Immunohistochemical evaluation of IFN-γ levels in sheep verminous pneumonia

ABSTRACT

Lungworms, a group of parasitic nematodes, are recognized as one of the major and most common parasitic pneumonia agents in ruminants worldwide. In this study, the expressions of interferon gamma (IFN-γ), which is an important pro-inflammatory cytokine, were evaluated by immunohistochemical methods in order to evaluate the immune response against parasitic agents in sheep naturally infected with different types of lungworms. The material for this study consisted of lung tissue samples obtained from 40 dead sheep brought for routine histopathological diagnosis to the Department of Pathology. In order to reveal the histopathological changes in the tissues, Hematoxylin and Eosin (H&E) staining was applied to the sections. Lung tissues were stained with IFN-γ commercial antibody using the Avidin-Biotin Peroxidase Technique (ABC) following the procedures of the manufacturer. Subpleural multifocal nodules of several mm in diameter were detected in the dorsal regions of the lung, especially in the caudal lobes. In the histopathological examination of the lungs, it was observed that the alveoli, bronchi, and bronchiole lumens were filled with adult forms, larvae, and eggs of the parasitic agents. Compared to the control group, the expressions of IFN-γ were significantly increased in the verminous pneumonia group. Overall, the study suggests that the Th1 response, as represented by increased IFN-γ expression, appears to play an active role in the immunity developed against lungworms in ruminants.

Keywords: Cytokine, IFN-γ, lungworms, sheep, verminous pneumonia

INTRODUCTION

Sheep pneumonia is one of the multifactorial problems that can be seen in sheep of all ages around the world and causes very important economic losses. Among the causes of sheep pneumonia, in addition to viral, bacterial, and fungal agents, verminous pneumonia caused by lungworms has remarkable importance (Can et al., 2018). Lungworms, a group of parasitic nematodes, are recognized as one of the major and common parasitic pneumonia agents for ruminants worldwide (Asmare et al., 2018; Fesseha et al., 2021).

Several species belonging to the superfamily Metastrongyloidea (*Muellerius capillaris*, *Prostrongylus rufescens*, *Cysocualus ocreatus*, *Neostrongylus linearis*, etc.) and Trichostrongyloidea (*Dictyocaulus viviparus* and *Dictyocaulus filaria*, etc.) can infect domestic animals such as cattle and small ruminants (de Macedo et al., 2021; Zafari et al., 2022). Small lungworms infect sheep by damaging the lung parenchyma and bronchioles. Species such as *Muellerius capillaris* and *Prostrongylus rufescens* cause a wide range of inflammatory responses and chronic eosinophilic granulomatous pneumonia (Hanks et al., 2021; Jabbar et al., 2013).

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Research Article

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Dictyocaulus filaria lives in the trachea, bronchi, and bronchioles and causes bronchitis (dictyocaulosis, also known as husk) in young animals (Mangiola et al., 2014). Infections involving multiple agents rather than a single species in the same lung are more common (Rehbein et al., 2022; Rehbein and Visser, 2002).

Clinical symptoms such as mucus discharge, cough, and tachypnea are seen in sheep infected with lungworms. Death occurs mostly as a result of severe infections. In addition, secondary complications in the lung cause bacterial pneumonia, weight loss, and low milk yield, especially in young animals (Kırcalı Sevimli et al., 2011).

Lungworms are found in regions with cold temperatures and heavy rainfall (Papadopoulos et al., 2004). Season, species, age, gender, irrigation, and grazing management are important factors affecting the risk of lungworm infection (Hanks et al., 2022).

It is known that cytokine responses that occur when a pathogen is encountered are very important in inducing and regulating immune reactions (Holmgren et al., 2014). Interferon Gamma (IFN-γ) produced by macrophages, dendritic cells, natural killer cells (NK), $CD4^+$ T helper cells (Th1) and a subset of CD8 + T cells, is a highly active pro-inflammatory cytokine (Kak et al., 2018; Samar et al., 2017). IFN-γ plays a central role in the inflammation and the induction of cell-autonomous immune responses (Beytut et al., 2011; Sasai and Yamamoto, 2019). IFN-γ acts by activating macrophages and increasing their phagocytic capacity (Fleury et al., 2018).

In this study, the expressions of IFN-γ, which is an important pro-inflammatory cytokine, were evaluated by immunohistochemical methods in order to evaluate the immune response against parasitic agents in sheep naturally infected with different types of lungworms.

MATERIALS AND METHODS

Animals

The material for this study consisted of lung tissue samples obtained from 40 dead sheep (average 4-5 years old, female, Morkaraman breed) brought for routine histopathological diagnosis to the Department of Pathology, Faculty of Veterinary Medicine of Kafkas University. According to the anamnesis information obtained from the owners, it was observed that the animals exhibited various clinical signs such as cough, nasal discharge, and loss of appetite and weight. The lung tissue samples from 10 healthy sheep (average 4-5 years old, female, Morkaraman breed) without any pathological lesions were also used for control purposes.

Histopathological examinations

Lung tissue samples from the sheep were fixed in a 10% formaldehyde solution. Serial sections of 5 µ thickness were taken from the paraffin blocks prepared following the routine tissue follow-up procedures. In order to reveal the histopathological changes in the tissues, Hematoxylin and Eosin (H&E) Staining was applied to the sections. The prepared sections were evaluated in detail by at least two pathologists under the light microscope, and the detected lesions were photographed.

Immunohistochemical examinations

Serial sections of 4 µ thickness taken from the paraffin blocks prepared from lung tissues were stained with IFN-γ commercial antibody using the Avidin-Biotin Peroxidase-Technique (ABC) following the procedures of the manufacturer. Information about the primary antibody used in the study is detailed in Table 1. Thermo Scientific Histostain IHC Kit (HRP, broadspectrum, REF: TP-125-HL) was used to

conduct all immunostainings. The color-revealing substrate, 3.3-diaminobenzidine tetrahydrochloride (DAB) solution (Thermo Scientific, REF: TA-125-HD), was dropped onto the sections and incubated for 15 minutes. The sections were washed with distilled water for 5 minutes, stained with Mayer Hematoxylin, and coated with Immu-mount.

Table 1. Information on primary antibodies used in immunohistochemical studies

Primary Antibodies	Pretreatment	Company and Catalog Numbers	Dilution	Incubation Condition
IFN-γ	Open Microwave	MyBioSource, MBS2091397, Polyclonal	1/100	Overnight, 4°C

After bloating, the prepared sections were evaluated under a light microscope (Olympus Bx53) and photographed using the Cell ^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyses of the captured photographs were made with the Image J program (1.51j8, Public Domain, Maryland, USA).

The evaluation of IFN-γ staining results was made with a grading system based on the number of positive cells in the areas examined with immune-positive reactions and reflecting most strongly the staining character. Three different fields from each case were examined with a 40x objective. The numbers of positively stained cells were recorded separately, and the average of these 3 fields was considered the average positive cell number of that animal. Scoring: (-) no immunoreactivity; (+) weak, 1-10% positivity; (++) moderate, 11–59% positivity; and (+++) severe positivity over 60% (Karakurt et al., 2023).

Statistical analysis

The program SPSS® (SPSS 26.0, Chicago, IL, USA) was used for the statistical analysis of the results. The Mann-Whitney U Test was used for the pairwise comparison of the control group and the verminosis group. The obtained results were given as the mean ± standard error (SE). In the evaluation of the results, the expression $P<0.05$ was considered statistically significant.

RESULTS

Macroscopic findings

Subpleural multifocal nodules of several mm in diameter were detected in the dorsal regions of the lung, especially in the caudal lobes. It was observed that the consistency of the nodules was soft in some cases and quite hard in others. The nodules were mostly gray-greenish. In addition, foci of intense bleeding were also found (Figure 1).

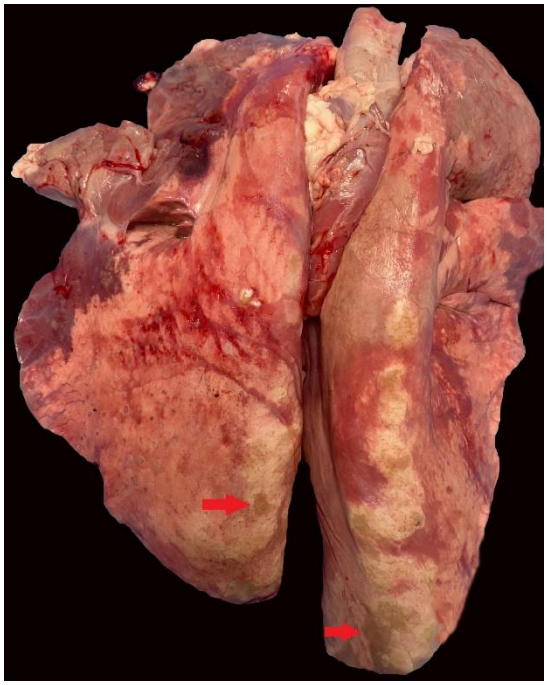

Figure 1. Lung, numerous grey-greenish-appearing nodules of varying size in the caudal lobes (red arrows).

Microscopic findings

No significant pathological changes were detected in the lung tissues of the control group (Figure 2a). In the histopathological examination of the lungs with verminous pneumonia, it was observed that the alveoli, bronchi, and bronchiole lumens were filled with adult forms, larvae, and eggs of the parasitic agents. Although a single type of parasitic agent was found in some cases, mixed infections were dominant in

most cases. In the lungs, parasitic granulomas consisting of agents and necrosis in the middle and foreign body giant cells, eosinophils, mononuclear cells, and a fibrous capsule localized around them were detected. It was observed that interalveolar septa were thickened due to connective tissue and inflammatory cell infiltration. Hyperplasia was observed in the bronchial and bronchiolar epithelium. The muscle layer around the bronchi and bronchioles was found to be hypertrophic (Figures 3-4-5).

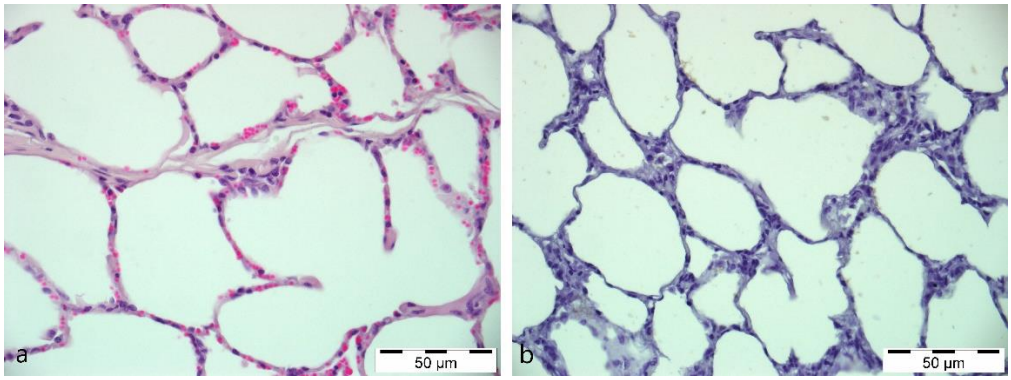

Figure 2. a: Lung, H&E, Control group, **b:** Lung, Immunohistochemistry, Control group.

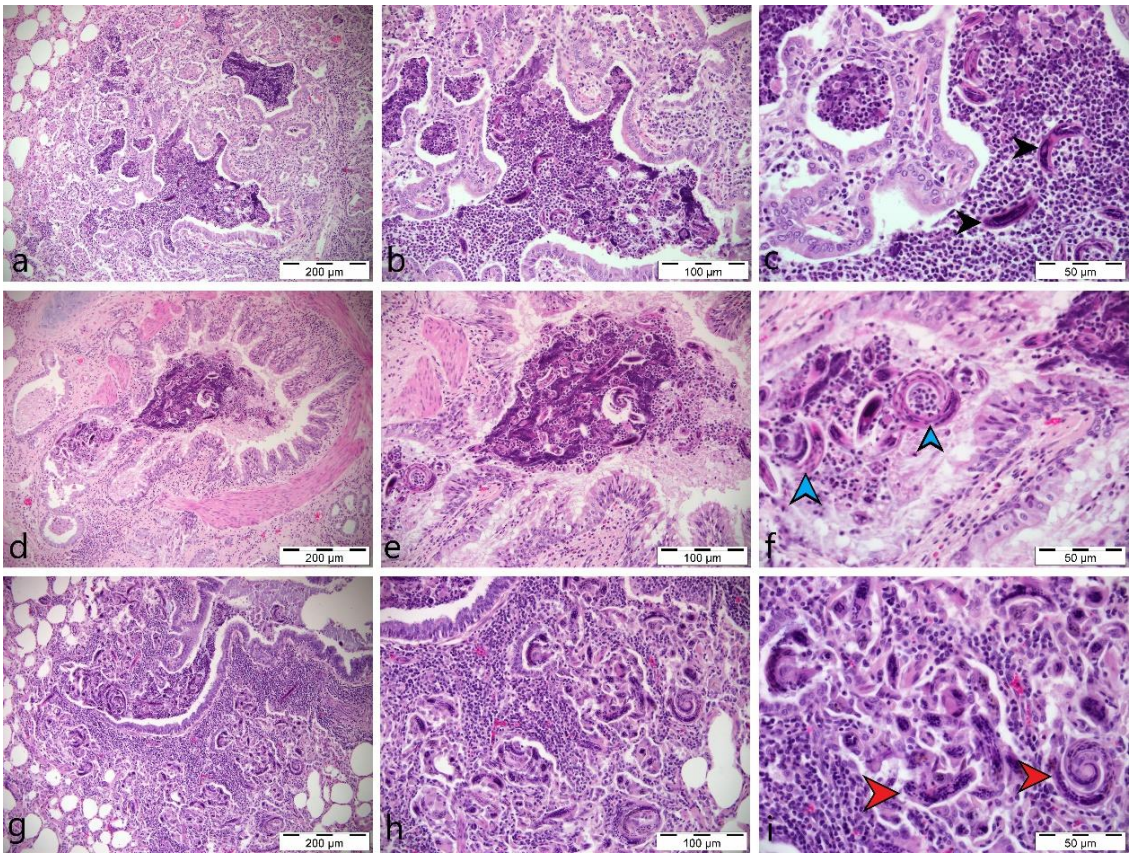

Figure 3. Lung, H&E, **a-b-c:** Appearance of the parasitic agents in the alveolar lumens (black arrowheads) in different magnifications, **d-e-f:** Appearance of parasitic larvae and eggs in the bronchial lumen (blue arrowheads) in different magnifications, **g-h-i:** Appearance of intense parasitic infiltration in the bronchiole lumens (red arrowheads) in different magnifications.

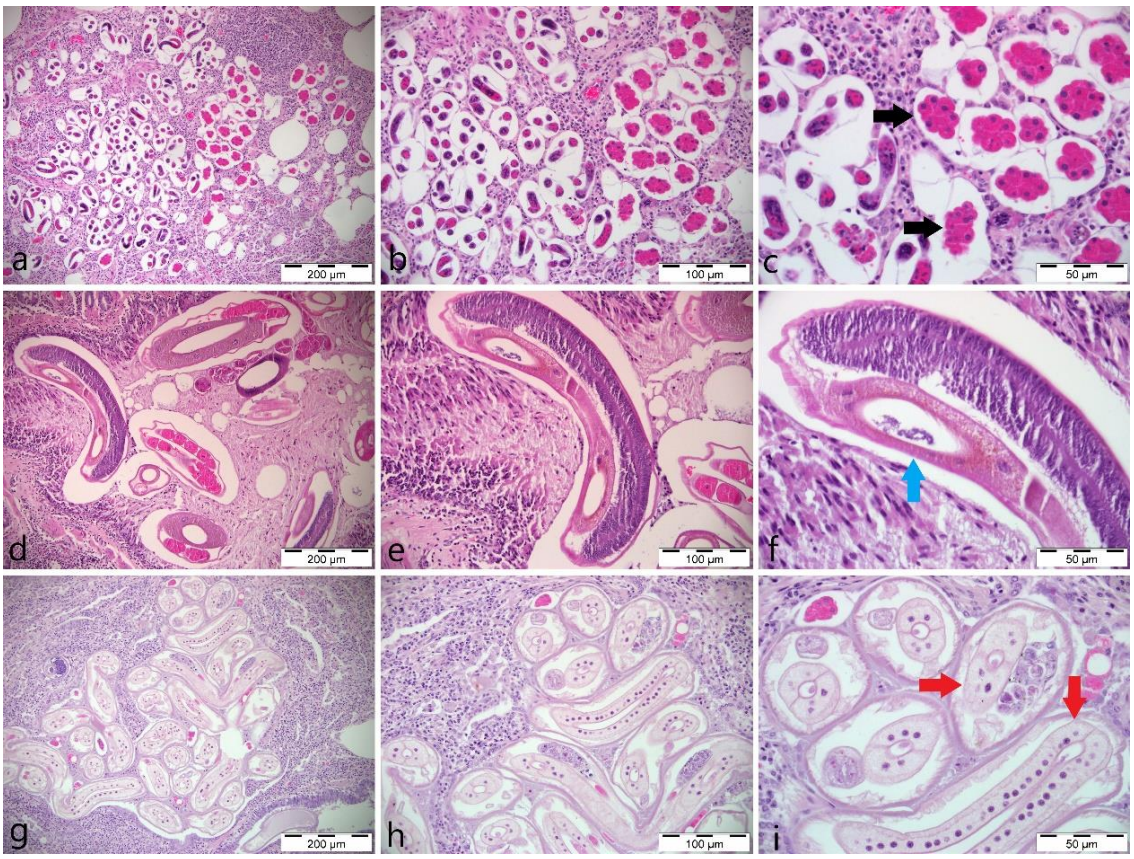

Figure 4. Lung, H&E, **a-b-c:** Appearance of the parasites located in the alveoli (black arrows) at different developmental stages and in different magnifications, **d-e-f:** Appearance of quite large parasites in the bronchial lumen (blue arrow) in different magnifications, **g-h-i:** Appearance of a large number and size of parasitic agents localized in the bronchiole lumens (red arrows) in different magnifications.

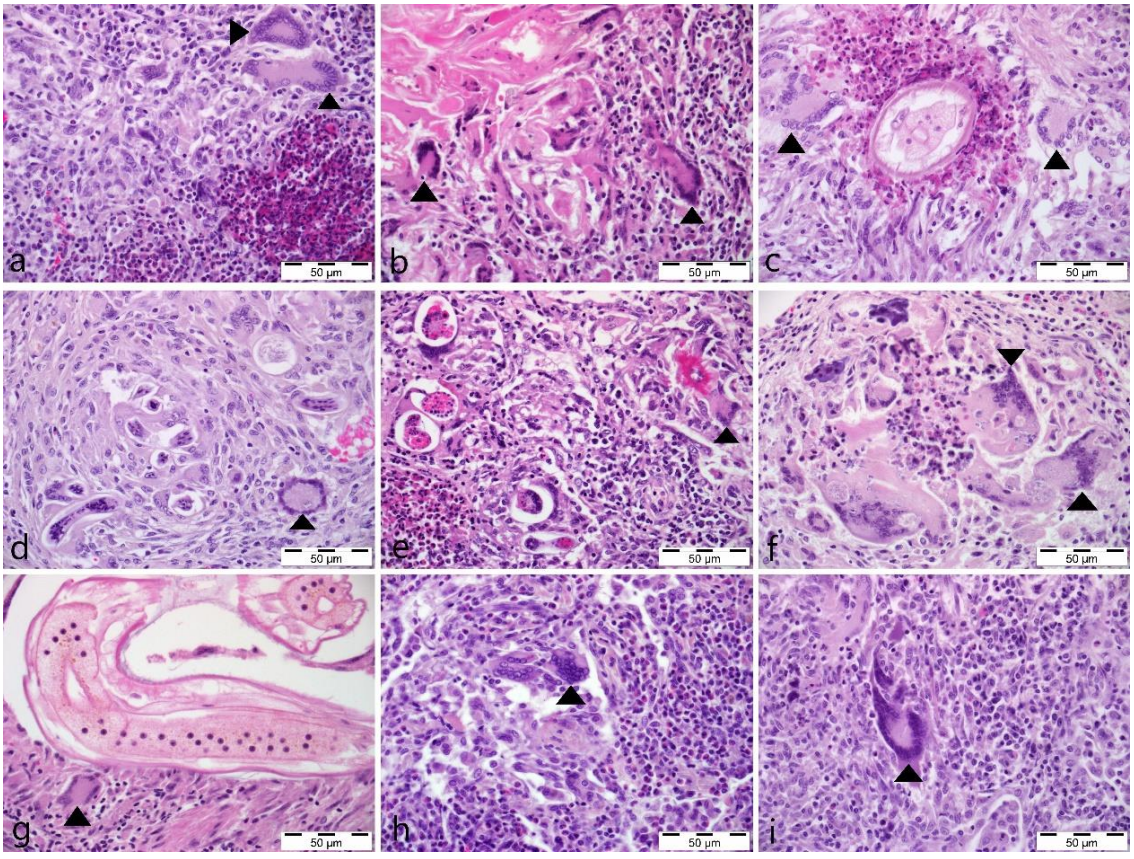

Figure 5. Lung, H&E, a-i: Multinucleated giant cells (arrowheads) localized around different parasitic agents.

Immunohistochemical findings

The immunopositivity scores of the control and verminous pneumonia groups are detailed in Table 2.

Table 2. IFN-γ immunopositivity scores of the groups

Groups	IFN-γ
Control	0.20±0.13
Verminous pneumonia	2.78±0.07
P value	<0.001

Compared to the control group (Figure 2b), the expressions of IFN-γ were significantly increased in the verminous pneumonia group. The IFN-γ positive stainings were mostly concentrated in the multinucleated giant cells localized around the parasitic agents and in the cytoplasm of the mononuclear cells around these giant cells. In addition to these cells, intracytoplasmic IFN-γ expressions were also present in the alveolar macrophages. The IFN-γ positive reactions were granular in form. It was remarkable that IFN-γ immunoreactivity was much more severe in cases with mixed infections compared to cases with a single type of parasitic agent. In cases where parasitic granulomas and the inflammatory cell infiltration were larger, IFN-γ immune-positive staining was equally intense (Figure 6).

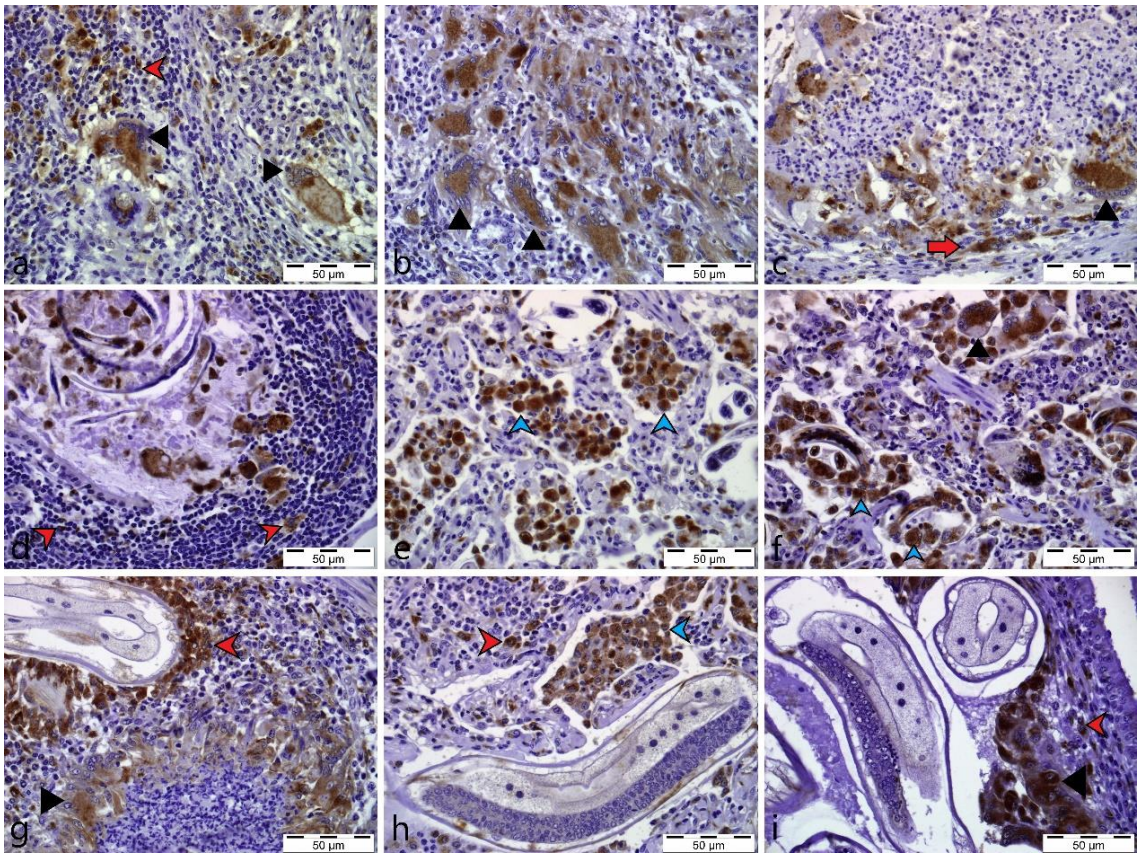

Figure 6. Lung, Immunohistochemistry, IFN-γ, **a-i:** Immune positive staining in multinucleated giant cells (black arrowheads), alveolar macrophages (blue arrows), and mononuclear cells (red arrows).

DISCUSSION

Lungworms cause significant economic losses, which emphasizes the importance of correct diagnosis methods, treatment, and control (Zafari et al., 2022). The clinical symptoms, the post-mortem lesions in the lung, the detection by the Baermann technique of agents in the first stage from fecal samples taken from animals, the FLOTAC and Mini-FLOTAC techniques, ELISA tests and bronchoalveolar lavage are

effectively used in the diagnosis of the disease (de Macedo et al., 2021; Fesseha and Mathewos, 2021; Hanks et al., 2021). In the current study, the macroscopic (Bouljihad et al., 1995; Gulbahar et al., 2009; Hashemnia et al., 2019; Panayotova-Pencheva and Alexandrov, 2010) and microscopic findings (Beytut et al., 2002; Kıran et al., 1993; Sağlam et al., 1998; Ülgen et al., 1997) were consistent with the literature data.

The cellular reaction in sheep to lungworms at various developmental stages includes the mobilization of eosinophils, mast cells, macrophages, and multinucleated giant cells that fill the alveoli, alveolar septa, and bronchioles (Gulbahar et al., 2009; Panuska 2006). Small lungworms cause long-term infections in their hosts, suggesting that the immunity to the disease is not efficiently stimulated (Rehbein and Visser, 2002). There are very few studies evaluating the immune response to lungworms in cattle (Holmgren et al., 2014; Johnson et al., 2005) and sheep (Gulbahar et al., 2009). Johnson et al., (2005) evaluated the mRNA expression of various cytokines such as IL-4, IL-5, IL-10, and IFN-γ in the lung parenchyma, tracheal rings in the bronchial bifurcation, and bronchial and caudal mediastinal lymph nodes on days 15, 22, and 43 post-infection with *D. viviparus*. They reported that the expression of IFN-γ increased due to the infection. Contrary to this study, Holmgren et al., (2014) reported that there was no significant increase in the levels of IFN-γ. Although there are indications that a mixed Th1/Th2 immune response is given with high (Th2-dependent) IgE and eosinophil levels against *D. viviparus* in cattle, detailed information is not available about the immune response against *D. filaria* (Mangiola et al., 2014). In the current study, it was observed that the IFN-γ levels were statistically increased in lung tissue samples from naturally infected sheep with various lungworm types compared to lung tissue samples obtained from uninfected healthy animals. This shows that, in addition to the Th2 immune response, in the immune response against lungworms, the Th1 response is also effective.

The control of parasitic diseases depends on the production of cytokines; the production of cytokines triggers cascade mechanisms, limiting the proliferation, survival, and invasion of parasites (Ram et al., 2020). It is known that the IFN family has important roles in the host immune response against infections, pathogens and various diseases. Parasitic infections in mammals are characterized by increased IFN-γ levels (Kim et al., 2019). In the literature reviews, IFN-γ has been found to have a protective effect against various parasitic infections such as Fasciolosis (Attia et al., 2022), Histomoniasis (Kidane et al., 2018), Coccidiosis (Kim et al., 2019), Leishmaniasis (Kak et al., 2018), Taeniasis (Fleury et al., 2018), Theileriosis (Ram et al., 2020), Toxocariasis (Samar et al., 2017) and Toxoplasmosis (Sasai and Yamamoto, 2019). In this study, it was determined that IFN-γ expressions increased directly proportional to the severity of the inflammatory response, especially against single or multiple types of lungworms. In addition, it was remarkable that IFN-γ immune-positive staining was intensified parallel to the size of the granulomas demarking the parasitic agents. This data also supported the pro-inflammatory effects of IFN-γ.

Interferon gamma is produced by professional antigen-presenting cells such as CD4+ T helper cell type lymphocytes (Th1), CD8+ cytotoxic lymphocytes, NK, monocytes and macrophages, dendritic cells, and B lymphocytes. IFN-γ is an important pro-inflammatory cytokine that plays a major role in the fight against viruses, intracellular bacteria, parasites, and tumors (Manirarora et al., 2022). It is known that IFN-γ supports cell-mediated immunity by activating the phagocytosis capacity of the macrophages (Kidane et al., 2018). The inducible nitric oxide synthetase (iNOS) isoform primarily activates lung macrophages, resulting in the production of nitric oxide (NO) from these cells. iNOS

expression is also induced by several cytokines, such as IFN-γ, IL-1, or TNF-α. In this study, IFN-γ positive staining was predominantly observed in the cytoplasm of multinucleated giant cells and alveolar macrophages. These immunohistochemical findings show that IFN-γ increases the phagocytosis capacity of giant cells and alveolar macrophages in the cellular defense developed against parasitic agents.

CONCLUSION

As a result, this remarkable increase in the levels of IFN-γ in sheep with parasitic infections compared to uninfected sheep reveals that IFN-γ is a very useful marker in the diagnosis of the disease and in determining the severity of the infection. Besides this, as a contribution to the literature data on the immunity developed against lungworms in ruminants, it is observed that, in addition to the Th2 response, the Th1 response plays an active role in IFN-γ, which is an important pro-inflammatory cytokine. In this respect, it has been concluded that the data obtained from the current study will contribute to the immunology of lungworms in sheep.

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Conflict of interest: The authors declared that there is no conflict of interest.

Ethical statement: The study was approved by the Local Ethics Committee on Animal Experiments of Kafkas University (KAU-HADYEK-2022/205).

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