precursor by a cysteine protease. This cysteine protease is also known as Caspase-1. The activation of caspase-1 needs a formation known as inflammasome and cytoplasmic pattern recognition receptor signaling in regulating the process. Therefore, these two steps are necessary for the secretion of *IL-1β* and subsequent activation of different receptors. Under exceptional conditions like during the higher neutrophilic inflammation, *IL-1β* can also be processed by other proteases [219].

It is interesting that the two major forms of *lL-1* (i.e. *lL-1*α and *lL-1β*) that can have identical biological effects by binding to the same receptors. In addition, it may exert other important functions including stimulation of synthesis of acute-phase reactants by the liver, stimulation of chemokine production by endothelial cells and macrophages, induction of endothelial cell adhesion molecules, and fever [220,221]. Because of the fact that they have a natural antagonist *IL-1Ra,* they have a strong effect on the proinflammatory cytokine production, and these molecules are discovered early, I*L-1α* and *IL-1β* are the most studied members among the all other members. Actually, a variety of autoinflammatory syndromes could result from the higher production of *IL-1β*. It is particularly true for the Cryopyrin-Associated Periodic Syndromes (CAPS) which is a monogenic condition which is the consequence of mutations in the

inflammasome receptor *NLRP3* that may trigger the production of *lL-1β* [222]. It has been shown that osteomyelitis can be induced by intestinal dysbiosis following an *IL-1β* dependent manner [223].

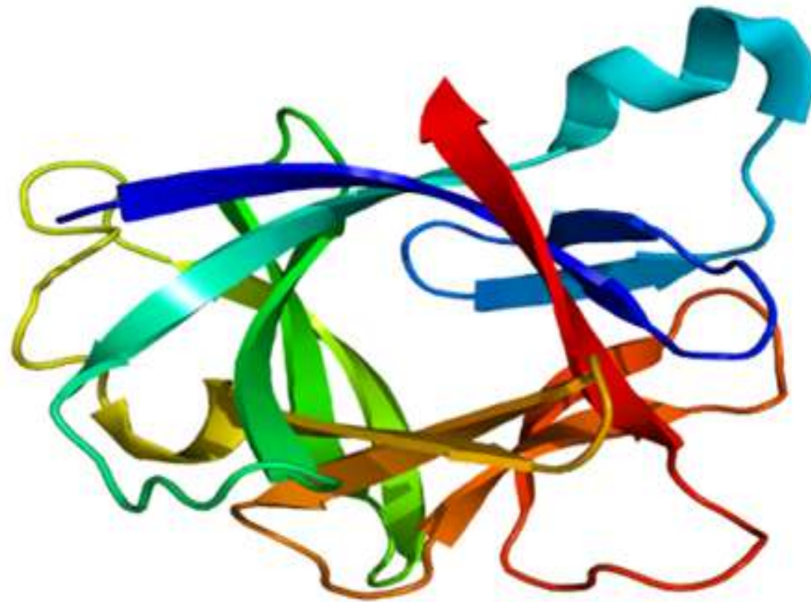

Figure 1.8. Crystal 2D structure of *IL-1β* Quoted from [224]

1.8.1.7.3. Interleukin 6 (*IL-6*)

Interleukin 6, commonly referred to as *IL-6*, is an important member of the interleukin family. It is interesting to note that *IL-6* may act as both that acts as both an anti-inflammatory myokine and a pro-inflammatory cytokine. In humans and other mammals, *IL-6* protein is encoded by the *IL6* gene [225]. To stimulate the immune response especially burns or other tissue damage leading to inflammation and against during pathogen-induced infection or after trauma, macrophages and T cells are secreting *IL-6*. It is noteworthy that *IL-6* has played an important role in fighting against infection. In addition, it has been shown that the mice lacking *IL-6* have exhibited lower resistance against bacterium Streptococcus pneumoniae [226].

It is interesting that in order to stimulate osteoclast formation, osteoblasts are secreting *IL-6*. However, the central question is how *IL-6* exerts its effect in the immune response against pathogen-induced infections? The answer is, mainly by performing the as an anti-inflammatory cytokine which is facilitated by the activation of *IL-1ra* and *IL-10*

and inhibition of *TNF-α* and *IL-1* expression of [227]. The important contribution of LI-6 is the induction of higher body temperature. Because IL-6 can cross the brain-blood barrier, in the hypothalamus, it starts the synthesis of PGE2, which subsequently alters the setpoint of the body's temperature. *IL-6* excites the metabolization of energy in fatty and muscle tissue which may result in [228]. In addition, in response to a variety of microbial molecules referred to as PAMPs, macrophages can secrete *IL-6*. These PAMPs bind to an important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) [229].

IL-6 exerts its effects via a cell-surface type I cytokine receptor complex. This cytokine receptor complex is composed of mainly two components i.e. the signal-transducing component gp130 (commonly known as CD130) and a ligand-binding *IL-6Rα* chain (commonly known as CD126). It is interesting to note that CD130 is not only a signal transducer for *IL-6*, rather it is a general signal transducer for a wide variety of cytokines such as cardiotrophin-1, *IL-11*, co-statin M, a ciliary neurotrophic factor, and leukemia inhibitory factor *(LIF)* [230]. To activating the receptor, *IL*-6 can trigger the formation of *IL-6R* and gp130 proteins complex during the interaction with its receptors. The intracellular regions of gp130 or CD130 can be brought together to start the cascade of signal transduction through Signal Transducers and Activators of Transcription (STATs), Janus kinases (*JAKs*), and certain transcription factors [231].

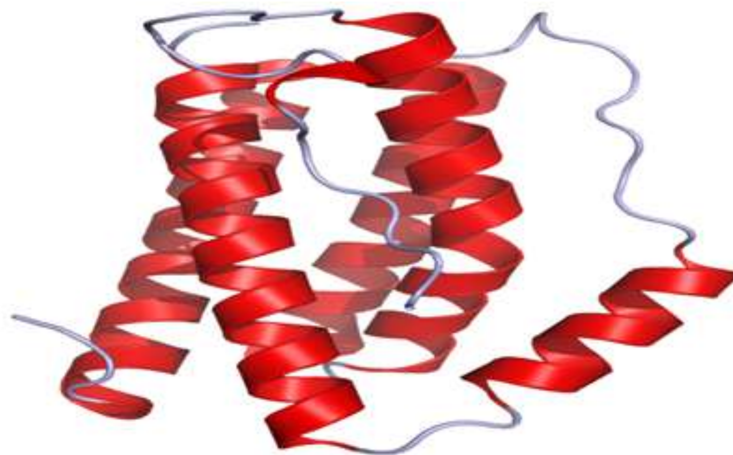

Figure 1.9. Crystal structure of *IL-6* Quoted from [232]

1.8.1.7.4. Interleukin 10

Interleukin 10 commonly known as *IL-10. IL-10* is one of the best-studied anti-inflammatory cytokines which is also described as human cytokine synthesis inhibitory factor (CSIF). In humans and other higher mammals, the gene *IL10* is responsible for the production of interleukin 10 [233]. The receptor complex of *IL-10* is composed of two receptors: two *IL-10* receptor-2 and two *IL-10* receptor-1 protein. STAT3 signaling is induced by *IL-10* binding through the phosphorylation of the cytoplasmic tails of *IL-10* receptor 1 + *IL-10* receptor 2 by *JAK1* and *Tyk2* respectively [234].

Being a homodimer, *IL-10* protein has many subunits. Interestingly, all of its subunits are approximately 178-amino-acid long [235]. *IL-10* is belongs to cytokine class-2 along with other cytokines including interferons type-III (*IFN*-lambda, also known as *IL-28A*, *IL-28B*, and *IL-29*), type-II (*IFN*-gamma), type-I (*IFN*-alpha, -beta, -epsilon, -kappa, -omega), a set of cytokines including *IL-19*, *IL-20*, *IL-22*, *IL-24* (Mda-7), and *IL-26* [236,237].

IL-10 protein is end-product of the translation of the *IL10* gene. It contains 5 exons and located on chromosome 1. Many cells can produce *IL-10*, however, the major producer is monocytes. The other producers are a certain subset of activated T cells and B cells, CD4+CD25+Foxp3+ regulatory T cells, mast cells, lymphocytes, namely type 2 T helper cells (TH2). When PD-1 triggers monocytes, these cells start to produce *IL-10* [100,233]. In a normal state, in the unstimulated tissue or cells the level of *IL-10* expression is minimal, however, upon immediately after stimulation of monocytes through pathogenic flora, higher expression of *IL-10* was observed [238].

In the process of inflammation and immunoregulation, *IL-10* exerts multiple pleiotropic effects. The expression of MHC class II antigens and Th1 cytokines is downregulated by *IL-10*. In addition, it also mediates effects on the macrophages by changing the abundance of co-stimulatory molecules. In addition, the antibody production, proliferation, and survival of B cell can be enhanced by the presence of *IL-10*. The activity of *NF-κB* can be blocked by it and the *JAK-STAT* signaling pathway is regulated by *IL-10* [239]. Almost three decades ago, In 1991, it has been demonstrated that the *IL-10* was involved in the suppression of CD4+ T cell activation, antigen presentation, and

cytokine secretion [240–242]. Additional experimentation and investigation have demonstrated that *IL-10* exclusively block the bacterial product and lipopolysaccharide (LPS) induced secretion of cytokines which is pro-inflammatory in nature including *IFNγ, IL-12*, *IL-1β*, and *TNFα* TLR triggered myeloid lineage cells [243,244].

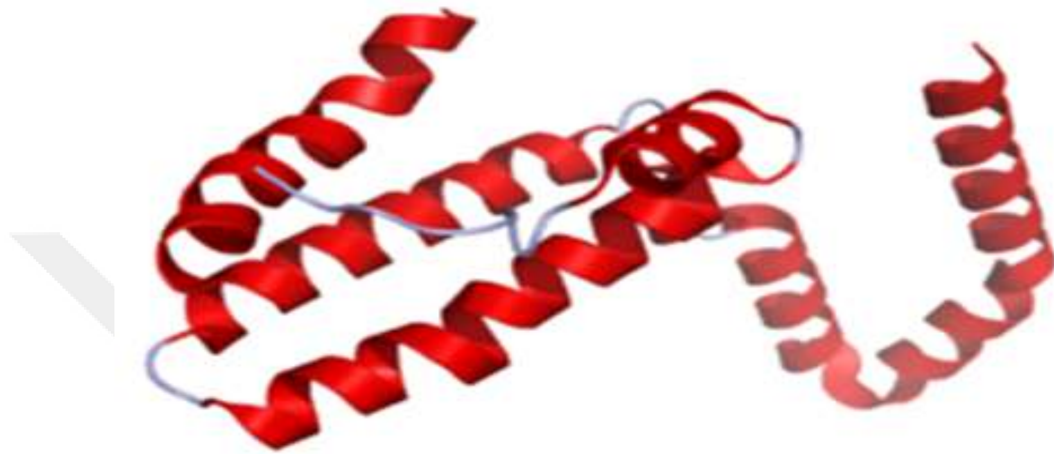

Figure 1.10. Crystal structure *IL-10* Quoted from [245]

1.8.1.7.5. Interleukin 8 (*IL-8*)

Interleukin 8 also known as chemokine (*C-X-C motif*) ligand 8 (*CXCL8*) or *IL8*. It is chemokine in nature. *IL-8* can be produced by any cells including endothelial cells, airway smooth muscle cells, epithelial cells, and macrophages [246]. *IL-8* protein is encoded by the *IL-8* gene which is located in chromosome 4q. The storage facilities in endothelial cells, the Weibel-Palade bodies, can store *IL-8* [247,248]. The interleukin-8 protein in human is encoded by the CXCL8 gene [249]. In culture, a 72 amino acid peptide is the major form secreted by macrophages [250].

In the cell surface membrane, there are several kinds of receptors present which can bind *IL-8*. However, CXCR2 and CXCR1 are the G protein-coupled serpentine receptors that have been studied extensively. It is important to note that the affinity and the expression of two receptors are different from one another (CXCR1 > CXCR2). The innate immune response and the immune reactions are mediated through *IL-8* by a chain of biochemical reactions [251].

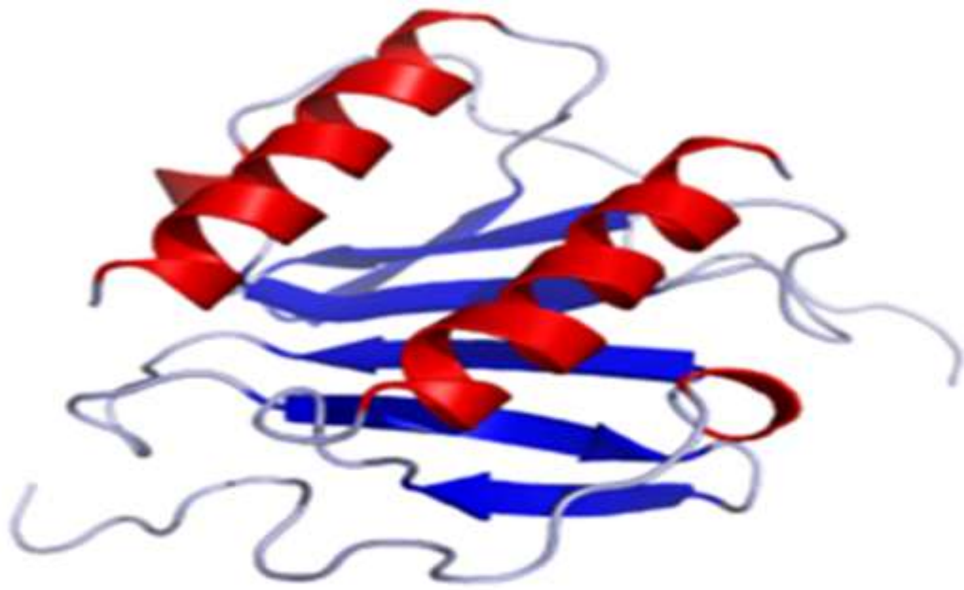

Figure 1.11. Crystal structure of *IL-8* Quoted from [252]

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

In this study, we treated the live animals with LPS and/or LTA in order to understand the molecular mechanism of innate immune response mediated by macrophages in sheep lung. We collected BAL fluid from slaughtered sheep in order to isolate the macrophages from the lungs. There are two stages of this study. In the first stage, we investigated the effects of LPS and/or LTA in stimulating the immune response in the sheep lung *in vivo*. In the second stage, we isolated the alveolar macrophages from sheep lung obtained from a slaughterhouse. To perform the animal and laboratory experiments, we have used several chemicals and kits according to the manufacturer's instruction. Kits, tools, chemicals, and animals were purchased from different resources and different manufacturers. We have used sterilized tips and tubes for preparation of samples and qPCR work. Moreover, buffers were prepared with deionized and demineralized water (ddH2O) as required and were autoclaved at 120°C for 20 minutes.

2.1.1. List of Animal's Breeds

Numbers of animals	Animals Breeds	Manufacturer	Aim of use
12	Akkaraman lambs/ Turkish lambs	The animals were purchased by Erciyes University through its accredited clients	Animals were used for *in vivo* study as host for LPS and LTA treatments
12	Romanov lambs/ Russian lambs	The animals were purchased by Erciyes University through its accredited clients	Animals were used for *in vivo* study as host for LPS and LTA treatments

2.1.2. List of Chemicals and the Biological Reagents

Chemicals and reagents	Manufacturer	Aim of use
Ethanol 70%	Emsure / Germany	Using for sterilizing and preventing contamination
RPM1640- Medium with L glutamine and sodium bicarbonate, R8758	Sigma Aldrich, United Kingdom	Used culture alveolar macrophages (AMs)
Fetal bovine serum, P30-8500	Sigma-Aldrich, Steinheim, Germany	Supplement @10% in cell culture medium to culture AMs
Antibiotic, anti-mycotic, P4333	Gibco® / USA	1% of total media contain from (AA). To avoid bacterial and fungal infection during culturing AMs cells
Trypsin + EDTA (0.25%), phenol red	Sigma-Aldrich /USA	Detach the cells from culture plate or flask
Trypan blue solution	Sigma- T8154- UK	Cell counting for cell culture
High Pure RNA Isolation Kit	REF. 11828665001, Canada	Extracting the total RNA
abm Easy Script Plus™, cDNA synthesis kit	Cat # G236, Germany	To reverse transcribe the total RNA into cDNA
Lightcycler® 480 SYBR Green I Master, version 13	Roche Diagnostics, GmbH, Mannheim, Germany, REF. 04707516001	Real-time quantitative PCR for target genes
Quantitative Real-Time PCR primers (forward and reverse)	Sentegen Biotechnology, 1B7-B8-1B9, Turkey	Quantify the target genes in response to different treatment
Escherichia coli 0111:B4, 1MG/ML, L4391 (LPS)	Sigma, Germany	To induce the effect of Gram-negative bacterial infection
Staphylococcus aureus, L2515 (LTA)	Sigma, Germany	To induce the effect of Gram-positive bacterial infection
Sharp scissors, Forceps	Turkey	Sample collection (lungs)
Translucent fabric of silk (gauze)	Local medical store, Kayseri Turkey	To filter the lungs lavage
1.5-ml microcentrifuge tubes	Isolab/ Spain	For cell collection and other lab works
Beybi Powder-free nitrile examination gloves Size L	Medical office, Turkey	Everyday laboratory works
1 mL pipette tips- Box- sterile	Isolab/ Spain	Everyday laboratory work (genetic, molecular and cell culture)
200 µL pipette tips- Box- sterile	Isolab/ Spain	Everyday laboratory work (genetic, molecular and cell culture)
100µL pipette tips- Box- sterile	Isolab/ Spain	Everyday laboratory work (genetic, molecular and cell culture)
10uL- long filter tips- Box-sterile	Isolab/ Spain	Everyday laboratory work (genetic, molecular and cell culture)
Corning® 50 mL Sterile centrifuge tubes	Isolab/ Spain	Sample collection, cell culture, and preparation of chemicals
6 well cell culture plate Corning 3516	Thermofisher, USA	To culture the cells (AMs)
96-well PCR microplate, Lightcycler type, white, 10 plates/box	Lot No: 14124, USA	Real-time quantitative PCR
Freezer BOX (paper) - for micro tubes 1,7 or 2 mL	Medical office, Turkey	Keep the tubes containing samples in the refrigerator or freezer

2.1.3. Preparation Buffers

Name of buffers	Preparation
Phosphate buffer saline 1X (PBS)	Prepared by adding 8.00g of NaCl, 0.2g of KCl, 1.44g Na2HPO4, 0.24g KH2PO4and were mixed to gather and then added and completed to 1000 ml by (dH_2O) distilled water, then was shaken gently and was sterilized by autoclaving in 120°C for 20mins and then UV for 30mins
RBCs lysis buffer	Prepared by adding 8.26g of NH4Cl, 1.19g of NaHCO3, 200ml of EDTA (0.5M, pH8), distilled water was added up to 100ml and pH has been adjusted in 7.3, the buffer was sterilized by autoclave to store up to 6 Months

2.1.4. List of Equipment used in Different Phases of this Experiment

Name of equipment	Manufacturer
CO2-incubator (MCO-19AIC)(UV)	Sanyo, Japan
Biological safety cabinet	Telstar
Centrifugation, D-78532 Tuttlingen	Hettich, NO: 0001466-04-00, Germany
Centrifugation, D-37520 Osterode am Harz	Sigma, NO: 150825, Germany
Microcentrifugation	Beckman Coulter/ Germany
Refrigerator centrifugation	Sigma 1-15pk, Germany
Thermocycler	Senso quest labcycler /Germany
Microscope, D-35578 Wetzlar	Leica: DMi1, NO: 419735, China
Water bath	Memmert GmbH / Germany
Hemocytometer	Fisher scientific / USA
lab shaker (vortex)	Thermofisher / USA
pH meter	Mettler Toledo/USA
Laboratory Oven	Panasonic biomedical / Japan
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific, DE, USA
Ultra-low freezer (-80oC)	Labotect GmbH, Göttingen, Germany
Biomedical freezer- MDF-U5312	Sanyo, NO: 11120695, Japan
Refrigerator (+4) °C	Siemens 360, EU
Roche Light cycler 480 real-time PCR Machine	Roche Life Science, Penzberg, Germany

2.2. Methods

The animals were reared in the Erciyes University Animal Rearing Unit. The *in vivo* experiments were carried out in strict accordance with the protocol approved by the Animal Welfare Committee of Erciyes University (Permit No. 17/009 on 15/02/2017). All the laboratory experiments were performed at the Genome and Stem Cell Center (GENKöK) in Erciyes University/ Kayseri province http://genkok.erciyes.edu.tr/2017/05.

2.2.1. The Design of *In Vitro* Experiment

In this study, several *in vitro* experiments have been designed to investigate the level of gene expression changes in alveolar macrophages cells exposed to LPS 10 ug/ml, LTA 10 ug/ml and with the combination of LPS 10 ug/ml + LTA 10 ug/ml treatments in different time points 4 and 24 hours.

2.2.1.1. Four Hours Experimental Groups (LPS 10 ug/ml), (LTA 10 ug/ml) and with the Combination of LPS 10 ug/ml + LTA 10 ug/ml

After the collection and purification, the alveolar macrophages, cells were stimulated with LPS 10 ug/ml, LTA 10 ug/ml and with the combination of LPS 10 ug/ml + LTA 10 ug/ml and left for four hours. After the culture period, cells were washed three times and cells were disassociated from the culture plate using 0.25% Trypsin EDTA and incubate for 5 minutes at 37°C. Following incubation, the culture plate was placed on ice for 15-20 min in order to complete disassociation from the culture plates. Once the cells were collected properly, other procedures such as isolation of total RNA, synthesis of cDNAs, and gene expression analysis through the real-time PCR technique were performed to check the mRNA expression levels for candidate genes in this study see Figure 2. 1.

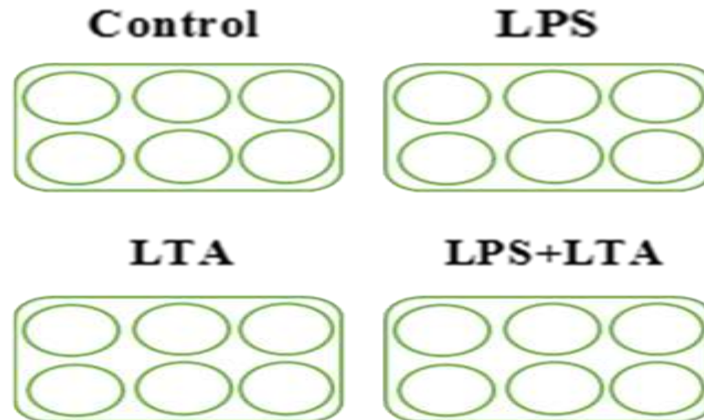

Figure 2.1. The experimental design of *in vitro* study 4 h time point

2.2.1.2. Experimental Groups in Twenty-Four Hours: (LPS 10 ug/ml), (LTA 10 ug/ml) and with the Combination of (LPS 10 ug/ml + LTA 10 ug/ml)

After collected and purified, the alveolar macrophages cells were stimulated with LPS 10 ug/ml, LTA 10 ug/ml and with the combination of LPS 10 ug/ml + LTA 10 ug/ml and alveolar macrophages were cultured for another 24 hours and followed the same procedure as we mentioned above to collect the cells. After the collection of cells, other steps have been done in order to purify the total RNA, cDNA synthesis and mRNA expression through the real-time PCR Technique to check the mRNA expression levels for the recommended genes in this study see Figure 2. 2.

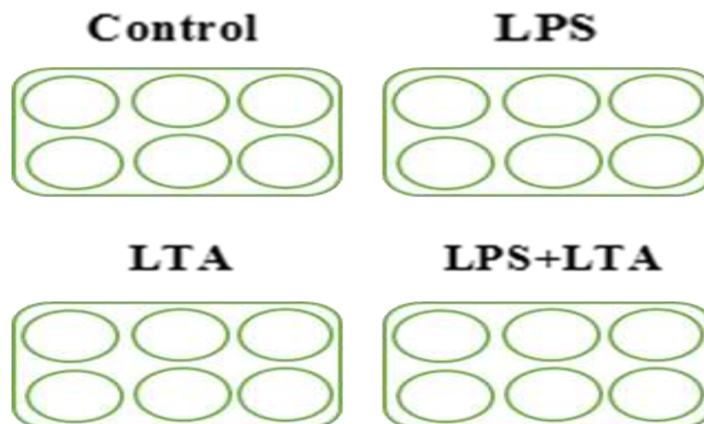

Figure 2.2. The experimental design of *in vitro* study 24h time point

2.2.1.3. Laboratory Works

2.2.1.3.1. Collection, Purification, and Culture of Alveolar Macrophages

The lungs from the slaughtered sheep were collected from a licensed local slaughterhouse in Kayseri province after the permission given by the slaughterhouse authority. Lungs opening was blocked using plastic rope and were immediately placed on ice box after the collection. Lungs were transported to the laboratory within one hour of collection and in the bronchoalveolar lavages (BAL) were obtained by adding 250 ml of cold and sterilized PBS (1X) into the lung for first wash and added other 250 ml of PBS (1X) for the second wash. The lung was palpate and shake gently for 2 to 3 mins. The BAL fluids were collected in a sterilized 50 ml falcon tube.

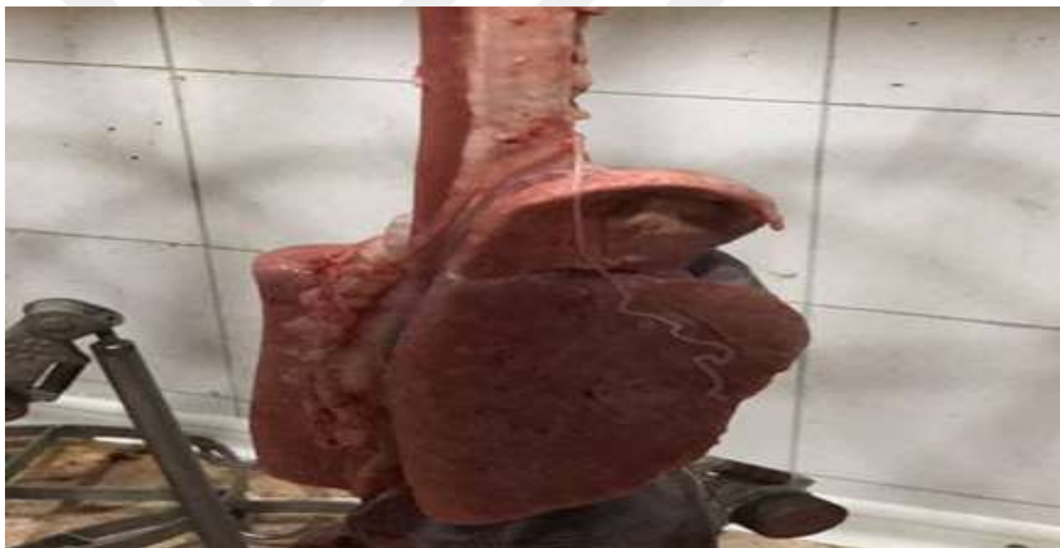

Figure 2.3. Lamb lung collected from license slaughterhouse

The tubes were stand for 5 min untouched in order to allow the fat cells appeared on the top of the tube. A plastic poster pipette was used to remove fat cells. In addition, the BAL fluids were filtered through sterile cotton (Gauze) to ensure the complete removal of visible fat cells, or any impurities left with the lavage (Figure 2.4). The tubes containing BAL fluids were then centrifuged at 400xg for 10 mins; and the supernatants were discarded, then the cells were transferred from the 50 ml falcon tubes into new 15 ml tubes. Then, the collected cells were adjusted with sterilized PBS (1X) to fill the 15 ml tubes and mixed gently.

Figure 2.4. Remove the fat cells and any impurities left with the BAL fluid

At that point, the cell mixture was centrifuged in 700xg for 5 min. After the centrifugation, the supernatant was discarded carefully. 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 in order to complete removal of erythrocyte contamination. Isotonicity was restored by adding PBS buffer (1X) to fill the 15 ml tubes. The cells with PBS were then centrifuged at 700 x g for 5 min at room temperature. Following the centrifugation, the supernatant was discarded carefully by not disturbing the pellet. At this time point, we resuspend the cells in 15 ml tubes by adding culture media (RPMI-1640- Medium with L-glutamine and sodium bicarbonate, R8758) supplemented with 10% fetal bovine serum (FBS) and 100 unit of penicillin-streptomycin and fungizone (combined together) and mixed gently. After that, cell counting was performed using trypan blue dye exclusion test. First, 100 µl of cell mixture was added to 100 µl of trypan blue dye. After that 300 µl of sterile and filtered PBS buffer (1X)) was added to the mixture and incubated at room temperature for 2 min. Approximately, 10 µl of trypan blue-treated cell suspension was taken and applied to the hemocytometer and both chambers underneath the coverslip. The solution was spread underneath the coverslip immediately due to the capillary force and waited for another 2 min to settle down the cells. An inverted lab microscope with a 40X objective was used to count the cells in each chamber and the average cells were recorded to identify the number of isolated cells. The blue stained cells are considered as dead cells whereas the live cells are unstained. A total of 1.6×10^6 live cells/mL were dispensed onto each well of the 6-wells cells culture dish (Corning 3516). At this point, plates

were incubated in an incubator at 37.0 °C and 5% CO_2 in a humidified environment for 4 hours to allow the alveolar macrophages to adhere to the bottom of the wells.

Figure 2.5. Incubation the BAL fluid for 4 h to adhere the AMs to the bottom of the wells

Then, after incubation, the culture plates were taken from the incubator into a laminar flow bench (Telstar) and immediately the old culture media with the flooding cells were discarded. After that, the adhered cells (AMs) were washed two times with sterilized and pre-warm PBS buffer (1X). Following the washing steps, 2 ml of freshly prepared warm culture media supplemented with 10% fetal bovine serum (FBS) and 100 units of penicillin-streptomycin and fungizone (combined together) was added to each well of the culture plate and AMs were checked under an inverted microscope (Leica Microscope, SN. 419735, China) using 40X objective and the percentage of viable AMs cells were >98%. After that, cells were directly exposed to LPS 10ug/ml (*Escherichia coli* 0111: B4, 1MG/ML, L4391, SIGMA, Germany), LTA (10ug/ml) (*Staphylococcus aureus*, L2515, SIGMA, Germany) and with the combination of LPS 10ug/ml + LTA 10ug/ml and incubated for 4 h and 24 h in the incubator at 37°C in a humidified environment as it was mentioned previously [28,253,254].

2.2.1.3.2. Harvest and Collect the Alveolar Macrophages (AMs) Cells

The AMs cells were harvested and collected after the incubation period of 4 h and 24 as described below:-

The old culture media was discarded from the plates, and the plates were washed two times with pre-warm, sterilized, and filtered PBS buffer (1X). After that, 750 µl of 0.25% Trypsin-EDTA were added to each well and plates were 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 mins and the plates were periodically shaken and checked under a microscope to ensure that cells (AMs) were detached from the plates. Once all the AMs are disassociated from the culture plate, 1.250 ml of warm culture media (Dulbecco's Modified Eagle's Medium-high glucose, Sigma Aldrich, D6429, UK) was added to each well to stop the functions of the Trypsin-EDTA. Then, the AMs cells were collected carefully from each well to 15 ml falcon tubes and centrifuged at 700xg for 5 mins at room temperature. Following the centrifugation, the supernatant was discarded and 1 ml of warm PBS (1X) was added to each falcon tubes and cells were mixed gently then transferred into a 2 ml microcentrifuge tubes. Finally, the mixed cells (AMs) were centrifuged at 700xg for 5 mins, then the PBS (1X) was removed carefully, and cells were stored immediately at (-80 °C) refrigerator for later use [25,27].

2.2.1.3.3. Total RNA Extracting from Treated Alveolar Macrophages (AMs) Cells

Total RNA was extracted from AMs cells (n = 3) by using high pure RNA isolation kit (REF 11828665001, Version12, Germany) according to the manufacturer's instructions with some modification adopted for cell samples. Briefly, the frozen AMs cells were taken out from the -80°C refrigerator and immediately resuspended in 200 µl sterilized PBS (1X) and mixed gently by pipetting for 15 sec. A 400 µl lysis-binding buffer was added to the mixture and vortexed for 30 sec. The entire sample was poured into the upper reservoir of the filter tube (max. solution input limit is 700 µl). All the filters tubes were placed into a standard tabletop centrifuge and were centrifuged at 8000 x g for 30 sec at room temperature. After that, the filter tubes were removed from the collection tubes, the flowthrough liquid were discarded, and the filters were again combined to the collection tubes. In sterilized reaction tubes, mixture was prepared for each reaction contain 90 µl DNase buffer + 10 µl DNase I, the solution was mixed by pipetting then it was pipetted on the glass filter fleece in the upper reservoir of the filter tubes and incubated for 15 min at room temperature to ensure the complete removal of DNA from the RNA sample. After that, 500 µl of wash buffer I was added to the upper

reservoir of the filter tube assembly and centrifuged at 8000 x g for 30 sec, then the flow through was discarded. The filter column was placed again to the used collection tube. After that, 500 µl of wash buffer II was added to the upper reservoir of the filter columns assembly and centrifuged at 8000 x g for 30 sec. The flow-through was discarded and the filter tube was combined with the used collection tube. Then, another 200 µl of wash buffer II was added to the upper reservoir of the filter tubes assembly and centrifuged for 2 min at maximum speed (approx. 13,000 x g) to remove any residual wash buffer II. Then, the collection tubes were discarded, and the filter tubes were inserted into a clean, sterile 1.5 ml microcentrifuge tubes. At that point, a 60 ul of elution buffer was added to the upper reservoir of the filter tubes and centrifuged the tubes assembly for 1 min at 8000 x g. Finally, the microcentrifuge tubes contain the total RNA and the concentrations, and the purity were checked for all samples by a BioSpec-nano Spectrophotometer (Shimadzu Biotech, Japan), then the isolated total RNA samples were stored at (-80)°C for later use.

2.2.1.3.4. cDNA Synthesis

For mRNA expression analysis the total RNA was reversed transcribed to cDNA using a commercially available cDNA synthesis kit (abm EasyScript Plus^{TM}, Cat # G236, Canada) according to the manufacturer's protocol. Previously isolated total RNA was thawed on ice. Because the concentration of total RNA was different in different samples, various amount of total RNA was taken from each sample and diluted with nuclease-free water in order to have equal input for all the samples. The total RNA input was 2 µg and the maximum amount of the solution must be 11.5 µl or less. Then, all the cDNA synthesis reagents have been mixed gently and kept on ice. In strip tubes 1 µl of Oligo [dT] (10 µM), 1 µl of random primer (10 µM) and 1 µl of dNTP Mix (10 mM) were mixed together with prepared tRNA from each sample. In this point, the total amount of the mixture must be 14.5 ul. After that, the mixture was mixed gently and incubated in the thermal-cyclers machine with 65°C for 5 mins and mixture was incubated on ice for 1 min. During the incubation period the second solution consisting of 4 µl of 5X RT buffer, 0.5 µl of RNase-OFF (40 U/µl) and 1 µl of RTase (200 U/µl) was prepared and kept on ice. After the initial incubation, samples were immediately placed on ice for 1 min. After that the second solution was added to each sample and mixed gently. The final volume of the mixture was 20 µl and the contents were

incubated in the thermal-cyclers machine for 10 min at 25˚C followed 15 mins at 50˚C. The reaction was terminated by heating for 5 mins in 85˚C. The concentration and purity of the complementary DNA have been determined using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan). Finally, the resulting cDNA was diluted with RNase free H_2O (two times floods: approximately 40µl) to have suitable concentration of cDNA and stored at -20°C for long-term use.

2.2.1.3.5. Real-Time Quantitative PCR(qPCR)

The real-time PCR reaction was performed in a Roche Light cycler 480 real-time PCR machine (Roche Life Science, Penzberg, Germany). The primer sequences of the genes that were used in this experiment was designed through Primer 3 online Software v.0.4.0 (http://frodo.wi.mit.edu/primer 3/) and the sequence of the primes are listed in Table 2.1. The genes we investigated in this experiment are *GAPDH* and *ACTB* as reference genes and *IL-1β*, *IL-6*, *IL-8*, *IL-10*, *TNFα*, *NF-κB*, *TLR2* and *TLR4* as target genes. Before running quantitative RT-PCR reaction, the suitability of endogenous control genes was confirmed by investigating the expression of GAPDH and ACTB expression among the experimental samples (data not shown). First of all, the cDNA and all other reagents related to quantitative real-time PCR were thawed on ice and then the 96 wells PCR microplate (Lightcycler- type, White, Lot No: 14124, USA) was put on the icebox and 2 µl of cDNA from each sample was distributed in each well of the 96 wells PCR microplate according to the experimental need. The last row of the PCR microplate was left for negative controls (2 µl of water instead of cDNA). After that, the PCR master mix reaction for each sample was prepared separately containing 10 µl of SYBR Green I Master (REF. 04707516001, version 13, Germany), 0.4 µl of forwarding primer, 0.4 µl of reverse primer and 7.2 µl of RNase free water for each reaction. 18 µl of master mix was added to each well giving a total reaction volume of 20 µl. Then, the plate was covered by the plastic cover (supplied by the company) with the help of a plastic sealing apparatus supplied with the kit. The sealed plate was protected from light by covering the plate with aluminum foil. After that, the 96 wells PCR microplate was vortexed gently for 10 sec and centrifuged for 25 sec at room temperature. The temperature and time settings for the real-time quantitative PCR steps are as follows: Preincubation: 2 min at 50˚C and for 10 min in 95˚C, 1 cycle; Amplification: 45 cycles in 95˚C and 60˚C withholding 15 and 60 seconds, respectively; Melt curve temperature

was programmed in 60˚C withholding for 15 seconds followed by 95˚C in continuous mode and 20 acquisitions (per ˚C). Relative expressions of each mRNA were analyzed using a comparative CT ($2^{\Delta\Delta ct}$) method.

Table. 2.1. List of primers that were used for qRT- PCR experiment

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

* bp: base pair.

2.2.2. *In vivo* Administration of LPS, LTA, and LPS+LTA

The animal trial experiments were performed at the Agricultural Farm Research Site of Erciyes University, Kayseri province. Twenty-four healthy live animals from two different breeds which are 12 Akkaraman lambs as a native breed and 12 Romanov lambs as an imported Russian breed were used in the study. All animals were first divided into two groups where each group consisting of four subgroups. Lambs were euthanized after 24 h of the treatments and lungs were collected and transferred immediately to the laboratory on ice within one h of collection. The whole procedure was carried out in strict accordance with the protocol approved by the Animal Welfare Committee of Erciyes University (Permit No: 17/009 on 15/02/2017) Figure 2. 7.

Figure 2.6. Akkaraman and Romanov lambs are in the Farm of Agriculture Faculty of Erciyes University

2.2.2.1. *In Vivo* Experimental Design

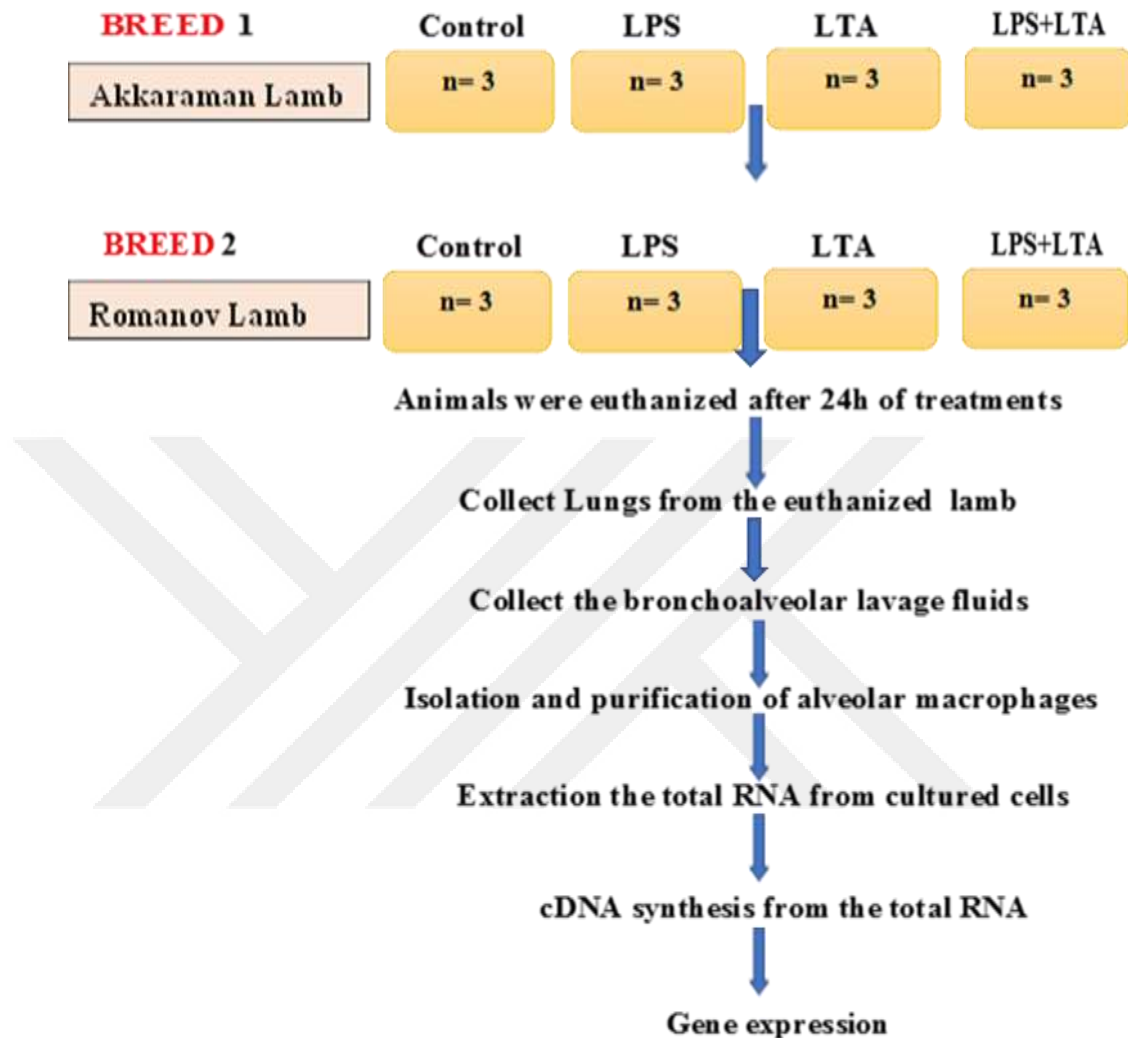

Figure 2.7. The experimental design for the *in vivo* study- 24 h the time point

2.2.2.2. Reagents and Preparation

In this trial *Lipopolysaccharide* (LPS) (*Escherichia coli* 055: B5) (L2880–100MG) and lipoteichoic acid (LTA) (*Staphylococcus aureus*) (L2515-25MG) were obtained from Sigma Analytical, USA and used to treat both lambs' breeds at the same time for 24 h.

2.2.2.2.1. *Escherichia Coli* 055: B5 (L2880–100MG) Preparation

100 mg of *Escherichia coli* (LPS) sigma was used in this experiment. 5 ml of sterilized Phosphate-Buffer Saline (PBS) was added to the 100 mg of LPS and shake carefully to obtain a concentrated solution. Then, 1 ml of the concentrated solution was diluted with

3 ml of PBS (1X) to have 5 mg (5000 µg) of LPS per 1ml of PBS (1X) (5 mg/ml). Finally, 20 µg of LPS from the diluted solution was administrated for each kg of live weight of the animal [255]. For example: For 24 kg of body weight (BW), we calculated the doses as follow:- 24 * 20 = 480 µg of LPS, we have 5000 µg per 1 ml therefore, 480 ÷ 5000 = 0.096 = 96 µl will be used for 24 kg BW.

2.2.2.2.2. *Staphylococcus aureus* (L2515-25MG) Preparation

25 mg of *Staphylococcus aureus* (LTA) sigma have been used in this experiment. 5 ml of sterilized Phosphate-Buffer Saline (PBS 1X) were added to the 25 mg of LTA and shake carefully to obtain a concentrated solution. Then, 1 ml of the concentrated solution have been diluted with 3 ml of PBS (1X) to have 5 mg (5000 µg) of LTA per 1 ml of PBS (1X) (5 mg/ml). Finally, 50 µg from the diluted solution was used per kg of animal [255]. For example. For 24 kg of body weight (BW); we calculated the dose as follow:- 24 * 50 = 1200 µg of LTA; we have 5000 µg per 1 ml, therefore, 1200 ÷ 5000 = 0.24 = 240 µl was used for 24 kg of BW.

2.2.2.3. Preparation of Animals

From the beginning of the experiment, 24 lambs were divided into Akkaraman group and Romanov group and each of those groups were divided into four sub-group (Control, LPS, LTA and the combination of LPS + LTA) groups and each group consists of three lambs according to the experiment design.

2.2.2.3.1. Weighing Animals

All the experimental lambs were phenotypically healthy and were three to four months old and their weight was ranged between 15 - 50 kg body weight (BW). In fact, the BW of the lambs was not considered as an effector in the in vivo study this is because doses of LPS or LTA were given to animals according to their individual body weight. However, animals were distributed evenly in the four groups according to their body weight to fix individual weight differences between animals. The weather conditions and dietary supplements were equal for all animals in this experiment.

Figure 2.8. Weighing lambs in agriculture station of Erciyes University

2.2.2.3.2. Administration of LPS, LTA and with the Combination of LPS + LTA to the Experimental Sheep

LPS (20 µg/kg) and LTA (50 µg/kg) solutions were prepared and administered through an intranasal aerosol to the animals (*in vivo* study). The first group consisting 6 lambs (n= 3 Akkaraman, n= 3 Romanov) were administered 20 µg of LPS per kg, the second group also consist of 6 lambs (n= 3 Akkaraman, n= 3 Romanov) were given 50 µg of LTA per kg, the third group consisting of 6 lambs (n= 3 Akkaraman, n= 3 Romanov) have been given the combination of 20 µg LPS + 50 µg of LTA per kg/ together, and the fourth group was the control group and consist of 6 lambs (n= 3 Akkaraman, n= 3 Romanov) and have been given sterilized PBS (1X) 500 µl per animal in the form of intranasal aerosol as sterile water for the amount of the substance concerned [255] as it can be shown in Table 2. 2.

Table 2.2. Akkaraman and Romanov groups according to LPS, LTA and with the combination of LPS + LTA treatments

Animals breeds	Control	LTA	LTA	LPS+LTA
Akkaraman	n = 3	n = 3	n = 3	n = 3
Romanov	n = 3	n = 3	n = 3	n = 3
Total number	24 animals			

The LPS or LTA was administered to all the 24 lambs through their intranasal aerosol by using adjustable pipette see Figure 2. 9.

Figure 2.9. Lambs were exposed by LPS, LTA and with the combination of LPS + LTA through an intranasal aerosol

After 24 h of intranasal administration of LPS, LTA or a combination of LPS and LTA, the rectal temperature and heartbeats were measured and recorded (Figure 2. 10). The rectal temperature and heartbeats were also measured and recorded before the drug administration. The readings are listed in Table 2. 3. All lambs from these two breeds were euthanized after 24 h of the treatments.in the license slaughterhouses in Kayseri province.

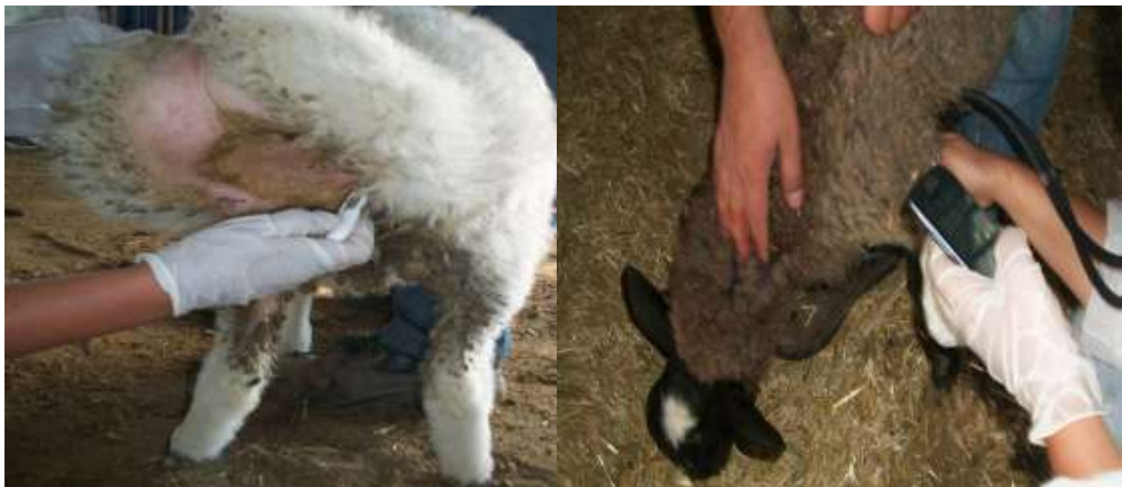

Figure 2.10. Rectal temperature and heartbeats were taken before and after 24 hours of the treatments

In the slaughterhouse, the animals were sacrificed according to the slaughterhouse recommendation. After the initial procedures, the lungs of the animals were collected

and held in an ice box carefully. After the collection from all animals, lungs were immediately transferred to the laboratory (Genome and Stem Cell Centre of Erciyes University). All the laboratory steps were done exactly the same as it was done for the *in vitro* study. The alveolar macrophages were collected, purified, harvested and total RNA was isolated, cDNA was synthesized, and real-time quantitative PCR was performed same as the *in vitro* experiments.

Table 2.3. The measurement of the weight, rectal temperature and heartbeats for all lambs before treatment

NU	Breeds	Weight/kg	Tempe before treatment/ ^{o}C	Heartbeats before treatment/mins
1	Akkaraman	32.6	40.2	154
2	Akkaraman	37	37.7	100
3	Akkaraman	30.4	40.1	90
4	Akkaraman	51	40.3	128
5	Akkaraman	45.8	39.4	128
6	Akkaraman	46.5	39.8	106
7	Akkaraman	42.6	39.6	124
8	Akkaraman	42.3	39.9	140
9	Akkaraman	40.7	40.1	150
10	Akkaraman	51.5	39.9	104
11	Akkaraman	55.4	40.3	114
12	Akkaraman	61.6	39.5	136
13	Romanov	15.1	39.8	110
14	Romanov	15.5	40.2	118
15	Romanov	25.4	39.5	100
16	Romanov	24.5	39.2	74
17	Romanov	20.3	40.1	98
18	Romanov	16.7	39.4	96
19	Romanov	32.2	39.2	106
20	Romanov	19.9	40	94
21	Romanov	20.4	39	108
22	Romanov	32.5	39.3	120
23	Romanov	20.3	39.3	120
24	Romanov	17.9	39.7	110

2.3. Statistical Analysis

A minimum of three biological replicates (n= 3) was used in all experiments in this study for (both *in vitro* and *in vivo* studies). For the statistical analysis and the graph preparation, Microsoft Excel (365, student version) 2016 was used. The microscopic pictures were captured randomly without any bias. To reduce the biases in the microscopic picture, a total of 5 fields were captured and analyzed. When two groups were compared (i.e., *IL-1β* /4 h vs *IL-1β* /24 h) the Student's t-test was used to detect the statistical differences between treatment groups. When the P value of ≤ 0.05, it is considered as significant. Data are expressed as mean ± SD of three biological replicates. As well as, gene-specific expression was measured relative to the geometric mean of the expression of a reference gene (GAPDH and ACTB). The delta Ct (ΔCt) values were calculated as the difference between the target gene and the reference gene:

$$\mathbf{\Delta Ct = \Delta Ct_{target} - \Delta Ct_{reference\ gene}}$$

The relative gene expression was calculated as $\mathbf{2^{(-\Delta Ct)}}$, and the fold change in expression between the treated and untreated AMs was calculated as $\mathbf{2^{(-\Delta Ct_{treated})} / 2^{(-\Delta Ct_{control})}}$ [256].

CHAPTER 3

RESULTS

3.1. Data Analysis According to the *In Vitro* Study

In this part of the study, we examined the AMs purifications and viability, mRNA expression levels of *TLR2* and *TLR4*, the mRNA expression levels of cytokines and chemokines compared with control and between the two different sheep breeds (Akkaraman and Romanov) when the AMs cells were exposed to LPS, LTA and with the combination of LPS + LTA in both in vitro and in vivo studies.

3.1.1. Collection and Purification of Alveolar Macrophages (AMs)

After the collection of BAL fluid, BAL cells were isolated and resuspended it in 6 well plates with the RPMI 1640 Medium and incubated in an incubator for 4 h. After the incubation period, all the macrophages cells adhered to the culture floor and non-adhered cells mostly polymorphonuclear cells (PMNs) and lymphocytes. In this way, it is possible to isolate macrophages with more than 92% purity. Cells were checked under the microscope (40X objective) after 4 h of culture period see Figure 3. 1

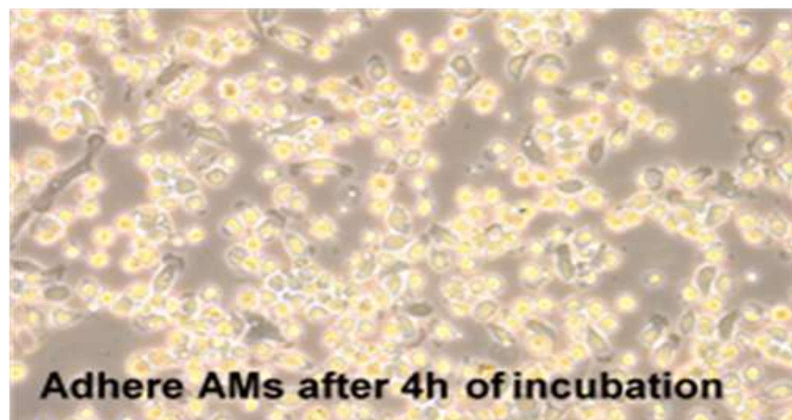

Figure 3.1. The adhered AMs cells after 4h of incubation (40X objective)

3.1.2. The Viability of Exposed Alveolar Macrophages Cells with LPS, LTA and with the Combination of LPS + LTA Treatments After 4 h and 24 h

After purification of the alveolar macrophage cells, they were exposed to LPS at 10 ug/ml, LTA at 10 ug/ml and with the combination of LPS at (10 ug/ml) + LTA at (10 ug/ml) for 4 h and 24 h according to the experimental design. To evaluate the phenotypic changes due to drug administration, we checked cells under an inverted microscope with a 40X objective. The representative pictures of 4 h and 24 h culture period are presented in Figures 3. 2 and Figure 3. 3 respectively. It is clear from the microscopic picture that at 4 h time point the number of dead cells is less as a lower number of floating cells are observed in each treatment. On the other hand, a higher number of dead cells appear in each group of cells after 24 h culture period. In addition, it seems there is no visible difference between the cells of control, LPS, and LTA treatment group after 4 h culture. However, a significantly lower number of cells were observed in LPS+LTA group compared to other groups.

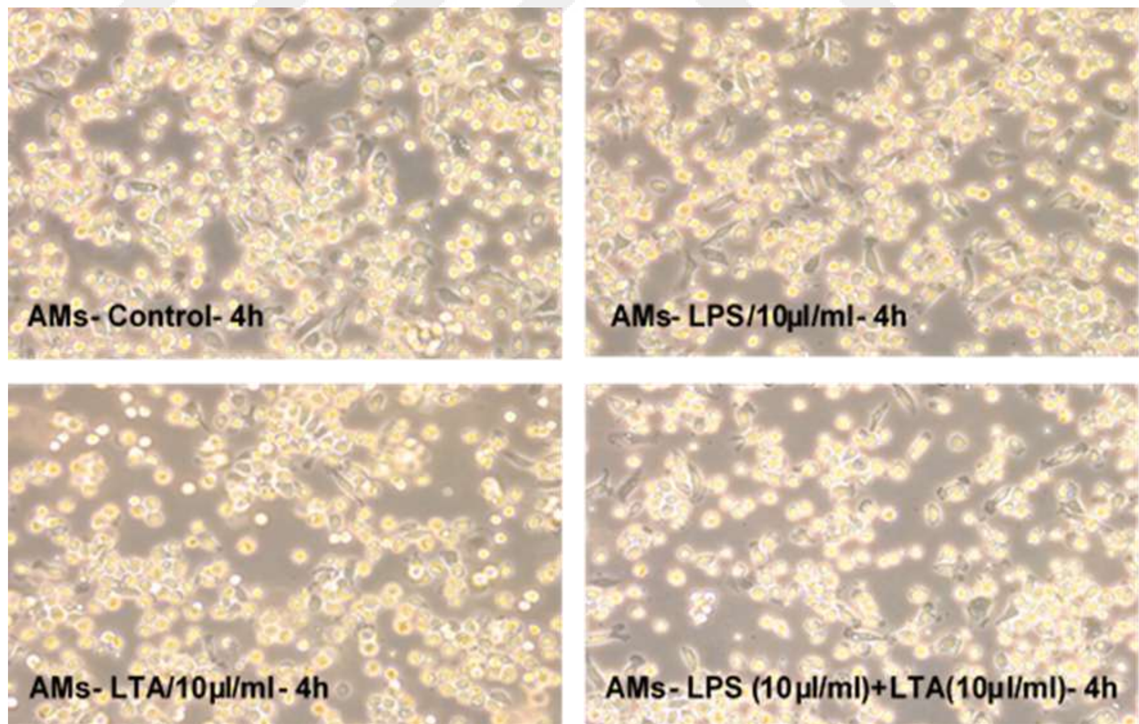

Figure 3.2. The viability of the AMs cells after 4 h of exposing to LPS and LTA and with the combination of LPS + LTA treatments (40X objective)

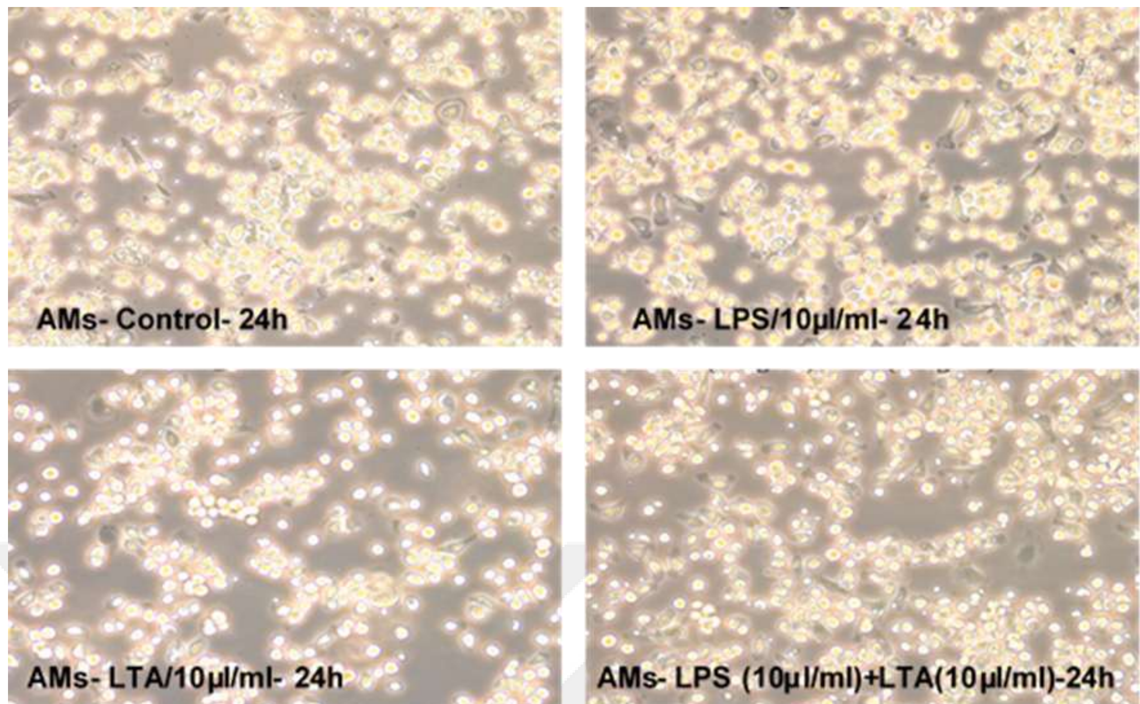

Figure 3.3. The viability of the AMs cells after 24 h of exposing to LPS and LTA and with the combination of LPS + LTA treatments (40X objective)

In fact, trypan blue exclusion demonstrated that there were no significant effects in the viability of AMs between treated samples or in the controls after 4 h and 24 h of incubation as it can be seen in Figure 3. 4. The mean percentages of viable cells for the 4 samples were 85.6, 86,84.8, 83.6, 83.2, 80.8 and for control, 89.6, 84.4 respectively.

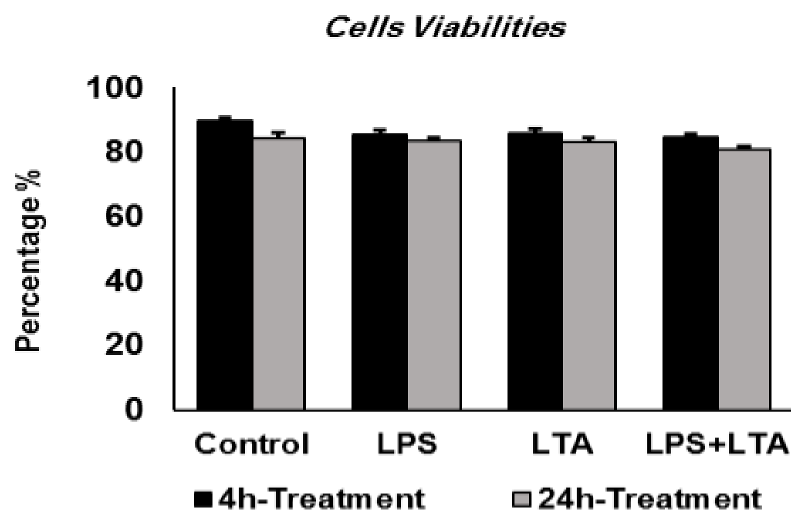

Figure 3.4. Effects of LPS, LTA and with the combination of LPS + LTA treatments stimulation on AMs cell viability. The results are expressed as the mean ±SEM (n= 3) percent cell viability

3.1.3. The mRNA Expression Levels of TLRs in Response to LPS, LTA and with the Combination of LPS + LTA Treatments After 4 h and 24h

In 4 h and 24 h in vitro study, the alveolar macrophages cells have been shown different mRNA expression levels of TLRs in response to LPS, LTA and with the combination of LPS + LTA treatments as described below.

3.1.3.1. mRNA Expression Levels of Toll-Like Receptor 2 (*TLR2*)

Exposure of alveolar macrophages cells (AMs) to LTA and with the combination of LPS + LTA treatments resulted in significant mRNA expression of *TLR2* compared with control in 4 h time point, whereas, treatment of AMs with LPS did not show a significant increase of mRNA expression of *TLR2* compared with control. On the other hand, in 24 h exposure of AMs to LPS and LTA did not present any significant increase in the expression of *TLR2* compared with control, while, with both LPS + LTA the treated AMs showed a significant increase of the expression of *TLR2* compared to control. Moreover, the treatment of AMs with LPS, LTA and with the combination of LPS + LTA in 4 h trial presented higher mRNA expression of *TLR2* than 24 h trial. As well as, there were no significant differences in the expression of *TLR2* between 4 h and 24 h trials in response to LPS, LTA and with the combination of LPS + LTA. as presented in Figure 3. 5.

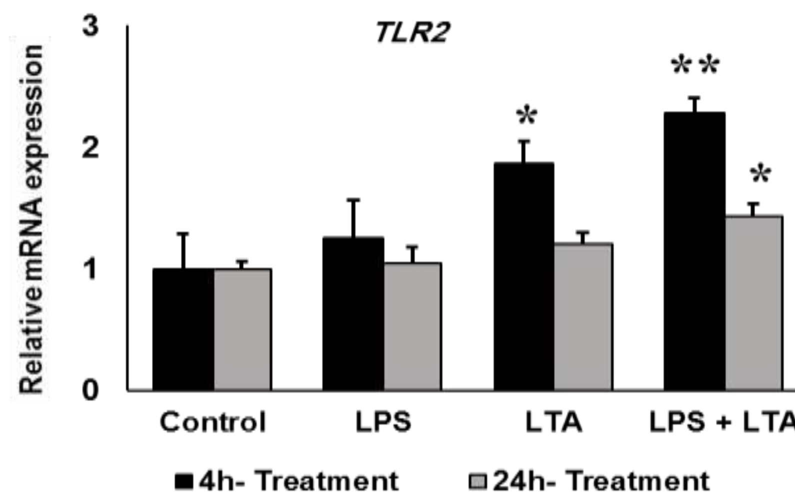

Figure 3.5. The mRNA expression levels of *TLR2* according to exposed AMs to LTA, LPS and with the combination of LPS + LTA treatments between different trials 4 h and 24h

Asterisks (*) indicate the significant mRNA expression levels differences between 4 h and 24 h trails compared with control in response to LPS, LTA and with both LPS + LTA treatments (*P<0.05)

3.1.3.2. The mRNA Expression Levels of Toll-Like Receptor 4 (*TLR4*)

Next, we checked the expression of *TLR4* in all groups of cultured cells. The expression analysis of real-time qPCR revealed that in 4 h exposure groups, the expression of TLR4 was significantly higher in the alveolar macrophages exposed to LPS and the combination of LPS + LTA compared to those of control group. However, there was no such expression was observed in the LTA treated alveolar macrophages. Furthermore, in 24 h exposure resulted in no significant changes in the expression of *TLR4* in the alveolar macrophages of LPS and LTA treated groups compared to control groups. However, the exposure of alveolar macrophages to a combination of LPS + LTA for 24 h showed a significant increase of mRNA expression of *TLR4* compared to control those of control groups. Furthermore, the treated AMs with LPS, LTA and with the combination of LPS + LTA in 4 h timepoint showed higher expression of mRNA of *TLR4* than 24 h time point. In addition, there were no significant differences in mRNA expression of *TLR4* between 4 h and 24 h trials in response to LPS, LTA and with the combination of LPS + LTA (Figure 3. 6).

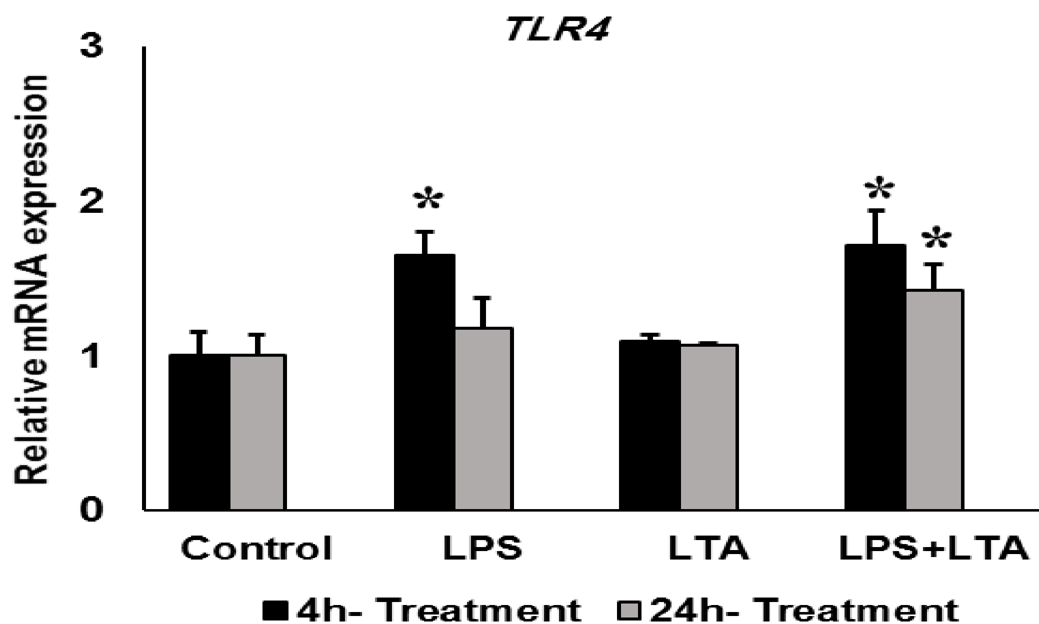

Figure 3.6. The mRNA expression levels of *TLR4* according to exposed AMs to LTA, LPS and with the combination of LPS+LTA treatments for 4 h and 24 h trials

3.1.4. The mRNA Expression of the Transcription Factor *NF-κB* in Response to LPS, LTA and with the Combination of LPS + LTA in 4 h and 24 h Trials

Exposure of alveolar macrophages to LPS, LTA and with the combination of LPS + LTA resulted in a significant increase in the expression of *NF-κB* compared with control in both 4 and 24h time points. It was observed that the stimulation of AMs cells with LPS, LTA and with the combination of LPS + LTA in 4 h timepoint showed comparatively higher expression of *NF-κB* than in 24 h time point. Furthermore, the treatment of AMs cells with LPS, LTA and with the combination of LPS + LTA treatments showed a significant increase of *NF-κB* mRNA expression between 4 h and 24 h trials. A well as 4 h trail showed higher expression levels in response to all treatments compared with the 24 h trail (Figure 3. 7).

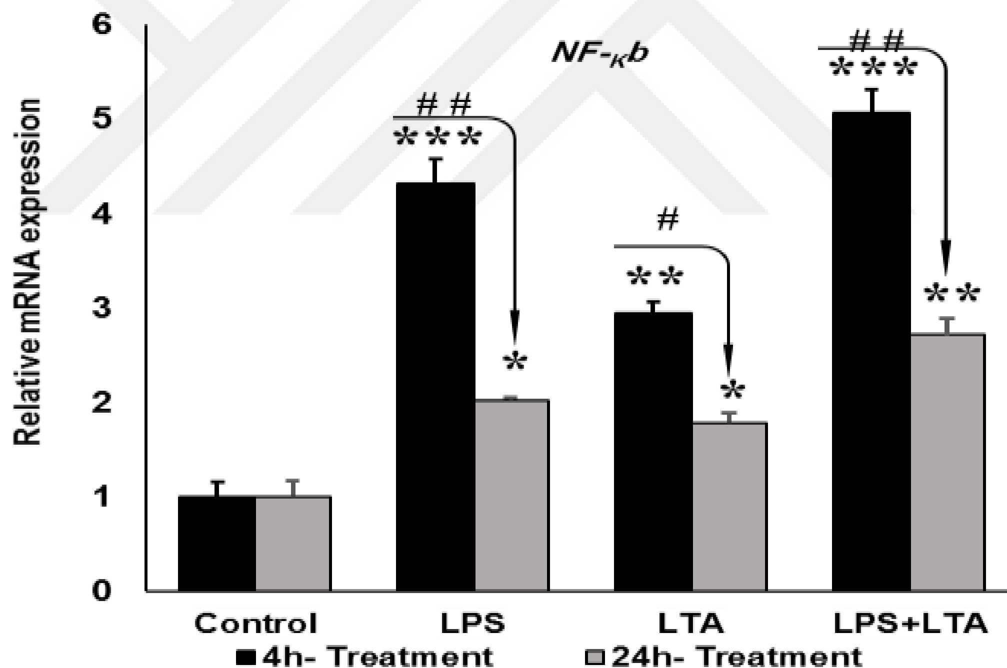

Figure 3.7. The mRNA expression levels of *NF-κB* in response to treated AMs with LTA, LPS and with combination of LPS + LTA treatments between different trials 4 h and 24h

Signs (#) refer to the mRNA expression levels differences between the two different timepoints 4 h and 24 h trials in response to LPS, LTA, and with the combination of LPS + LTA (*P<0.05)

3.1.5. Cytokines mRNA Expression Levels in Response to LPS, LTA and with the Combination of LPS + LTA Treatments Between Different Timepoints 4 h and 24 h Trials

The alveolar macrophages cells (AMs) showed different expression levels of cytokines in response to LPS, LTA and with the combination of LPS + LTA treatments between 4 h and 24 h trial. In this study, we have checked the expression of cytokines in two parts as pro-inflammatory cytokines and anti-inflammatory cytokines as below-

3.1.5.1. Pro-Inflammatory Cytokines

It's a kind of signaling molecule (a cytokine) which is secreted from immune cells such as helper T cells (Th) and macrophages, and other cell types that promote inflammation [257]. In this study, we aimed to investigate the expression of *IL-1β*, *IL-6,* and *TNFα* as pro-inflammatory cytokines.

3.1.5.1.1. *IL-1β* mRNA Expression Levels in Response to LPS, LTA and with the Combination of LPS + LTA Treatments Between Different Timepoints 4 h and 24 h Trials

Stimulation of alveolar macrophages with LPS, LTA and the combination of LPS + LTA in both 4 h and 24 h trials resulted in a significant increase in the expression of *IL-1β* compared with control. In addition, the treatment of AMs cells with LPS, LTA and with the combination of LPS + LTA in 4 h trial showed a higher expression of *IL-1β* than 24 h trial. Moreover, the expression of *IL-1β* in both 4 h and 24 h was significantly increased with the treatment of LPS and the combination of LPS + LTA treatments than LTA treatment alone or control. Furthermore, there were significant differences of *IL-1β* expression between 4 h and 24 h treatment in response to LPS and LTA stimulation, whereas, there were no significant differences of *IL-1β* mRNA expression between both trials 4 h and 24 h in response to the combination of LPS + LTA treatment (Figure 3. 8).

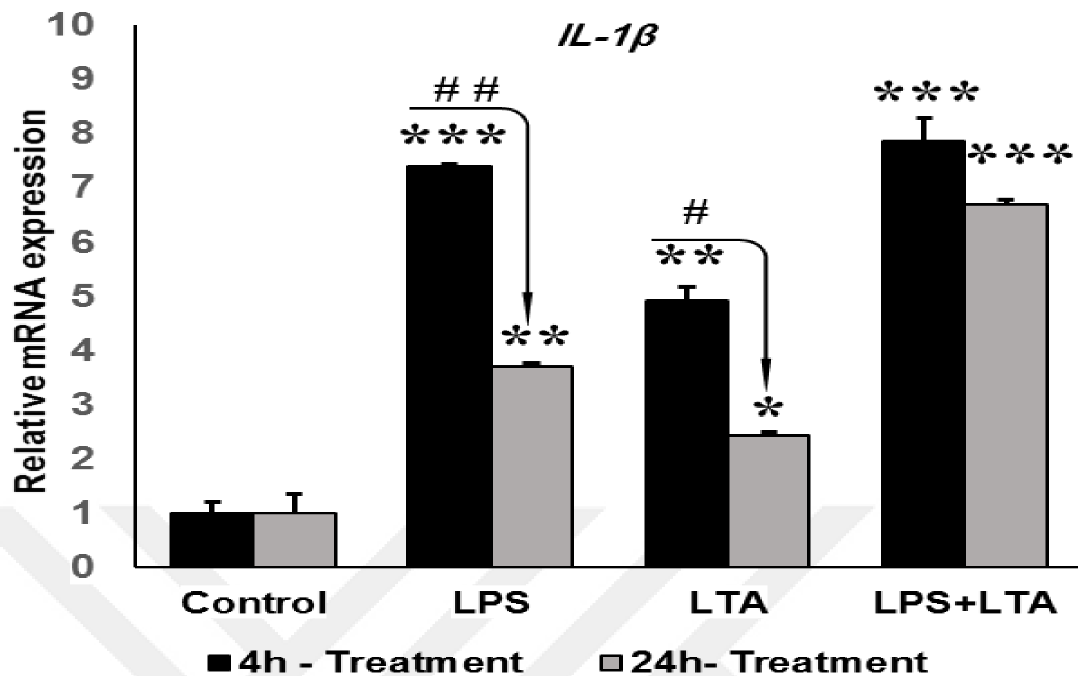

Figure 3.8. The mRNA expression levels of *IL-1β* according to the treated AMs cells with LTA, LPS and with the combination of LPS + LTA treatments between different trials 4 h and 24 h

3.1.5.1.2. Expression of *IL-6* mRNA in Response to LPS, LTA and with the Combination of LPS + LTA in 4 h and 24 h Timepoint

The expression of *IL-6* mRNA was significantly increased when the AMs cells were exposed to LPS, LTA and with the combination of LPS + LTA treatments in both time points, 4 h and 24 h compared to the control group. Interestingly, stimulation of alveolar macrophages with LPS, LTA and the combination of LPS + LTA in 4 h time point exhibited higher expression of *IL-6* than 24 h time point. Moreover, when the AMs cells exposure to LPS and the combination of LPS + LTA showed a significant increase in the expression of *IL-6* in 4 h and 24 h timepoints. However, no significant increase was increased of *IL-6* was observed in the AM cells treated with LTA (Figure 3. 9).

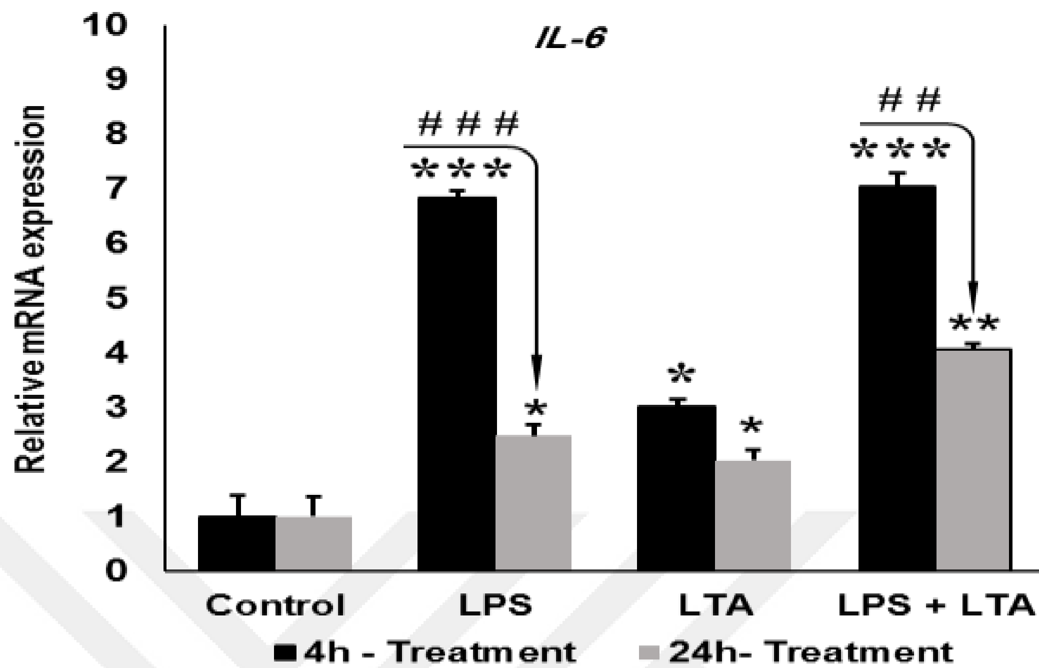

Figure 3.9. The mRNA expression levels of *IL-6* in response to treated AMs with LTA, LPS and with the combination of LPS + LTA treatments between different trials 4 h and 24 h

3.1.5.1.3. *TNFα* mRNA Expression in Response to LPS, LTA and the Combination of LPS + LTA Treatments in 4 h and 24 h Timepoints

Stimulation of AMs cells with LPS, LTA and the combination of LPS + LTA treatments resulted in a significant increase in the expression of *TNFα* compared with control in both 4 h and 24 h treatments. In addition, the expression of *TNFα* was significantly higher in the cells exposed to LPS and the combination of LPS+LTA at 4 h time point compared to those of 24 h time point. However, no such difference was observed in the alveolar macrophages of the LTA treated group. The highest fold change was observed in the AM cells treated with LPS and a combination of LPS+LTA at 4 h time point. In all cases, the expression of *TNFα* was significantly increased compared to those of the control group (Figure 3. 10).

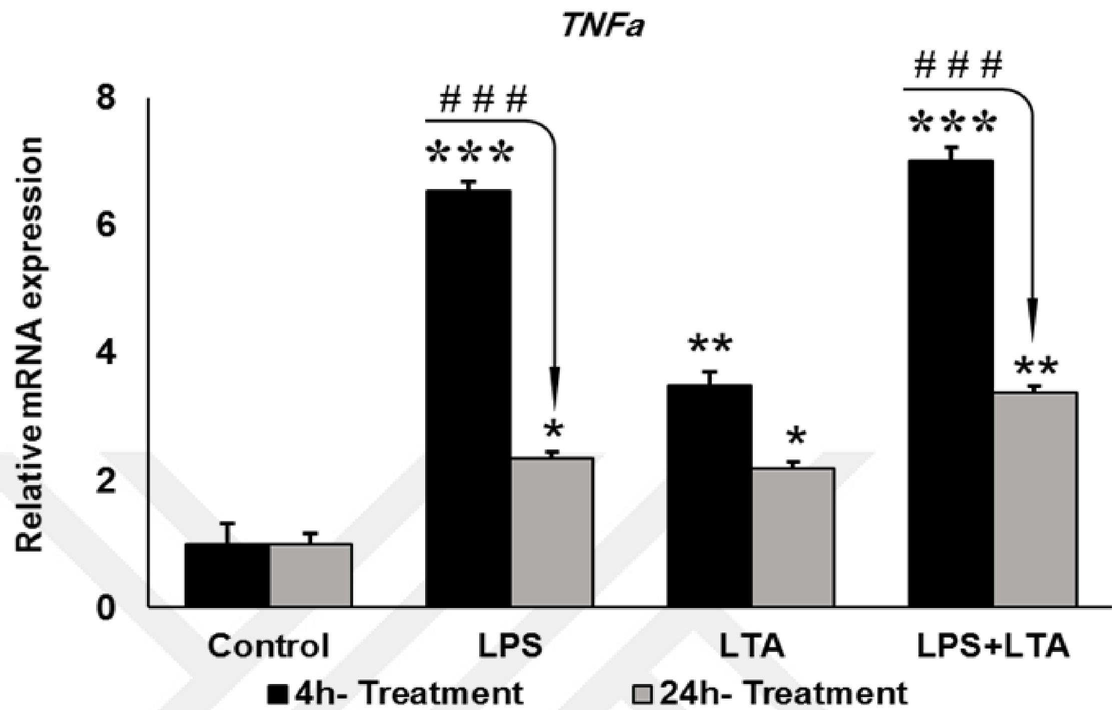

Figure 3.10. The mRNA expression levels of *TNFα* in response to treated AMs with LTA, LPS and with the combination of LPS + LTA treatments between different time points 4 h and 24 h trials

3.1.5.2. Anti-Inflammatory Cytokines

In fact, anti-inflammatory cytokines are a series of immunoregulatory molecules which regulator the pro-inflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the immune response [258].

3.1.5.2.1. Expression of *IL-10* mRNA in Response to LPS, LTA and the Combination of LPS + LTA Treatments in 4 h and 24 h Timepoints

After being stimulation with LPS, LTA and a combination of LPS + LTA in 4 h time point, the AMs cells showed a significant increase in the mRNA expression of *IL-10* compared with control. In both 4 h and 24 h, the expression of *IL-10* was higher in the LPS+LTA treated AM cells. The lowest expression increase was observed in the LTA group compared to control. On the other hand, the mRNA expression of *IL-10* increased moderately in LPS treated AM cells compared to those of the control group. In addition,

the stimulation AMs cells with LPS, LTA and with the combination of LPS + LTA treatments the 4 h timepoint showed a significantly higher expression of *IL-10* mRNA than in 24 h time point. Furthermore, there were significant differences in the mRNA expression of *IL-10* between 4 h and 24 h trials in response to LPS + LTA treatment. Although a decreased expression pattern was observed in the expression of *IL-10*, there was no significant difference in the expression of *IL-10* in the cells treated with LPS or LTA at 4 h time point compared to 24 h time point (Figure 3. 11).

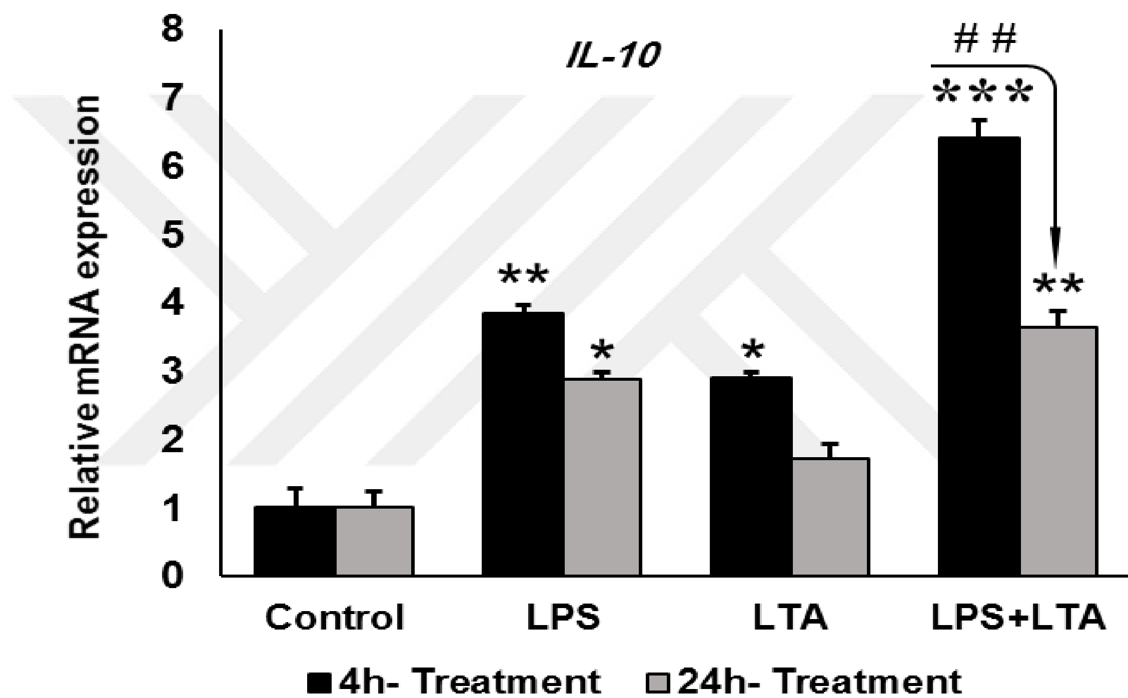

Figure 3.11. The expression levels of *IL-10* mRNA in response to LTA, LPS and the combination of LPS + LTA in 4 h and 24 h timepoints

3.1.5.3. Chemokines mRNA Expression Levels in Response to LPS, LTA and the Combination of LPS + LTA Treatments in 4 h and 24 h Timepoint

Exposed alveolar macrophages cells (AMs) resulted in differential expression of chemokine genes in response to LPS, LTA and the combination of LPS + LTA between different time points such as 4 h and 24 h timepoints.

3.1.5.3.1. Expression of *IL-8* mRNA Levels in Response to LPS, LTA and a Combination of LPS + LTA in 4 h and 24 h Trials

4 h exposure of AMs cells to LPS, LTA and a combination of LPS+LTA treatments resulted in a significant increase in the expression of mRNA level of *IL-8* compared to control. It is interesting to note that the expression of *IL-8* did not show a significant increase in LPS and LTA treated cells compared to that of the control group at 24 h time point. However, the expression of *IL-8* was significantly increased in the cells of LPS and LTA treated cells at 4 h time point compared to the cells of the control group. The maximum increase in the expression of *IL-8* was observed in the AM cells exposed to LPS+LTA at 4 h time point. In both 4 h and 24 h time point, the expression of *IL-8* was lower in LTA treated AM cells compared with other treatments. In addition, there were significant differences in the expression levels of *IL-8* between 4 h and 24 h trials in response to LPS and a combination of LPS + LTA treatments, but there were no significant differences mRNA expression of *IL-8* with LTA between 4 h and 24 h trials (Figure 3. 12).

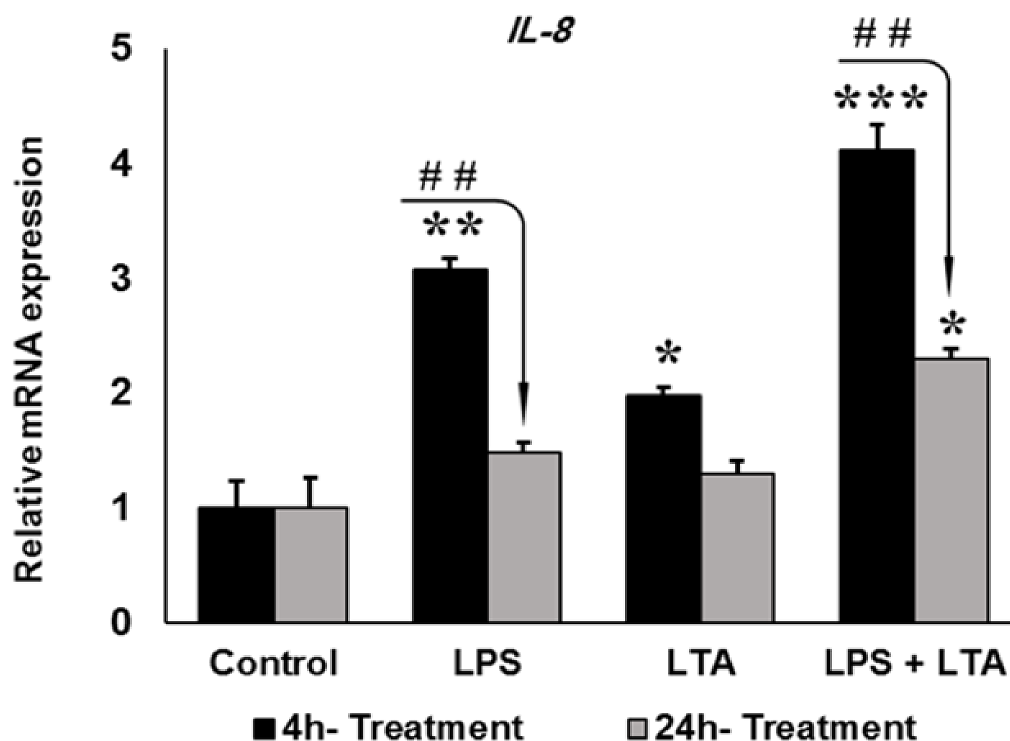

Figure 3.12. The mRNA expression levels of *IL-8* in response to treated AMs with LTA, LPS and with the combination of LPS + LTA treatments between different time points 4 h and 24 h trials

3.2. Results of the *In Vivo* Experiments

3.2.1. The Performance of Akkaraman and Romanov Lambs after Exposed to LPS, LTA and a Combination of LPS + LTA Treatments After 24 h

In fact, there were some phenotypic symptoms we have observed in the animals after 24 h of being exposed to LPS at 20 µg/kg, LTA at 50 µg/kg or a combination of LPS at 20 µg/kg + LTA at 50 µg/kg. These symptoms include sneezing, coldness, lack of movement, redness of the eyes and drooling the tears from the eyeballs and the tendency of the animal to stagnate near sources of food and drink, as well as, they had stocked each other (Figure 3. 13). These symptoms were observed in both Akkaraman and Romanov breeds, however, the exotic Romanov breed suffered the most compared to the native Akkaraman breed.

Figure 3.13. Animals performance after treated with LPS, LTA and with the combination of LPS + LTA

Furthermore, for all animals undergone some measurements before treatments and also after the animals were exposed to LPS (20µg/kg), LTA (50µg/kg) and with the combination of LPS (20 µg/kg) + LTA (50µg/kg). These measurements were the same for all animals and these include rectal temperatures, heartbeats and the live weight of animals. Interestingly, there were no significant differences between the two measurements, as the results presented in Table 3. 1.

Table 3.1. 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

NU	Breeds	Treatments	Weight/kg	Doses/µl/kg	Tempe after treatment ^{o}C	Heartbeats after treatment/mis
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

3.2.2. The Differences in the mRNA Expression Levels of TLRs in the Alveolar Macrophages of Akkaraman and Romanov Lung in Response to LPS, LTA and a Combination of LPS + LTA Treatment

When Akkaraman and Romanov lambs were exposed to LPS (20 µg/ kg), LTA (50 µg/ kg) and with the combination of LPS (20 µg/ kg) + LTA (50 µg/ kg) treatments., the alveolar macrophages cells were collected from the lungs after they were euthanized. Then, the total RNA was purified, cDNA was synthesized, and mRNA expression was quantified through quantitative Real-Time PCR (qRT-PCR) technique. The acquired results which showed a differential expression of TLRs in response to the different treatments after 24 h of the treatments are described below-

3.2.2.1. The Expression of Toll-Like Receptor 2 (*TLR2*) mRNA for Akkaraman and Romanov Lambs After 24 h of Treatments

Akkaraman lambs that were exposed to LPS and LTA treatments did not present any significant mRNA expression of *TLR2* compared with control, while, only with the combination of LPS + LTA the treated lambs showed significant mRNA expression of *TLR2* compared with control. On the other hand, Romanov lambs showed significant mRNA expression of *TLR2* compared with control in LPS, LTA and with the combination of LPS + LTA treatments. Additionally, Romanov lambs presented higher mRNA expression of *TLR2* compared with Akkaraman lambs when they were exposed to LPS, LTA and a combination of LPS + LTA treatments. Moreover, there were significant differences in mRNA expression of *TLR2* between Akkaraman and Romanov lambs in LPS, LTA and a combination of LPS + LTA treatments (Figure 3. 14). It clearly indicates that the Romanov breed which is not adapted with the harsh environment may suffer much more from the disease with a similar infection level than that of native Akkaraman breed. It means that Akkaraman breed is more resistant to the infection than Romanov.

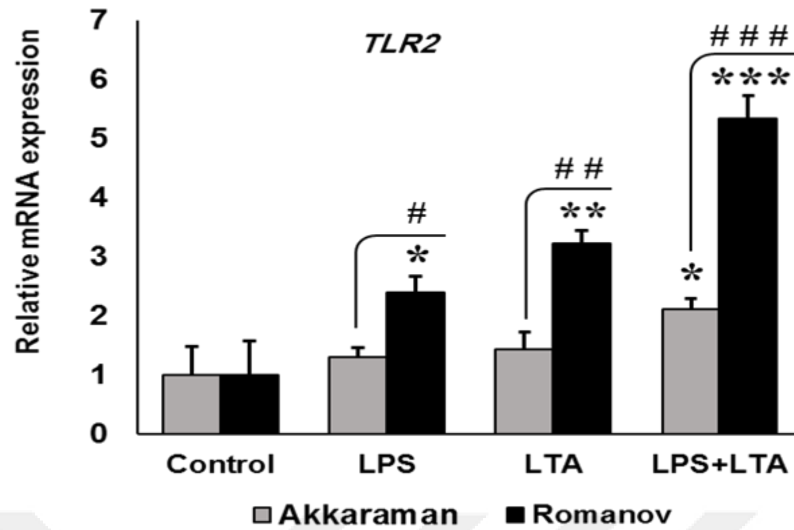

Figure 3.14. The mRNA expression differences of *TLR2* between Akkaraman and Romanov lambs exposed to LPS, LTA and a combination of LPS + LTA treatment

3.2.2.2. The mRNA Expression Levels of Toll-Like Receptor4 (*TLR4*) for Akkaraman and Romanov Lambs After 24 h of Treatment

There was a significant increase in the expression of *TLR4* mRNA compared to control when the Akkaraman lambs were exposed to a combination of LPS + LTA treatment. However, the LPS and LTA treatment did not result in any significant increase in the expression of *TLR4* compared with control. On the other hand, Romanov lambs showed a significant increase in the expression of *TLR4* compared with control in LPS, LTA and with the combination of LPS + LTA treatments. Moreover, Romanov lambs presented higher mRNA expression of *TLR4* compared with Akkaraman lambs when they were exposed to LPS, LTA and with the combination of LPS + LTA treatments. Furthermore, there were significant differences in mRNA expression of *TLR4* between Akkaraman and Romanov lambs in LPS, LTA and with the combination of LPS + LTA treatments (Figure 3. 15).

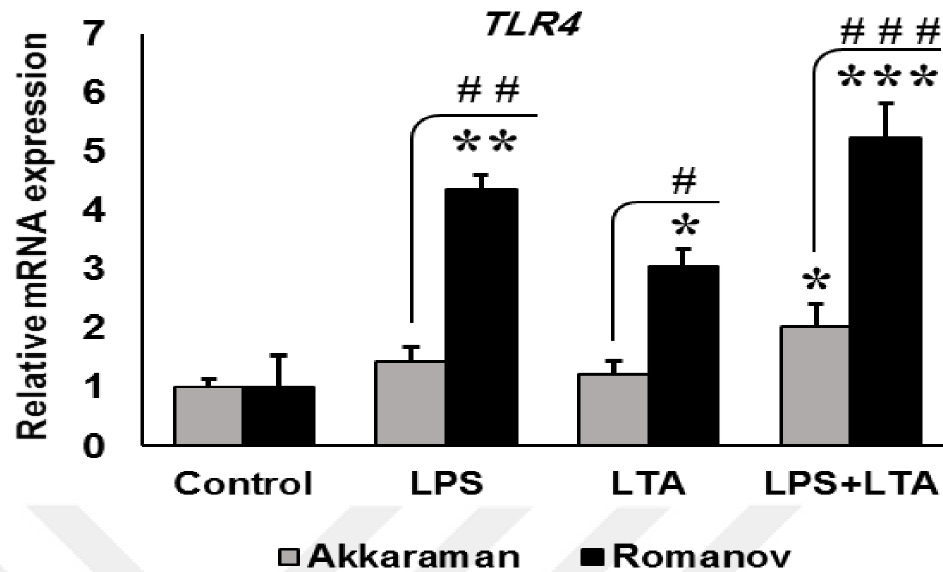

Figure 3.15. The mRNA expression of *TLR4* in the alveolar macrophages of Akkaraman and Romanov lambs that were exposed to LPS, LTA and with the combination of LPS + LTA for 24 h.

3.2.3. The mRNA Expression of the Transcription Factor *NF-κB* in Response to LPS, LTA and a Combination of LPS + LTA in the Alveolar Macrophages of Akkaraman and Romanov Lams After 24 h of Treatment

It is important to note that only the expression of *NF-κB* was significantly increased in both Akkaraman and Romanov lambs in response to LPS, LTA and to the combination of LPS + LTA treatments. However, the highest level of expression was observed in the alveolar macrophages derived from the lung of Romanov sheep treated with LPS+LTA for 24 h (approximately 8-fold increase). On the other hand, the lowest expression of *NF-κB* was observed in the alveolar macrophages obtained from the lung of Akkaraman sheep which was treated with only LTA (approximately 2-fold increase). In general, Romanov lambs showed comparatively higher expression of *NF-κB* in response to LPS, LTA and with a combination of LPS + LTA treatments compared to that of Akkaraman lambs. It is also interesting to note that there was a significant increase in the expression of *NF-κB* levels in the alveolar macrophages of Romanov lamb compared to Akkaraman lamb in response to LPS, LTA and a combination of LPS + LTA treatment (Figure 3. 16).

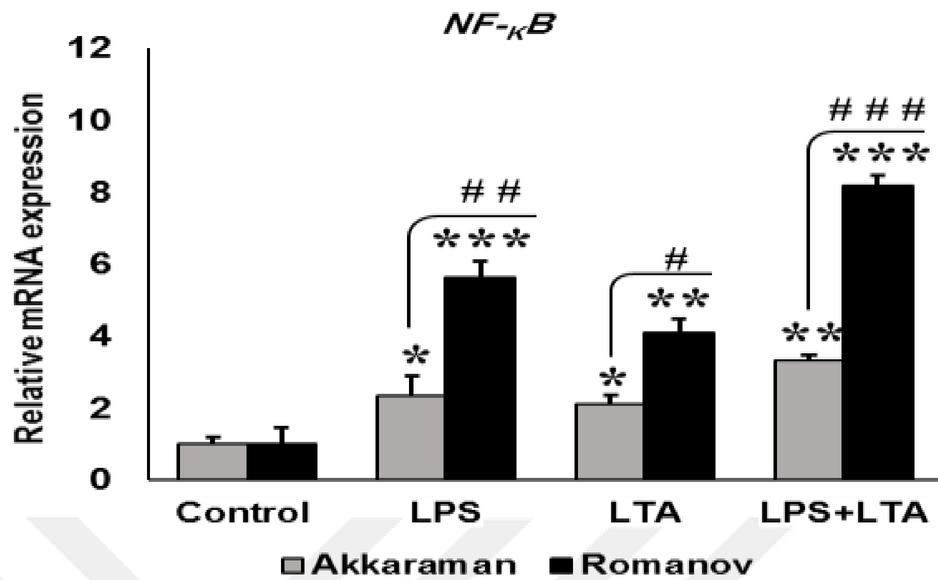

Figure 3.16. The mRNA expression level of *NF-κB* mRNA in Akkaraman and Romanov lambs in response to LPS, LTA and a combination of LPS + LTA treatments after 24h

3.2.4. Differential Expression of Cytokines in Akkaraman and Romanov Lambs in Response to LPS, LTA and a Combination of LPS + LTA After 24 h of Treatment

Akkaraman and Romanov lambs showed a different level of mRNA expression of cytokines when they exposed to LPS, LTA and a combination of LPS + LTA treatments after 24 h of treatment. The results are described below-

3.2.4.1. Pro-Inflammatory Cytokines

3.2.4.1.1. The mRNA Expression Differences of *IL-1β* Between Akkaraman and Romanov Lambs After 24 h of Treatment

Stimulated Akkaraman lambs with LPS and LTA treatments resulted in no significant increase in the expression the levels of *IL-1β* mRNA in the alveolar macrophages compared to that of the control group. However, the administration of a combination of LPS + LTA resulted in a significant increase in the expression level of *IL-1β* in the alveolar macrophages compared to that of the control group. On the other hand, when Romanov lambs were stimulated with LPS or LTA or a combination of LPS + LTA, in all cases, it resulted in a significant increase in the expression of *IL-1β* mRNA in the

alveolar macrophages compared to that of the control group (Figure 3.17). In general, the expression was higher in the alveolar macrophages derived from Romanov sheep in all treatments compared to that of Akkaraman sheep. The highest expression of *IL-1β* was observed in the alveolar macrophages of Romanov lamb when they were treated with a combination of LPS+LTA for 24 h. In addition, Romanov lambs showed higher mRNA expression of *IL-1β* than the alveolar macrophages of Akkaraman lambs in response to LPS, LTA and with the combination of LPS + LTA treatments after 24 h of the treatment. Moreover, there was a significant difference in the level of mRNA expression of *IL-1β* in the alveolar macrophages derived from Akkaraman and Romanov lambs in response to LPS, LTA and with the combination of LPS + LTA treatment after 24 h of the treatments (Figure 3. 17).

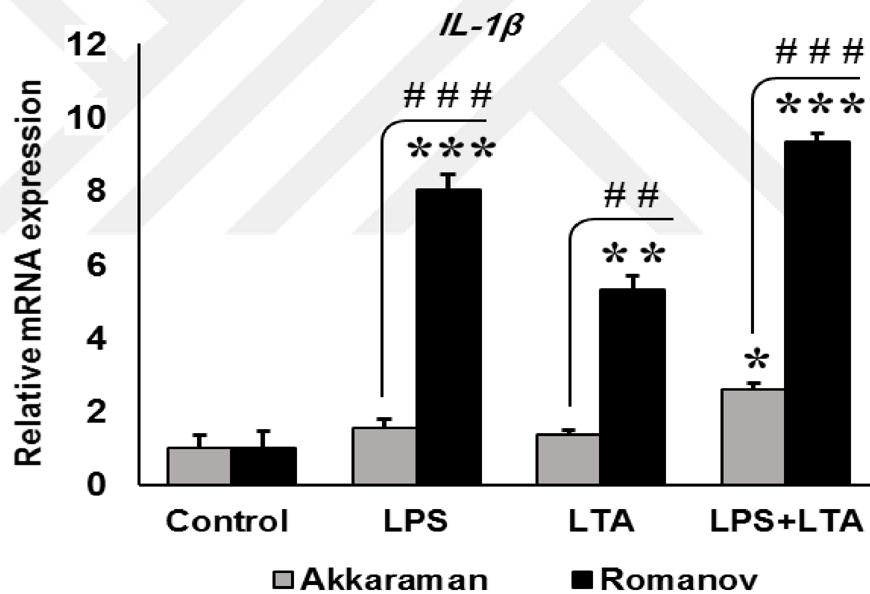

Figure 3.17. The differential mRNA expression levels of *IL-1*β in the alveolar macrophages of Akkaraman and Romanov lambs treated with LPS or LTA or a combination of LPS + LTA after 24 h of treatment

3.2.4.1.2. The mRNA Expression Levels of *IL-6* for Akkaraman and Romanov Lambs After 24h of the Treatment

Similar to other pro-inflammatory cytokines, *IL-16* showed no significant increase in the expression in the alveolar macrophages derived from Akkaraman lamb treated with LPS or LTA for 24 h (Figure3.18). However, when they were treated for 24 h with a

combination of LPS+LTA, the expression of *IL-16* was significantly increased in the alveolar macrophages of Akkaraman lamb compared to that of control lamb. In addition, Romanov lambs exposed to LPS, LTA and with the combination of LPS + LTA showed significantly higher mRNA expression of *IL-6* in the alveolar macrophages of all treatments compared to that of control. Additionally, alveolar macrophages of Romanov lambs exhibited higher expression of *IL-6* mRNA than that of Akkaraman lambs in response to LPS, LTA and a combination of LPS + LTA after 24 h of treatment. Like other cytokines, the expression of *IL-16* was significantly higher in the alveolar macrophages derived from the Romanov lambs treated with a combination of LPS and LTA, while the lowest expression was observed in the LTA treated alveolar macrophages of Akkaraman lamb (Figure 3. 18).

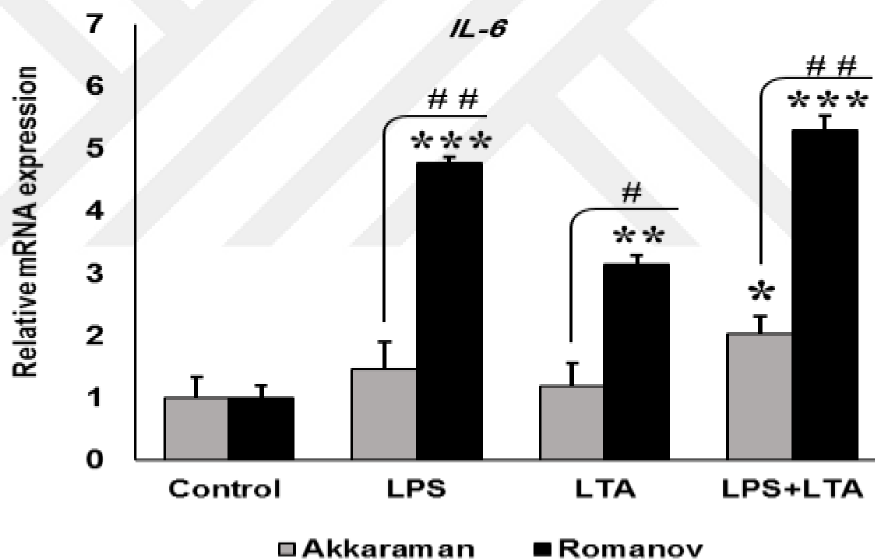

Figure 3.18. The differential mRNA expression of *IL-6* in the alveolar macrophages derived from Akkaraman and Romanov lambs treated with LPS or LTA or a combination of LPS + LTA after 24 h

3.2.4.1.3. The mRNA Expression of *TNFα* in the Alveolar Macrophages of Akkaraman and Romanov Lambs Exposed to LPS or LTA or a Combination of LPS + LTA for 24 h

The expression of *TNFα* was significantly increased in the alveolar macrophages derived from Akkaraman lambs treated with LPS and a combination of LPS+LTA. However, in the LTA treated groups the expression of *TNFα* in alveolar macrophages of Akkaraman lambs was not significantly increased, although an increase-trend was

observed. On the other hand, the expression of the *TNFα* was significantly increased in the alveolar macrophages compared to that of control derived from Romanov lambs exposed to LPS or LTA or a combination of LPS + LTA. In addition, compared to Akkaraman, the Romanov alveolar macrophages expressed a significantly higher level of *TNFα* into LPS or LTA or a combination of LPS + LTA treatment after 24 h period. It should be noted that there was significant difference increase in the expression level of *TNFα* between both breeds when they were stimulated with LPS, LTA and with the combination of LPS + LTA after 24 h of treatment (Figure 3. 19). The highest level of expression increase in terms of fold change was observed in the alveolar macrophages of Romanov lambs treated with a combination of LPS+LTA (approximately 8-fold change). On the other hand, the lowest level of expression was observed in the alveolar macrophages of Akkaraman lambs treated with LTA (approximately 1.5-fold change) (Figure 3.19).

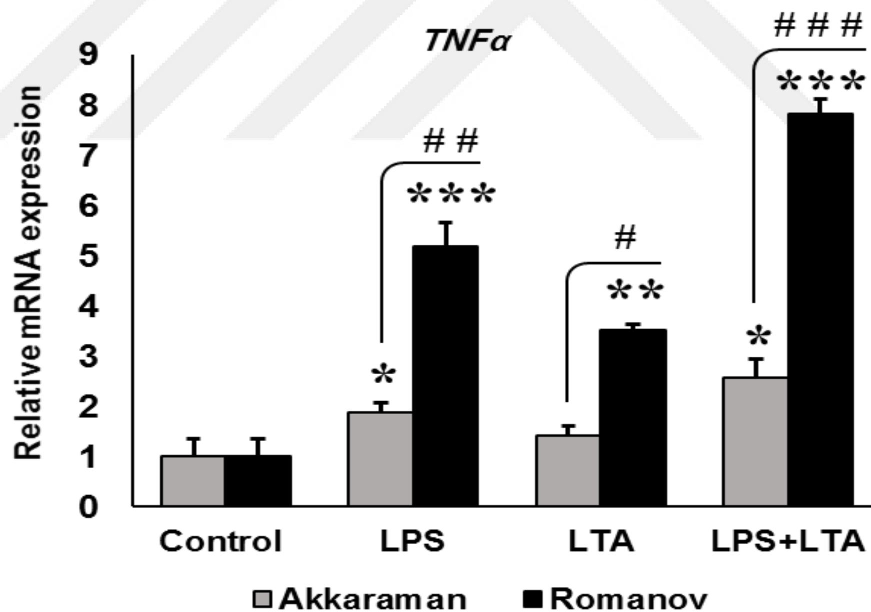

Figure 3.19. The mRNA expression levels of *TNFα* in Akkaraman and Romanov lambs in response to LPS or LTA or a combination of LPS + LTA after 24 h of treatment

3.2.4.2. Anti-Inflammatory Cytokines

3.2.4.2.1. The mRNA Expression Levels of *IL-10* for Akkaraman and Romanov Lambs After 24 h of Treatment

Akkaraman lambs that are treated with LPS or a combination of LPS+LTA resulted in a significant increase in the expression of *IL-10* mRNA level in the alveolar macrophages compared to that of control lambs (treated with a similar amount of sterile PBS). However, no significant increase in the expression of *IL-10* was observed in the alveolar macrophages of Akkaraman lambs treated with LTA compared to control group. On the other hand, Romanov lambs treated with LPS or LTA or a combination of LPS+LTA resulted in a significant increase in the expression of *IL-10* in alveolar macrophages of all treatment groups compared with control. When Romanov lambs stimulated with LPS, LTA and with the combination of LPS + LTA they exhibited comparatively higher expression of *IL-10* mRNA levels compared to that of Akkaraman lambs after 24 h of treatment. Similar to other genes, the highest level of *IL-10* expression was observed in the alveolar macrophages of Romanov lambs treated with a combination of LPS+LTA (approximately 6-fold change, compared to control). On the other hand, the lowest level of expression increase was observed in the alveolar macrophage cells of Akkaraman lambs treated with LTA (approximately, a 1.5-fold increase compared to control) (Figure 3. 20).

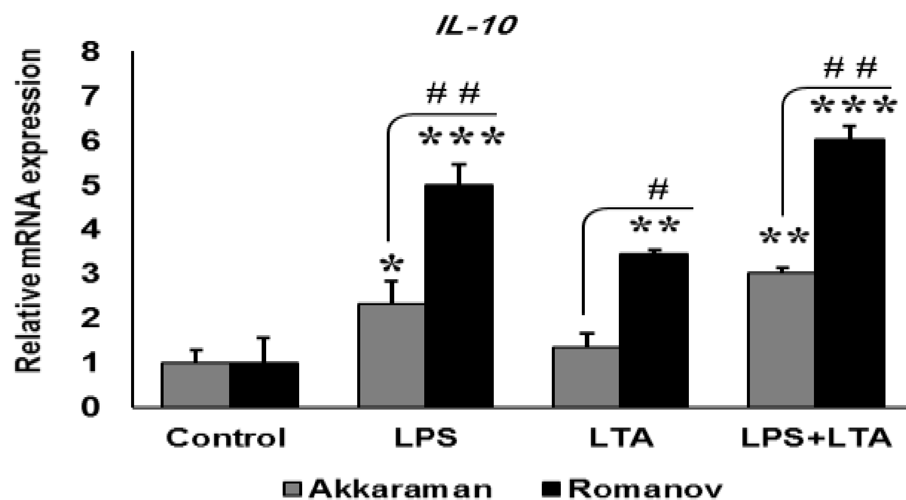

Figure 3.20. The mRNA expression of *IL-10* in the alveolar macrophages of Akkaraman and Romanov lambs in response to LPS or LTA or a combination of LPS + LTA after 24 h of treatment

3.2.5. Expression of Chemokines in the Alveolar Macrophages of Akkaraman and Romanov Lambs in Response to LPS or LTA or a Combination of LPS + LTA After 24 h of Treatment

Akkaraman and Romanov lambs were exposed to LPS or LTA or a combination of LPS+LTA treatments resulted in differential expression of chemokine genes which is presented below-

3.2.5.1. Expression of *IL-8* mRNA in the Alveolar Macrophage Cells of Akkaraman and Romanov Lambs Treated with LPS or LTA or a Combination of LPS + LTA After 24 h of Treatment

There was a significant increase in the mRNA expression of *IL-8* the alveolar macrophages of Akkaraman lambs treated with LPS or a combination of LPS+LTA. Like other genes, stimulation of Akkaraman lambs with LTA resulted in no such increase in the expression of *IL-8* in the alveolar macrophages compared to those of control. On the other hand, Romanov lambs exposed to LPS or LTA or a combination of LPS+LTA treatment showed a significant increase in the mRNA expression of *IL-8* in the alveolar macrophages compared to that of control. In addition, when the Romanov lambs were exposed to LPS or LTA or a combination of LPS + LTA, the expression of *IL-8* in alveolar macrophages was significantly higher than that of Akkaraman lambs in response to after 24 h of treatment. Furthermore, *IL-8* resulted in a significant difference in the mRNA expression levels in Akkaraman and Romanov lambs in response to LPS or LTA or a combination of LPS + LTA after 24 h of the treatment (Figure 3. 21). Like other cytokines and chemokines, the highest expression of *IL-8* was observed in the alveolar macrophages of Romanov lambs exposed to a combination of LPS+LTA treatment for 24 h (approximately, 6.5-fold increased compare to control). On the other hand, the lowest expression of *IL-8* was observed in the alveolar macrophage cells of Akkaraman lambs treated with LTA (approximately, 2-fold higher compared to the control group) (Figure 3.21)

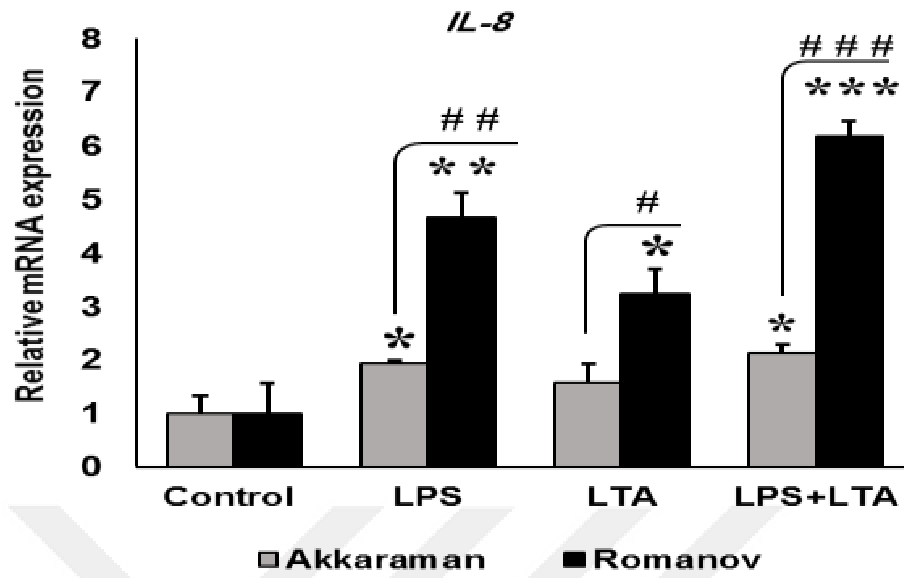

Figure 3.21. The differential expression levels of *IL-8* mRNA in Akkaraman and Romanov lambs stimulated with LPS or LTA or a combination of LPS + LTA for 24 h of treatment

CHAPTER 4

DISCUSSION AND CONCLUSION

Since the start of domestication, sheep are an integral part of agriculture in many parts of the world. In the last century, there was a satisfactory development in the genetics of sheep production. Now, we have high and faster meat producing sheep, fine quality wool producer sheep varieties. However, during this selection process, we only focused on the production traits rather than focusing the production + disease resistance traits. As a result, today we have high producing sheep but highly prone to a wide variety of diseases. Therefore, for the farmers and producers are concentrating the focus on the production of sheep and processing of its products such as milk, meat, and wool are certainly concerned about the economically important disease including respiratory disease. This study was conducted to shed light on the molecular mechanisms of immune response in sheep lung in both *in vitro* and *in vivo* setups. The key hypothesis of the current study was that "it is possible to use the genetic information of the innate immune system as the main assistant in sheep selection against respiratory diseases. Diseases in respiratory system are one of the most important and infectious diseases that have a direct impact on the animal's health and production, and this cause an economic loses for farmer and producers [259]. In fact, small ruminants, particularly foreign lamb breeds in Turkey, suffer from respiratory diseases due to the different environmental conditions and lower adaptive capacity of the animal itself. Therefore, identify underlying immunogenetic mechanisms associated with resistance to respiratory diseases that could enhance the effectiveness of selection for sheep against respiratory diseases as its the premium choice of both farmers and producers in terms of economic point of view.

In the current study, our main focus is to investigate the expression of genes related to the immune system in the alveolar macrophages (AMs) after the stimulation with LPS or LTA or a combination of LPS+LTA treatments by using *in vitro* and *in vivo* studies. We have investigated the expression of genes in two time points (4 h and 24 h) for the *in vitro* experiments, however, for the *in vivo* experiment, we have chosen only one time point (24 h). After the exposure of the alveolar macrophages both in *in vitro* and *in vivo*, we have investigated many of the important genes related to the innate immune system. The expression of genes that we investigated is *TLR2*, *TLR4*, *IL1β*, *IL6*, *IL8*, *IL10*, *NF-κB* and *TNFα* in each trial according to the time point and different breeds based on the previously published work [28,260].

The cell viability results through trypan blue test confirm that there were no significant differences in the viability of alveolar macrophages cells due to different treatments (LPS or LTA or a combination of LPS+LTA) compared to the control group. In addition, there were no differences in the viability of the alveolar macrophages in different time points as well. In the current study, we did not investigate directly the apoptosis caused by the treatments. The results of the microscopic photographs and the viability test indicating that the apoptosis induced cell death may not be a major issue when the alveolar macrophages were cultured with LPS or LTA or a combination of LPS+LTA treatments for 4 h and 24 h. It is worth to note that we have used two reference genes in the current study, *GAPDH,* and *ACTB*, whose expression pattern was stable and consistent in the alveolar macrophage cells after different treatments.

Stimulation of Alveolar Macrophages (AMs) and the Viability

For the phagocytosis of respiratory pathogens or other external environmental pollutants, alveolar macrophages (AMs) are the major category of cells in the airway that are necessary for the proper activation and function of the innate immune defense. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) both are able to induce the immune response through triggering the production of inflammatory cytokines and therefore enhance the phagocytosis rate of macrophages which is autocrine in nature [261]. In the current experiment, firstly, we focused on the investigation of immune response in the alveolar macrophage cells when they are exposed to LPS @10 ug/ml or LTA @10 ug/ml or a combination of LPS + LTA treatments at two different time points

(4 h and 24 h) for *in vitro* study; whereas, for *in vivo* study we used LPS 20 µg/kg, LTA 50 µg/kg and with the combination of LPS 20 µg/kg + LTA 50 µg/kg through the differences in the expression level of a number of immune response-associated genes in two trials. We have seen that alveolar macrophages showed a significant increase in the expression of immune-related genes when exposed to LPS or LTA or a combination of LPS + LTA in both *in vitro* and *in vivo* studies. However, at the same time, we found that alveolar macrophages exhibited a higher increase in the gene expression with 4 h trial than in 24 h time point. It has been shown in many studies through kinetic experiments using mice, pigs, and cows that the onset of the immune response against a variety of viral or bacterial stimulus is earlier time points than 24 h. Furthermore, studies related to immune response kinetics have demonstrated that the expression of several genes returns to its basic level of expression after 48 h of stimulation suggesting that homeostasis is restored at that time [262–264]. These findings suggesting that the cellular homeostasis is restored mostly after 48 h of infection. Therefore, in our study, for the *in vitro* studies, we have chosen an earlier time point (4 h, when the immune response is higher) and a later time point (24 h, when the immune response is comparatively low but not in the homeostasis). It is important to note that the cytokines produced due to the stimulation through LPS or LTA or a combination of LPS + LTA induced the immune response in the immune-related cells in a time- and dose-dependent manner [265].

***In Vitro* Study**

Expression Changes of TLRs in Response to LPS or LTA or a Combination of LPS + LTA Treatments

It is an established fact that *TLR2* and *TLR4* are working as primarily bacterial sensors. Through the interaction with either membrane-bound or soluble coreceptors, they are also able to identify the parasitic and viral components. Particularly, these features give them the ability to diversify the recognition of their ligands and widened their role in immune surveillance. lipopolysaccharides (LPS) and lipoteichoic acid (LTA) response have been observed in human macrophages where the core transcriptional response was conserved between many different species of bacteria and bacterial components such as LPS and LTA and this response was predominantly mediated by *TLR4* and *TLR2*

signaling [266,267]. The findings of the current study showed that in 4 h trial the exposure of AMs to LTA and with the combination of LPS + LTA treatments present a significant gene expression of *TLR2* compared with control. However, the 24 h trial shows that the stimulated AMs only with the combination of LPS + LTA treatment present a significant change in the expression of *TLR2* gene in the alveolar macrophages compared to that of the control group. The results of this experiment indicated that both LTA and LPS acted as immuno-stimulatory compounds. The results of our study are in accordance with a previous study where they showed that the immune cells are activated by LTA via *TLR2* [101]. Moreover, the treated AMs with LPS or LTA or a combination of LPS + LTA treatments in 4 h trial report higher mRNA expression of *TLR2* than 24 h trial. Rosenberger et al [268] suggested that LPS and LTA play an important role in early response to bacterial infection 4 h. Moreover, there were no significant differences of mRNA expression between the two trials 4 h and 24 h in response to LPS, LTA, and with both LPS + LTA treatments. The previous study showed that the early response to LPS and LTA (the first few hours) is dominated by TLRs signaling and other signaling mechanisms have either weaker effects or influence the smaller proportion of cells [269].

In this study, we observed that the stimulation of alveolar macrophages from Akkaraman or Romanov lambs with LPS or a combination of LPS + LTA treatments resulting in a significant increase in the mRNA expression of *TLR4* compared to that of control in 4 h trial. However, when the alveolar macrophages cells were exposed to different treatments for 24 h, only the combination of LPS + LTA treatment showed a significant increase in the mRNA expression of *TLR4* compared to that of control. It is clear from our results that the synergistic effects of LPS and LTA induce a stronger immune response in sheep alveolar macrophages *in vitro* which is evident through the expression of *TLR4*. It has also been shown that *TLR4* signaling pathway can induce the production of cytokines, the expression of related genes as well, in a wide variety of cells when they are exposed to Gram-negative bacteria and other pathogenic compounds to induce the disease-resistant effects [270,271]. Interestingly, in the present study, we have found that the expression of *TLR4* mRNA was higher when the alveolar macrophage cells were exposed to LPS or LTA or a combination of LPS + LTA treatments in 4 h trial than 24 h time point. The findings of our study are closely

matched with previously published findings where the authors showed that higher expression of *TLR4* occurs when the cells are exposed to LPS which further rapidly activate the *TLR4* signaling pathway. The rapid activation of the *TLR4* pathway immediately initiates an immune response to fight against infection and invasion induced by pathogens. This process is crucial for the induction of resistance against pathogens and to keep the internal environment in a balanced condition. In addition, nuclear factor-kappa B (*NF-κB*) can be activated by *TLR4* through both *MyD88*-dependent or *MyD88*-independent pathways [217,272].

Expression of Transcription Factor *NF-κB* mRNA in Alveolar Macrophages Exposed to LPS or LTA or a Combination of LPS + LTA

NF-κB is considered to be one of the most important components of the inflammatory immune response that is involved in the regulation of more than 150 genes related to immunity. Several types of cancer, inflammation, adaptive and innate immune responses, and stimuli of various pathogens can activate *NF-κB*. It has also been shown that lowering the abundance of *NF-κB* activation is an important strategy for anticancer therapies and anti-inflammation [273,274]. In our present study, we found that the stimulation of AMs using LPS or LTA or a combination of LPS + LTA significantly increase the mRNA abundance of *NF-κB* compared to that of control in both 4 h and 24 h time points. As well as, this study showed that there were significant differences in mRNA expression of *NF-κB* between 4 h- 24 h trials in all treatments, this might be because of the time point differences between the two experiments. In fact, in our study, it was apparent that 4 h trial has shown higher mRNA expression of *NF-κB* than 24 h trial in response to LPS, LTA, and with the combination of LPS + LTA treatments. *NF-κB* -dependent genes induced by *IL-1β* whose expression may peak within hours of stimulation and lead to the identification of secondary effects. Moreover, *NF-κB* showed high mRNA expression in earlier hours and it returns to basal activity after 24 h [196].

The mRNA Expression Levels of Different Cytokines

IL-1β

Many studies have presented that mRNA expression of several genes could be rapidly induced by *IL-1β* in a variety of cell types, including macrophages that can be induced

by both LTA and LPS [275,276]. It is important to note that *IL-1β* is one of the most important members of the cytokine family which upon activation immediately activate defense mechanism related cascade to protect against both Gram-positive and Gram-negative bacterial infection [277,278]. The findings of our study completely agree with this statement. In our study, we found that the expression of *IL-1β* mRNA was significantly increased when alveolar macrophages were treated with LPS or LTA or a combination of LPS + LTA for both trials 4 h and 24 h compared to control groups. It is worth to note that the expression of *IL-1β* was higher in the alveolar macrophages exposed to LPS or a combination of LPS + LTA treatments compared to both control and LTA treatment in 4 h and 24 h trials. However, in both 4 and 24 h time points, the highest expression of *IL-1β* was observed when the alveolar macrophages were exposed to a combination of LPS+LTA. These results are completely aligned with the results of a previous study where the authors reported that in case of Gram-negative bacterial infection the expression of *IL-1β* was higher compared to Gram-positive bacterial infections [279].

Moreover, in the current study, we have seen that the expression of *IL-1β* was different in 4 h and 24 h time points in response to LPS and LTA treatments, but not with LPS + LTA treatment. It has been reported that the main function of *IL-1β* is essential pro-inflammatory cytokine which possesses multiple properties and affects almost all kind of cells. It is important to note that *IL-1β* acts as a mediator of inflammation, which is acute in nature, by initiating systemic and local responses [280,281]. As well as, our results demonstrated that the expression of *IL-1β* mRNA was significantly elevated in 4 h trial than in 24 h trial. In fact, a previous study, it has been shown that the short time (4 h) stimulation by LPS favors the higher expression of *IL-1β* [275]. Perhaps *IL-1β* could be responsible for the changes of the majority of gene expressions in macrophages at the time of inflammatory reactions.

IL-6

In the case of host immune response to pathogenic organisms, *Interleukin 6* (*IL-6*) is considered as an important mediator of the immune cascade. Based on circumstantial evidence, it has also been proposed that it is one of the major circulating endogenous pyrogens which is responsible for the activation of central nervous system (CNS)

mechanisms during fever caused by inflammation and infection. It is important to note that although *IL-6* is crucial for the fever induced by LPS, it is not able to induce fever alone. It appears that it may act as an important agent during fever in association with *IL-1β* [282,283]. In the present investigation, we find that the mRNA expression of *IL-6* was significantly high when AMs treated with LPS or LTA or a combination of LPS + LTA treatments compared to control groups in both trials 4 h and 24 h time points. In addition, the results of this study confirm that stimulation of alveolar macrophages using LPS or LTA or a combination of LPS + LTA treatments shows higher mRNA expression of *IL-6* in 4 h trial than 24 h trial. A previous study demonstrated that a dramatic enhancement of the concentration of circulating *IL-6* (a 22-fold increase compared to basal level) just after 2 h of the administration of LPS [228]. In addition, Alapirtti and colleagues showed that there was a significant increase in the level of *IL-6* at every time points that have been checked from 3 h to 24 h after the index seizure (IS) compared to the control condition [284].

At the time of immune response, the upregulation of *IL-6* is one of the distinctive feature factors. It is true that the expression of *IL-6* enhanced dramatically due to LTA and LPS stimulation [285]. The present study reports that stimulation of AMs cells with LPS and with the combination of LPS + LTA treatments present significant differences in the expression of *IL-6* mRNA in the trial of 4 h and 24 h time points. Previous studies have indicated that the mice deficient in *IL-6* gene succumb from Gram-positive infection earlier than that of the control wild type mice which reinforce the idea that *IL-6* is the central member to initiate the defense mechanisms against bacterial. Furthermore, the rate of mortality was high *IL-6*–knockout mice upon the administration of live *Escherichia coli*, which is associated with the higher number of bacterial organs [286,287].

TNFα

Being a cytokine, *TNF-α* is involved in the *TLR4* pathway and consider to be a primary agent of the inflammatory response. It has been reported that the administration of LPS resulted in inflammation by producing inflammatory cytokines by macrophages including *TNF-α* [288]. The results of our study clearly showed that the expression of *TNFα* mRNA was significantly increased by LPS, LTA, and LPS + LTA treatments

compared with control in both trials 4 h and 24 h. As shown in one previous study that during stimulation through LPS administration, the increased expression of *TNF-α* was necessary to mediate the immune response [289]. As well as, our study indicates that there was significant differences mRNA expression of *TNFα* between both trials 4 h and 24 h when AMs was exposed to LPS and with both LPS + LTA treatments. Additionally, our study clearly shows that 4 h trial presented higher mRNA expression of *TNF-α* than 24 h trial when AMs stimulated with LPS, LTA, and with the combination of LPS + LTA treatments. The findings of our study are similar to what is observed in previous studies [290,291] where the investigators found that there was a significant increase in the expression level of *TNF-α* at 0.5 hours and the expression reached to a peak at 2 h time point. However, the expression of *TNF*-α mRNA dramatically decreased until 4 h time point and reached to the basic level after 24 h of incubation. Basically, the expression of *TNF*-α was highest in 2 hours after stimulation. It is important to note that the higher levels of *TNF-α* are not good for cells or tissue because it may induce tissue damage through an activation of endogenous mediators associated cascades. This indicates that internal mechanisms are strictly controlling the immune response and the differential transcription patterns could be associated with types of cells [292].

IL10

Interleukin-10 (*IL-10*) was initially characterized as an inhibitory factor for the synthesis of cytokines. It was initially believed that the mechanism of *IL-10* inhibition of cytokine production was related to the suppression of the capacity of producing antigen-presentation of dendritic or macrophage cells. Later studies confirmed that *IL-10* also played a crucial part to block the production of cytokines [237]. Our results demonstrate that the treated AMs with LPS or LTA or a combination of LPS + LTA treatments resulting in significant increase in the expression of *IL-10* mRNA compared to that of control group in 4 h trial, while, with the 24 h treatment the significant increase in the expression of *IL-10* mRNA compared with control was with LPS and a combination of LPS + LTA treatments only. The study conducted by Moreno and colleagues reported that LPS induced *IL-10* production by peripheral blood mononuclear cell (PBMC) and particular microsatellite polymorphisms are correlated [293]. In addition, it has also been reported that in response to the stimulatory pathogens

or protein compound, a variety of cells can express *IL-10* which indicating that the expression of IL-10 can be regulated in different cell (T cells and monocytes/macrophages) types through different mechanisms [240].

It is important to note that *IL-10* may partially inhibit the activities that are triggered by other cytokines, notably *IFNγ-*, *IL-2*, *TNFα-*, *IL-4*, and also *NF-κB* activation by activated monocytes/ macrophages. Despite *IL-10* may affect T cell responses, it is involved in the enhancement of the survival of T cells, B cells, tumor cells, and the promotion of the development of B1 cells and, paradoxically, played an important role in the induction of apoptosis in chronic B cell leukemia cells [294–297]. Furthermore, our findings also report that 4 h trial presented higher mRNA expression of *IL-10* than 24 h trial with all treatments. As well as, our study presents that there was significant differences mRNA expression of *IL-10* between 4 h and 24 h trials in response to the combination of LPS + LTA treatment. In contrast to our and other's findings, Moore *et al.* reported that 7 h after the activation of the monocytes through LPS may result in lower levels of *IL-10*, and the maximal production of *IL-10* may be observed in 24 h to 48 h after activation [240].

IL8

Interleukin 8 (*IL-8*) is one of the important chemokines which can stimulate and attract leukocytes to accumulate at sites of inflammation, and both *TNF-α* and *IL-1β* can increase the release and secretion of *IL-8* in a dose- and time-dependent manner [298]. Many studies showed that mRNA induction and protein release of *IL-8* have been associated with the activity of a variety of cell types including endothelial cells, mononuclear cells, and airway epithelial cells. Moreover, it has been recently reported that Lipopolysaccharide (LPS) stimulates the expression of *IL-8* and induce inflammatory response in many cells [299–301]. Indeed, results from our study show that in 4 h trial, the stimulation of AMs with LPS, LTA and with the combination of LPS + LTA treatments resulting in significant mRNA expression of *IL-8* compared with control, whereas, in 24 h trial the stimulated AMs show significant gene expression of *IL-8* compared with control only with the combination of LPS + LTA treatment. Furthermore, our study indicates that stimulated AMs with LPS and with the combination of LPS + LTA treatments present significant differences in mRNA

expression of *IL-8* between 4 h and 24 h trials. Additionally, in this study, we find that in 4 h trial, the exposure AMs to LPS, LTA and with the combination of LPS + LTA treatments present higher mRNA expression of *IL-8* than in 24 h trial. This is in accordance with previous findings which indicate that the production of *IL-8* secreted at high levels between 4 h and 8 h after activation of the monocytes [302,303].

***In Vivo* Study**

Sheep and lamb meat are a valuable source of nutrients, high-priced, and highly popular in most parts of the world and as well as in Turkey. Recently, for many reasons including arid climate, unbalanced slaughter and many kinds of diseases; the numbers of sheep have been decreased. Indeed, respiratory diseases, considered to be one of the most important diseases that have a direct effect on sheep health and may cause mortality. Therefore, there are now accumulating attempts to develop genetic markers related to the disease resistance capacity of animals which potentially contributing to the development of disease-resistant breeds which can continue its productive performance in the presence of pathogenic organisms. The science and scientific techniques are improving rapidly. We are doing and using such technologies which we could not even think 20 years back. Therefore, it is important to combine the knowledge and perform integrated studies that consider functional and quantitative genomics, a collection of large-scale data and prediction epidemiology which could open a new window to the breeders for selecting sheep with a higher resistance against a wide variety of infectious diseases including respiratory diseases [304].

To understand the adaptive and innate immunological responses, it is highly important to uncover the underlying mechanisms of the gene expression regulation in macrophages in the host during the immune stimulation through pathogens and stimulants of various origin. It is worth to note that the tissue macrophages and blood mononuclear cells are the major source of cytokines and are the principal responders against any pathogen like viral or bacterial proteins. Because of this vicious circle of the reaction related to inflammation, it may be perpetuated [305,306].

In fact, in the second part of this study (*in vivo* study), we investigated the gene expression of stimulated alveolar macrophages (AMs) with LPS (20 µg/ kg), LTA (50

μg/ kg) and with both LPS (20 μg/ kg) + LTA (50 μg/ kg) treatments by using two different breeds which are Akkaraman and Romanov lambs to see the differences in the mRNA expression levels of *TLR2*, *TLR4*, *IL1β*, *IL6*, *IL8*, *IL10*, *NF-κB* and *TNFα* between these two different breeds after 24 h of the treatments. It has been shown that Gram-positive bacteria primarily recognized by the host immune cells by identifying extracellular soluble toxins or a membrane protein called peptidoglycans, *lipoteichoic* acid (LTA) while the Gram-negative bacteria can be recognized by the host immune cells by identifying LPS or membrane-associated endotoxin or their shed [307].

TLR2

As we have mentioned earlier in this study that in initiating the innate immune response, toll-like receptors (TLRs) play a central role. Previous studies reported that *NF-κB* can be activated by *TLR4* and *TLR2* which later induce the expression of co-stimulatory molecules and inflammatory cytokines in humans. It has also been reported that proinflammatory signaling in macrophages induced by bacteria-associated toxins is mediated by *TLR2* [308]. In the current study, we showed that the stimulation of Akkaraman lambs only with the combination of LPS + LTA treatment resulting in a significant increase in the expression of *TLR2* mRNA in alveolar macrophages compared to that of the control group. While we find that the Romanov lambs showed a significant increase in the expression of *TLR2* mRNA compared to control in response to LPS or LTA or a combination of LPS + LTA treatments. As well as, Romanov lambs present higher mRNA expression of *TLR2* than Akkaraman lambs when they were exposed to LPS, LTA and with the combination of LPS + LTA treatments. Moreover, the results of our study referred to there were significant differences in the expression patterns of *TLR2* mRNA in the alveolar macrophages of Akkaraman and Romanov lambs in response to all treatments. Studies have shown that mainly the agonists present in *TLR2* induce the inflammatory cytokine production process dendritic cells and macrophages, which further suggests that *TLR2* has cell type-specific role against antiviral responses [309], that means that the higher expression of *TLR2* genes resulting in more inflammatory cytokines production as well.

Interestingly, the results of our study showed that the infection of Akkaraman lambs with the combination of LPS + LTA resulting in a significant increase in the expression

of *TLR4* mRNA in the alveolar macrophages in comparison with the control. However, Romanov lambs present a significant increase in the expression of *TLR4* mRNA compared to control in LPS or LTA or a combination of LPS + LTA treatments. It has been shown previously that the *NF-κB* pathway is responsible for the activation of immune cells through the receptors of *TLR2* and *TLR4*. Thus, Gram-negative bacteria produced LPS is involved in the immediate production of inflammatory cytokines via cellular activation and the activation of *NF-κB* via *TLR4* [310]. The cytokines produced upon *NF-κB* activation resulted in an increase in the expression of *TLR2* while resulted in a down-regulation of *TLR4* mRNA. Additionally, the results obtained from our recent study show that Romanov lambs present high and significant mRNA expression of *TLR4* compared with Akkaraman lambs in all treatments. In another study, Deng and colleagues reported that there are several types of Gram-negative bacteria that may cause economically important diseases in sheep [311]. In fact, it is true that most of the common Gram-negative bacterial infections are responded by *TLR4* which is an important member of the Toll-like receptor (TLR) family. It is important to note that the inflammatory response induced by the activation of *TLR4* might act as a linkage between the adaptive and innate immune systems. It is worth to note that *TLR4* can recognize Gram-negative bacterial LPS and the recognition leads to the NO production that induces the inflammatory cytokines release and finally promotes the infiltration of inflammatory cells. The previous study reported that the response of macrophages against LPS is mediated by *TLR4*, and the overexpression of *TLR4* resulted in an increase in the level of expression of *TNF-α*, *IL-6*, and *IL-8* [312]. In addition, some other studies showed that *TLR2* may be involved in the activation of *NF-κB* following interaction of macrophages with LTA from the cell walls of Gram-positive bacteria, LPS from Gram-negative bacteria, and lipoarabinomannan (LAM) from mycobacterial species [313].

NF-κB

It is a well-accepted notion that *NF-κB* plays a crucial part in the adaptive and innate immune responses. Study on Mongolian sheep indicates that the activation of *NF-κB* resulted in the higher expression of cytokines that are pro-inflammatory in nature, chemokines, and antimicrobial peptides, which are supposed to be an important mediator or effector of the adaptive and innate immunological system [314]. Our study

results, present that the stimulation of both breeds (Akkaraman and Romanov) lambs with LPS, LTA and with the combination of LPS + LTA resulting in significant mRNA expression of *NF-κB* compared with control after 24 h of treatments. As well as, in this study, we find that Romanov lambs show high and significant mRNA expression of *NF-κB* compared with Akkaraman lambs in response to LPS or LTA or a combination of LPS + LTA after 24 h of treatments. It has been shown by several studies that the stimuli of different pathogens and environmental pollutants resulted in the activation of *NF-κB* which initiate a rapid activation of different targets by activating the production of a wide variety of gene products including *iNOS, COX-2*, and cytokines that involved in adaptive and innate immunological responses, cancer, and inflammation [197,315]. Therefore, the higher expression of *NF-κB* in the alveolar macrophages of Romanov sheep in response to LPS or LTA or a combination of LPS or LTA possibly indicating 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 higher level of other inflammatory cytokines. In addition, the lower expression of *NF-κB* in the alveolar macrophages of the Akkaraman sheep in response to different treatments indicating that this sheep is stronger and have well developed adaptive and innate immune response which can easily handle the infection induced by Gram-positive and Gram-negative bacteria-induced infection.

IL-1β

It has been reported that in initiating the acute phage innate immune response, the macrophages are the main effector cells. It is interesting to note that the *IL-1β* which is produced by macrophages exerts an autocrine effect the cells which induced a change in the transcriptomic level of the cells that is induced by the higher expression of cytokines. For instance, *IL-1β* played an important role in the defense mechanism cascade against a wide variety of bacterial infections, and also, its involvement in the familial auto-inflammatory syndromes induced pathogenesis, and the blocking of *IL-1β* in systemic diseases reduces *IL-6* levels [316]. In this study, we find that the treated Akkaraman lambs with the combination of LPS + LTA showed a significant increase in the expression of *IL-1β* mRNA in comparison to the control. Whereas, the treated Romanov lambs with LPS, LTA and with the combination of LPS + LTA presented a significant increase in the expression of *IL-1β* mRNA compared with control. As well

as, the findings of our study show that Romanov lambs present higher expression of *IL-1β* mRNA than Akkaraman lambs in response to LPS or LTA or a combination of LPS + LTA after 24 h of the treatments. Moreover, our data demonstrate that there was a significant difference in the mRNA expression level of *IL-1β* between Akkaraman and Romanov lambs in response to LPS or LTA or a combination of LPS + LTA after 24 h of the treatments. Recent studies reported that *IL1β* is a highly inflammatory cytokine response by mediating its own 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 [280,317].

IL-6

During the innate immune response, the differentiation and infiltration of activated macrophages are induced by *IL-6* through upregulating the expression cell adhesion molecules [318]. In this study, we find that the stimulated Akkaraman lambs only with the combination of LPS + LTA resulting in a significant enhancement in the expression of *IL-6* mRNA compared to the control group. While, exposure Romanov lambs to LPS, LTA and with a combination of LPS + LTA show a significant abundance of mRNA expression of *IL-6* in the alveolar macrophages compared to that of control. As it has been shown in one previous study under LPS stimulation, *IL-6* increasing of expression levels were necessary for immune response [289]. Moreover, our data further demonstrate that Romanov lambs present higher and significant mRNA expression levels of *IL-6* than Akkaraman lambs in response to LPS, LTA and with the combination of LPS + LTA after 24h of the treatments. In fact, our findings are in agreement with the previous study that *IL-6* expression has been suggested as a diagnostic marker of Gram-negative bacterial sepsis [319]. As well as, *IL-6*-deficient animals were unable to induce neutrophilia in comparison with control animals, and this defect could be reversed upon exogenous *RIL-6* administration, and also the *IL-6*-deficient mouse during listeriosis, a gram-positive bacterial infection. Therefore, *IL-6* may be important in a general aspect of innate immunity to a variety of bacterial infections [286].

TNFα

Study on sheep showed that the transient expression of *TNF-α* helped to prevent over inflammatory reaction [311]. As well as, a line of studies indicated 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 which reside in many tissues and trigger an array of innate immune responses upon encounter of invading pathogens. And the reduction of *TLR4* level in cells by half correlated with 50–60% inhibition of *TNF-α* production induced by 1–1000 ng/mL of sLPS and rLPS, which means cytokines were generated in *TLR4*-dependent manner [320,321]. In fact, our study shows that the treated Akkaraman lambs with LPS and with the combination of LPS + LTA present a significant higher expression of *TNFα* mRNA in the alveolar macrophage cells compared to that of control.

However, the results of this study present that the treated Romanov lambs with LPS, LTA and with the combination of LPS + LTA present significant mRNA expression of *TNFα* compared with control. Moreover, exposure Romanov lambs show high and significant mRNA expression of *TNFα* compared with the Akkaraman lambs in response to LPS, LTA and with the combination of LPS + LTA after 24 h of treatments. Ro *et al.* have reported that *TNF-α* dominated acute and excessive release of proinflammatory cytokines can be triggered by the LPS [290]. On the other hand, one of the previous studies has been shown that the higher level of *TNF-α* might be involved in tissue damage through modulating a cascade of endogenous mediators and to avid serious tissue damage induced overreaction, there are internal mechanisms playing either negative role in *TLR4*. As well as, it has been reported that the level of *TNF-α* transcription returned to the basic level after 24 h of stimulation [322].

IL-10

It has been shown in both *in vivo* and *in vitro* studies that the stimulation of immune cells using neutralizing antibodies revealed pleiotropic and recombinant cytokine resulted in an activation of B-cells, T-cells, *IL-10*, mast cells and provides supporting results and data to understand the significance of *IL-10* in *in vitro* by examining different *in vitro* activities [323,324]. 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 pneumonia, and Klebsiella pneumonia [325]. Indeed, the results of this study present that, when the Akkaraman lambs stimulated with LPS and with the combination of LPS + LTA resulting in significant mRNA expression of *IL-10* compared with control. However, we find that the stimulation of Romanov lambs with LPS, LTA and with the combination of LPS + LTA present a significantly higher expression of *IL-10* mRNA compared to the control. As well as, our results present that when Romanov lambs stimulated with LPS, LTA and with the combination of LPS + LTA they show high and significant mRNA expression level differences of *IL-10* compared with Akkaraman lambs after 24h of treatments. It has been shown by study that the phagocytosis of E. 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 [326].

IL-8

Being a pro-inflammatory chemokine in nature, *IL-8* is involved in the neuroprotection which has been demonstrated in vitro. This chemokine is secreted by microglia, astrocytes, and endothelial cells. Moreover, several cytokines including *IL-l* and *TNF-α* have the capacity to induce the secretion and expression of *IL-8* in astrocytes [327,328]. The results obtained from this study show that there was significant mRNA expression of *IL-8* compared with control when Akkaraman lambs are exposed to LPS or a combination of LPS + LTA. Whereas, we find that when Romanov lambs are exposed to LPS or LTA or a combination of LPS + LTA showed a significantly higher expression of *IL-8* mRNA in the alveolar macrophages in comparison with that of the control group. In fact, it has been shown that there is primarily two functions of *IL-8* such as activating neutrophils and induce the production of chemotaxis in certain cells [311]. This is in agreement with a previous study where the authors indicated that macrophages and other cells such as endothelial cells, airway smooth muscle cells, and macrophages can produce *IL-8* [329]. The results of our study indicated that the Romanov lambs present high and significant mRNA expression levels differences of *IL-*

8 compared with Akkaraman lambs in exposure to LPS or LTA or a combination of LPS + LTA after 24 h of the treatment. Many studies have indicated the involvement of *IL-8* in most acute and chronic inflammatory diseases and several infections which is acute in nature can alter the hemodynamics and the clotting and fibrinolytic systems in ways that can precipitate ischemic events [330,331].

4.1. Conclusion

In conclusion, sheep are one of the most important animals that use for human consumption and play a significant role in the world industries. So, disease free healthy animals are the premium choice of farmers and producers in terms of economic point of view. Obviously, *in vitro* study clearly showed that stimulation of alveolar macrophages with LPS, LTA and with the combination of LPS + LTA in 4 h trial presented higher and significant mRNA expression of *TLR2*, *TLR4*, *NF-κB, IL-1β*, *IL-6*, *IL-8*, *IL-10* and *TNF-α* than 24 h trial. On the other hand, *in vivo* study showed that the exposure Romanov lambs to LPS, LTA and with the combination of LPS + LTA treatments for 24 h presented higher and significant gene expression levels of *TLR2*, *TLR4*, *NF-κB*, *IL-1β, IL-6, IL-8, IL-10, and TNF-α* than Akkaraman lambs. Indeed, Akkaraman lambs showed more for diseases resistant than Romanov lambs and that was clear from the differences between their gene expression levels.

Overall, the evidence from our study suggests that Akkaraman lambs as it's a native Turkish sheep breed showed higher resistant to respiratory diseases than Romanov lambs, this was clear from the higher and significant mRNA expression levels presented in Romanov lambs in response to all treatments compared with Akkaraman lambs after 24 h of treatments. Finally, having knowledge of respiratory diseases in molecular and genetic level highlighted in this study will enable researchers to identify underlying immunogenetic mechanisms associated with resistance to respiratory diseases that could enhance the effectiveness of selection for sheep against respiratory diseases.

4.2. Suggestions for Future Research

Based on the results of our research, we suggest some future possible research ideas. Although there is some research published in the scientific journals that investigated the effects of LPS or LTA in other domestic animals including cattle and pig, there is no substantial study which can be used to understand the molecular mechanisms of the immune response against Gram-positive or Gram-negative bacteria the alveolar macrophages of sheep. This is the first study that investigated the effects of LPS or LTA or a combination of LPS+LTA in inducing the immune response in the alveolar macrophages in both *in vitro* and *in vivo* condition. This study provides substantial information about the expression of several immune-related genes in both conditions. In addition, the effects of these chemicals have not been investigated in two different breeds of sheep. Therefore, to our information, the current study is the first to provide insight into the *in vitro* and *in vivo* effects of LPS or LTA or co-stimulation using LPS + LTA in innate immune responses in the AMs cells in two different sheep breeds. Although our study gives substantial information about the genetics of immune response against two types of bacterial agents in the alveolar macrophages of two different breed in both *in vitro* and *in vivo* condition, there is still room to investigate further in order to understand the complete innate immune mechanism in the sheep lungs. Therefore, we suggest that the following area can be investigated in future studies-

- Investigation of the expression of candidate immune-related genes in different time points between 2 and 24 h of the exposure with LPS or LTA or a combination of LPS + LTA.
- Investigation of the whole TLR pathway against Gram-positive and gram-negative bacteria.
- Investigate the role of TLRs pathway signaling molecules, cytokine production and the effect of pathogen-associated molecular patterns (PAMPs) on AMs cells in response to LPS, LTA and the combination of LPS + LTA in different time points.

- Finally, and most importantly, we want to suggest a study investigating the whole genome and miRome to identify suitable molecular targets that can be used as markers in the selection of disease resistant sheep.

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CURRICULUM VITAE

PERSONALITY: -

Full name: AHMED QASIM NAJI

Surname: NAJI

Date of Birth: **17/10/1973**

Relationship Status: Married and I have three children (one boy and two girls)

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ACADEMIC ACHIEVEMENTS:-

2019: Erciyes University / Ph.D. / Turkey/English Program

2013: The University of Western Australia/ Master's degree/ Australia

1997: Baghdad University/ bachelor's degree in Agricultural Science/ Iraq

1991: AL- Fayhaa High School/ Highschool/ Iraq/ Babylon Province

Specialization: In Animal Science/ Animal Breeding and Genetics

WORK EXPERINCE: -

2014: Improve the preference of dairy cows and ewes' production- the Babylon Research Station- Province of Babylon.

2013: The director of agricultural equipment- Province of Babylon.

2012: The director of the Studies and Research division in the directorate of cultivation of Babylon- Province of Babylon.

2009-2012: I was doing my Master's degree in the University of Western Australia/ Australia/ Perth.

2007-2009: An employee in an animal resource department- project section in the directorate of cultivation of Babylon- Province of Babylon.

2004- 2007: The director of agricultural and guidance in Al- Muradiya- the Agriculture and training and extension- Province of Babylon.

2002- 2004: An employee in Babylon Agriculture Directorate-department of guidance and Agriculture Corporation- Province of Babylon.

Spoken languages: -

Arabic: - Reading - Talking - Listening - Writing

English: - Reading - Talking - Listening - Writing

Turkish: - Under Learning Courses

Experiences, certifications and training courses:-

1. Training course to improve the performance of dairy goats/ Baghdad.
2. Training course on feed conversion efficiency in the dairy cows/ Baghdad.
3. Training course in Genetic Engineering and Biotechnologies/ Baghdad.
4. Training course in animal breeding under intensive feed system/ Babylon.
5. Training course in internet –local ruling support project/ Babylon.
6. Training course in first safety- engineering college- Sultanate Oman.
7. Training course in guidance, participation of ARDI Organization.
8. Training course for guidance -Irbil.
9. Training course in leading and learning- Irbil.

Social activities:

1. A member in agricultural engineers' association
2. A Member of the Iraqi Red Crescent Society - Babylon Branch
3. A member of university teachers and academics gathering in Babylon

ACADEMIC PARTICIPATION

1. I have been choosing as one of the best students at the University of Western Australia/ Australia and was published under link (https://www.youtube.com/watch?v=QfEB-r0i0D0)
2. Participated in the international Symposium held at the Erciyes University in May 2017. (Genetic Analysis of Brown Hare Populations from Iran and Iraq Based on Mitochondrial COXI Sequences)

HOBBIES:

- Read books and articles
- Play soccer
- Swimming

REFERENCES:-

Available on Request

Interesting in:-

Genetics, Animal Breeding, Immune System