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Original Article

Immunoregulatory Effects of Somatic Extract of *Toxocara canis* on Airway Inflammations in Murine Model

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Abstract

Background: The immunomodulatory role of many parasites is well-documented. The current study designed to assess the immunoregulatory effects of the somatic extract (SE) of *Toxocara canis* on murine model of airway inflammations.

Methods: The experiment was performed in department of parasitology of Tarbiat Modares University, Tehran, Iran from November 2018 to May 2019. Totally 30 female BALB/c mice divided into one control group and two experimental groups (10 mice in each group). The ovalbumin (OVA) group was sensitized with OVA in alum, while the SE group was administered with SE and OVA in alum intraperitoneally. The control group was injected with PBS in alum. Then, SE and OVA groups were intranasally challenged with OVA for three consecutive days and the control group encountered with PBS at the same time. One day after the last challenge, real-time PCR and histopathology survey were conducted on isolated lung tissues.

Results: The gene expression of IL-25, IL-33, TNF- α and TLR-4 in SE group was significantly lower than OVA group ($P < 0.05$). The level of IL-10, TGF- β and IFN- γ were considerably higher than the OVA group ($P < 0.05$). The inflammation was reduced in SE group, as the total cell number of bronchoalveolar lavage fluid was less than OVA group. Based on the histopathology findings the inflammation was decreased in SE group compared to the OVA group.

Conclusion: Although, an inhibitory effect of SE of *T. canis* on airway inflammations was detected, there is still a long way ahead regarding the indication of the precise mechanisms.



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Introduction

Toxocariasis is a cosmopolitan neglected tropical disease which is common around the world and it is considered as a public health problem. Nematodes of the family Toxocaridae including *Toxocara canis*, *T. cati* and *Toxascaris leonina* are the main parasitic agents and carnivores specially cats, dogs and jackals are the definitive hosts for this infection (1).

Eggs are excreted in the feces of the main host and then they develop under optimal conditions. After the ingestion of embryonated eggs by human, larva do not turn into the mature stage, and they can cause to different kinds of clinical signs such as ocular larva migrans syndrome (OLM), visceral larva migrans syndrome (VLM), neurological toxocariasis (NT), and covert toxocariasis in human (2–4).

Airway inflammations are common around the world and approximately 400 million people are affected by them. They include different types of disorders such as asthma, chronic obstructive pulmonary disease and also chronic rhinosinusitis (5). The typical characteristics of airway inflammations and Helminthic Infection are high level production of Th2 cytokines (Interleukin-4 (IL-4), IL-5, IL-10, IL-13, IL-25 and IL-31), eosinophil infiltration, growing level proliferation of CD4+ T cells and expanded production of IgE, resulting in tissue damage (6–9).

The role of helminthic infections in modulation of immune responses have been revealed via investigations of many autoimmune and inflammatory diseases such as Crohn's disease, inflammatory bowel disease (IBD), multiple sclerosis (MS), rheumatoid arthritis (RA), Type 1 diabetes, malignancies and airway inflammations. In addition, the potential role of helminthic infections in modulation of Th2 responses in some allergic disorders have been discovered in previous studies (10–12).

Helminth therapy is currently known as a new treatment approach for a broad range of

airway inflammations. According to the previous study on individuals who were infected with helminthic infections during childhood, helminthic infections have a prolonged maturation and often occur chronically, as a results of which they can optimize the Th1 and Th2 responses (13).

However, existing disputes about the immunoregulatory role of *T. canis* in airway inflammations, motivated us to design the present study to evaluate the expression of IL-25, IL-33, thymic stromal lymphopoietin (TSLP), tumor necrosis factor alpha (TNF- α), toll-like receptor-4 (TLR-4), IL-10, transforming growth factor β (TGF- β) and Interferon- γ (IFN- γ) cytokines in the lung tissue of murine model of the airway inflammations encountered with somatic extract (SE) of *T. canis*.

Materials and Methods

Preparation of T. canis somatic extract

The current study was conducted in department of parasitology of Tarbiat Modares University, Tehran, Iran from November 2018 to May 2019. Helminths were collected from the intestine of road-killed stray dogs and the morphological structures were recognized under a light microscope. Subsequently they were washed several times and homogenized with sterile phosphate-buffered saline (PBS) containing antibiotics penicillin and streptomycin (Gibbco: BL1036). A tissue homogenizer with an ice bath were used to fragment the helminths. They were then exposed to ultrasonic waves and centrifuged at 10000 rpm for 30 min (14). The supernatants were isolated and passed through a 0.2 μ m filter. The Bradford assay was conducted to determine the total protein concentration. Ultimately, the obtained extract was stored at -80 °C.

Animal model for induced airway inflammation

A total of 30 female pathogen-free BALB/c mice aged 6–7 wk were considered and purchased from the Razi Vaccine and Serum Research Institute (Karaj, Iran). To evaluate the effect of somatic extract (SE) of *T. canis* on allergic airway inflammations, mice were divided into one control group and two experimental groups (ten mice in each group). All procedures were approved by the Ethics Committee for Animal Experiments, Tarbiat Modares University, Tehran, Iran (ID:IR.TMU.REC.1396.565).

Airway challenge with ovalbumin and immunization

The first sensitization was performed via intraperitoneal injection (IP) for all groups of mice in days (0, 2, 4, 6, 8, 10, 12 and 14). In

summary, the Ovalbumin (OVA) group, was administered with 10 mg/ml of OVA (Grade V; Sigma Aldrich, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Alum; Sigma Aldrich). The SE group was immunized with 20 µg SE of *T. canis* along with 10 mg/ml OVA in 1mg of alum and the control group was injected with 0.5 ml PBS in 1mg of alum. Forty days after the first sensitization, the mice in groups of OVA and SE were encountered with 2, 20 and 200 µg dosage of ovalbumin in 20 µl saline intranasally for three days apart. The control group were exposed with PBS intranasally at the same intervals and 24 h after the last challenge all groups of mice were euthanized with CO₂ gas (Fig. 1).

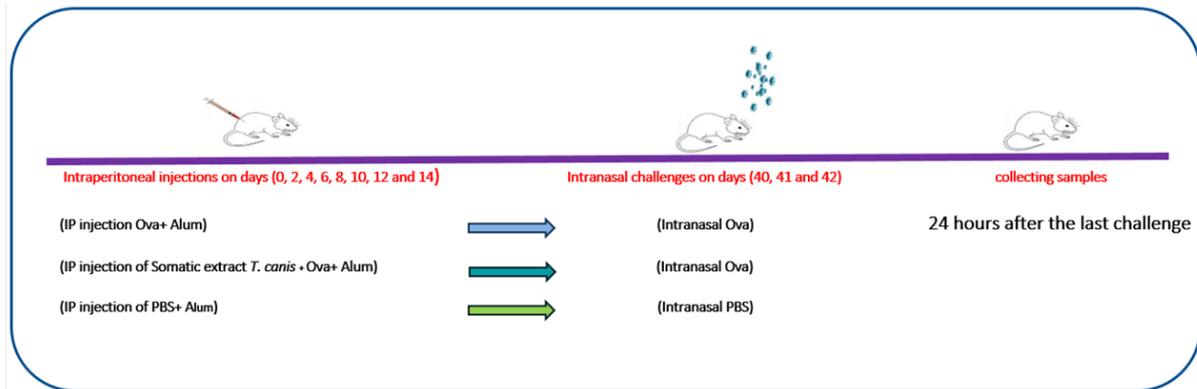


Fig. 1: The schematic protocol for the development of allergic airway inflammations in mouse model

Histopathological determination of lung inflammation

For histopathological evaluation, lungs were removed and divided in two sections. A piece of the right lung tissue was stored at –80 °C to preserve RNA integrity and then prepared for qPCR. The remaining lung tissue was fixed in 10% buffered formalin for 24h at room temperature, embedded in paraffin and sectioned into 5µm slices. The Lung sections were stained with hematoxylin & eosin (H&E) according to routine procedures. The intensity of inflammatory cells infiltration as well as goblet cells metaplasia was evaluated by a his-

topathologist and scaled to four scores with 0 as “no cell infiltration, no activated goblet cells”, 1 “mild”, 2 “moderate”, and 3 “severe bronchial cellular infiltration” (15).

The isolation of bronchoalveolar lavage fluid (BALF)

The samples of BALF were isolated about 24 h after the last exposure. The total cell counts in BALF samples evidenced the pathological disorders in allergen-sensitized mice in the experimental groups. The mice were sacrificed with CO₂ gas and their lungs were cannulated for extraction of cells from BALF.

The BALF samples were obtained by injecting 1 ml of PBS into the tissue of lungs. They were then stained with Giemsa-Wright (Sigma, Germany) and the total cell count was determined with haemocytometer.

RNA Isolation and cDNA Synthesis

Briefly, the lung tissue specimens were directly lysed with 100 µl of Trizol Lysis Reagent and total RNA was isolated according to manufacturer's instructions (Invitrogen, Karlsruhe, Germany). A NanoDrop™1000 spectrophotometer (Thermo Fisher Scientific, USA) was used to quantify extracted RNA. The synthesis of cDNA was carried out based on manufacturer's protocols (GeneALL, Korea).

Quantitative Real-Time PCR Assays

Real-time PCR assays were performed using a RotorGene 6000 instrument (Corbett life science, Sydney, Australia). The expression level of IL-10, TGF-β, TNF-α, TLR-4, IL-25, IL-33, TSLP and IFN-γ was monitored by qPCR. The reaction mixture (25 µl) contained 20-50 ng of total RNA-derived cDNAs, 1000 Nm from each of the forward primer and reverse primer. Some of the primers were designed by the authors and the rest were taken from literatures (Table 1). 12.5 µL of RealQ Plus 2X MasterMix (SYBR Green without ROX, AMPLIQON). The thermal cycle conditions were as follows: 15 min at 95 °C, followed by 40 cycles at 95 °C for 15s, 60 °C for 30s and 72 °C for 30s. The ΔΔCT method of relative quantification was used to determine the fold change in expression (REST 2008 software).

Table 1: Set of primers and probes used in this study to measure pro-inflammatory and anti-inflammatory cytokines

Target gene	Amplicon size(bp)	Sequence(3'-5')	Direction	References
TSLP	128	CAATCCTATCCCTGGCTGCC TGTGCCATTTCCCTGAGTACCGT	Forward Reverse	This primer was designed by the authors
IL-33	132	TTGGCTTACGATGTTGTGGA AGGAAAGAGCAAGGCTGAAT	Forward Reverse	This primer was designed by the authors
IL-25	108	GGCATTCTACTCAGGAACGGA GGTGGAGAAAGTGCCTGTGC	Forward Reverse	This primer was designed by the authors
TNF-α	175	CATCTTCTCAAAATTCGAGTGACAA TGGGAGTAGACAAGGTACAACCC	Forward Reverse	(26)
IL-10	92	GCGCTGTCATCGATTTCTC GACACCTTGGTCTTGGAGCTTATTAA	Forward Reverse	(26)
TGF-β	101	TGTCACAACCTCAGCCAACAGG CAACCAGCCTCCTAAACACCC	Forward Reverse	(27)
TLR-4	201	ACCTGGCTGGTITACACGTC CTGCCAGAGACATTGCAGAA	Forward Reverse	(26)
IFN-γ	85	CCTGAAAGAAAGCAGTGTCT TTTGTCAITTCGGGTGTAGTC	Forward Reverse	(28)
Mouse GAPDH	102	ATGGACTGTGGTCATGAGC ATTGTCAGCAATGCATCCTG	Forward Reverse	(26)

Statistical analysis

Statistical analysis was performed using SPSS (Chicago, IL, USA) Statistics 22 software. All the results were represented as the means±SD. The one-way analysis of variance (ANOVA) was used followed by post hoc Dunnett's test to determine the significant differences among all groups. *P*-values of less than 0.05 were considered to be statistically significant.

Results

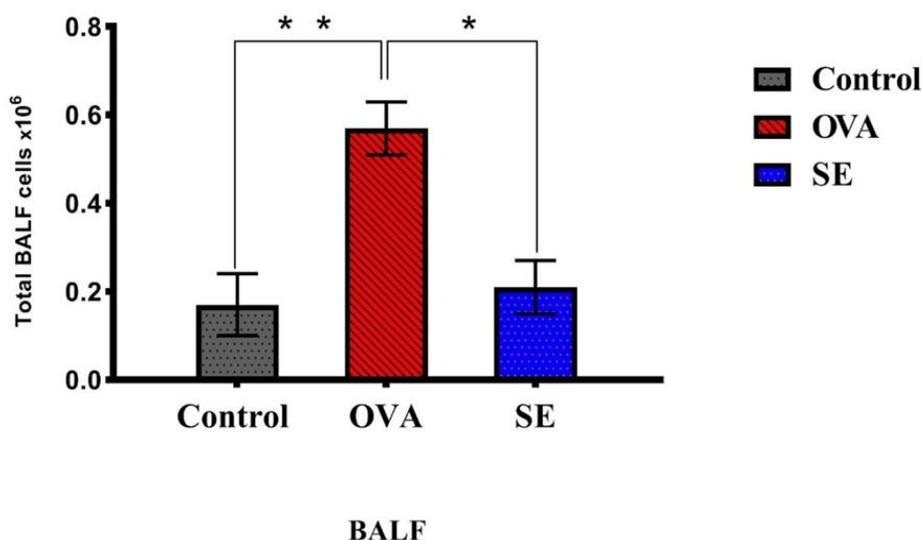


Fig. 2: The increased number of eosinophils was significant in BALF samples of OVA group. **P* < 0.05 OVA versus all other groups
OVA: Ovalbumin, SE: Somatic extract

Histopathological findings

Different pathological changes were observed in all experimental groups of the present study.

After 24 h of challenge with OVA, an allergic airway inflammation was developed in mice which was characterized by intense bronchial cellular infiltration along with predominant eosinophils in lungs tissue. In the SE group, reduced inflammatory cells infiltration and a mild hyperplasia of goblet cells was detected. The infiltration of inflammatory cells

Survey of BALF

OVA challenged group showed the highest total number of cells which can be explained by the high number of eosinophils, the intense secretion of airway mucus as well as infiltration. In SE group, low numbers of eosinophils were detected in BALF, even after challenging with OVA, revealing that SE group presented no eosinophilia in the airway. The count of eosinophils was significantly reduced in SE group (*P* < 0.05 by one-way ANOVA test) (Fig. 2).

was considerably normal in the control group, while it was developed in the OVA group with a high level of metaplasia of goblet cells.

The lung pathology was observed in PAS-stained sections of all the experimental groups. OVA group showed pathological signs of pulmonary allergic inflammation compared to *T. canis* SE and control groups. These changes included eosinophil and mononuclear cell infiltration around airways and vessels, goblet cell metaplasia as well as mucus secretion in bronchi (Fig. 3).

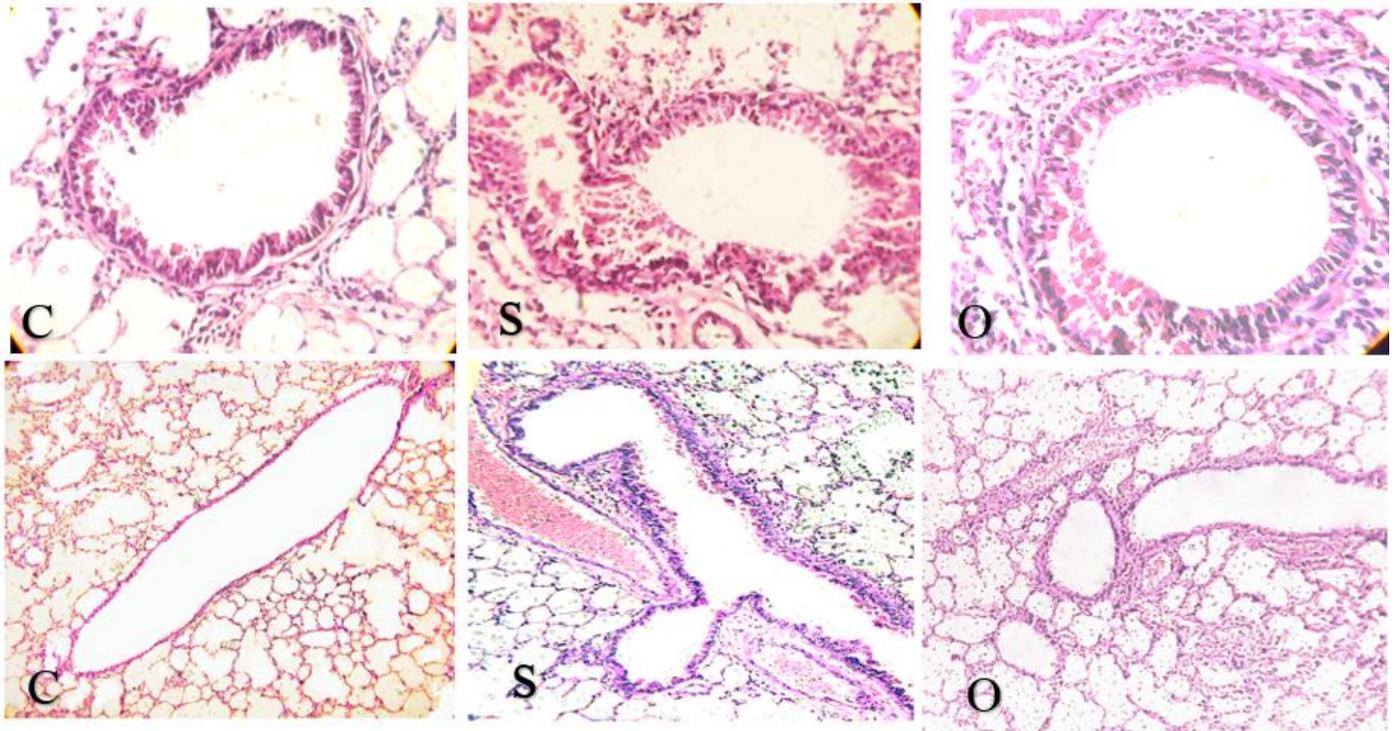


Fig. 3: Histopathology of lung in different groups: Their Upper photos are with magnification of X100, and lower ones with magnification of X40.

- C: Control group (no infiltration of inflammatory cells)
- S: SE group (mild cell infiltration and activated goblet cells)
- O: Ovalbumin group (severe bronchial cellular infiltration)

The expression of cytokines in lung epithelial tissue of mice

Real-time quantitative PCR (qPCR) was conducted on cDNA libraries to determine the expression of IL-25, IL-33, TSLP, TLR-4, TNF- α , IL-10, TGF- β and IFN- γ cytokines in lung specimens of mice from all experimental groups. Our results showed that OVA upregulated the gene expression of IL-25, IL-33, TLR-4, TNF- α and TSLP, but it downregulated the gene expression of TGF- β , IL-10 and IFN- γ in OVA group ($P < 0.05$). However, SE downregulated the expression of IL-25, IL-33, TNF- α , TLR-4 and TSLP, nevertheless it upregulated the gene expression of IL-10, TGF- β and IFN- γ in SE group ($P < 0.05$). In spite

of the low level of the gene expression and reduction of TSLP in SE group, there was no significant difference observed between OVA and SE group (Fig. 4 and 5).

Based on the data provided in Table 2, the lowest level of the gene expression in SE group was related to IL-25, but this amount was the highest in OVA group. Our results showed that the gene expression of TGF- β , IL-10 and IFN- γ was increased in SE group in comparison with the OVA group ($P < 0.05$). The highest gene expression in SE group was related to the IL-10, TGF- β and IFN- γ . While the lowest level of gene expression of IL-10, TGF- β and IFN- γ was detected in OVA group (Table 2).

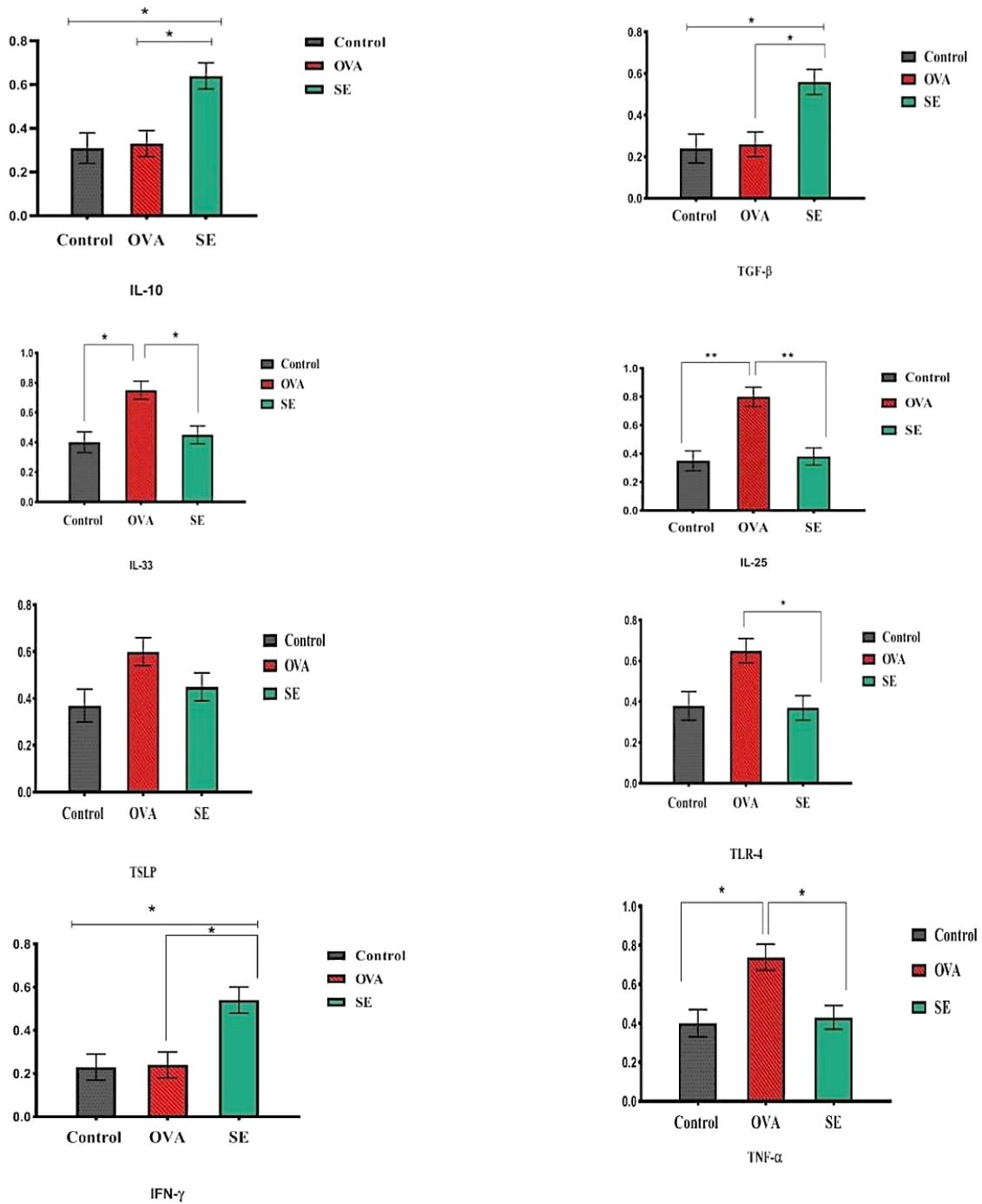


Fig. 4: The analysis of cDNA by qPCR to determine the expression of IL-25, IL-33, TSLP, TLR-4, TNF- α , IL-10, TGF- β and IFN- γ cytokines. The *P*-value is represented in three experimental groups (**P* < 0.05; ***P* < 0.01; ****P* < 0.001)

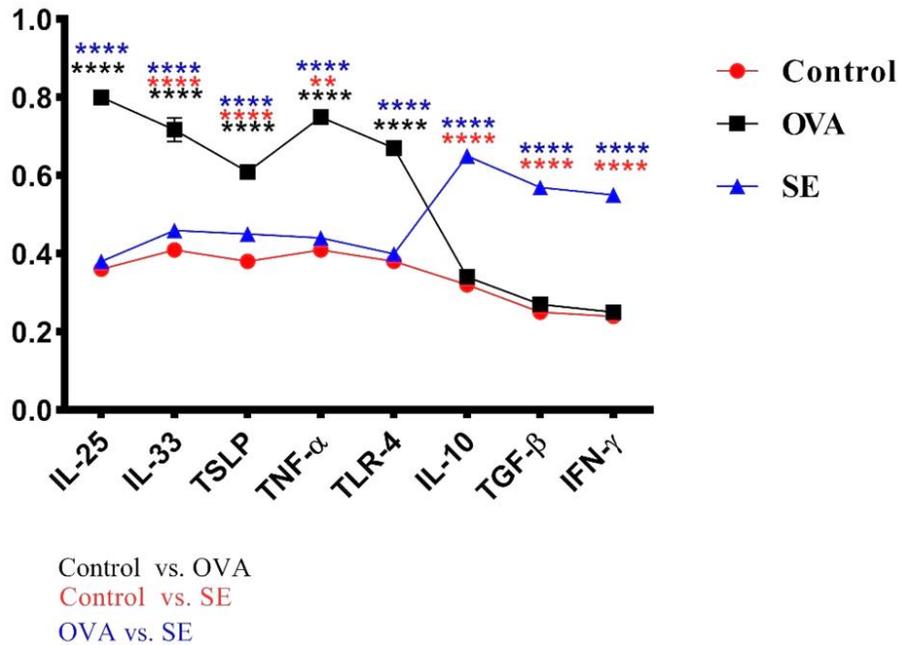


Fig. 5: Comparison of gene expression in all groups; each index is represented with assigned color in legend (it was considered statistically significant when * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$)

Table 2: Cytokine production in all groups of mice

Variable	Ovalbumin (Mean±SD)	Somatic extract (Mean±SD)	Control (Mean±SD)
IL-25	0.8±0.01	0.38±0.01	0.36±0.01
IL-33	0.7±0.01	0.46±0.01	0.41±0.01
TSLP	0.61±0.01	0.45±0.01	0.38±0.01
IL-10	0.34±0.01	0.65±0.01	0.32±0.01
TGF-β	0.27±0.01	0.57±0.01	0.25±0.01
TNF-α	0.75±0.01	0.44±0.01	0.41±0.01
TLR-4	0.65±0.01	0.40±0.01	0.38±0.01
IFN-γ	0.24±0.01	0.54±0.01	0.23±0.01

Discussion

Based on “hygiene hypothesis”, epidemiologic evidence suggests a steady increase in occurrence of inflammatory disorders in developed countries, coupled with significantly reduced infectious diseases in such areas (16).

Parasites can survive in the host body and escape its immune system via a wide range of molecular mechanisms. Previous studies re-

vealed that some of the phylogenetically-related species of Ascarididae family including *Ascaris lumbricoides*, *A. suum* and *T. canis* have different impact on the immune system (17,18). As some signs of airway inflammations are detectable during the helminth infections, there is substantial evidence from various parts of the world that populations profoundly infected with helminths are rarely affected by allergic diseases (19).

Given the controversial point of view on the immunoregulatory role that SE of *T. canis* plays in airway inflammations, in this study, we measured the expression of specific cytokines (IL-33, IL-25, TSLP, TNF- α , TLR-4, IL-10 and TGF- β) in mice exposed to somatic extract of *T. canis*. Our observations are consistent with previous studies demonstrating that the SE of parasites such as *T. canis* can regulate the immunological pathways. Antigenic extracts of parasites can induce the production of cytokines and chemokines (20). The current study also showed that treatment with SE products of *T. canis* could reduce epithelial cell lesion and metaplasia, smooth muscle hypertrophy as well as hyperplasia of goblet cells.

In our study, the gene expression level of IL-25 and IL-33 was significantly decreased in SE group which is similar to a study on mice infected with *Heligmosomoides polygyrus*, the release of IL-1 β got elevated and a chronic infection occurred following decline of type 2 immunity cytokines such as IL-33 and IL-25 (21). Despite the decline in level of TSLP expression in SE group, there was no significant difference between OVA and SE group. Furthermore, the absence of TSLP in *Trichuris muris* infected mice results in decreased immune responses of Th2 and subsequently leads to an increase in IL-12, IFN γ , and IL-17 expression level (22).

The gene expression of TNF- α was significantly decreased in SE group in the current study which is parallel with findings of the previous studies regarding this phenomenon. A survey was conducted on mice infected with embryonated eggs of *T. canis* and their results proved that the production of IL-12 and TNF- α got lessen in both peritoneal exudate macrophages and spleen adherent cells (23).

The reduced level of gene expression of TLR-4 in SE group is in accordance with the results of an in vitro study on *Schistosoma mansoni* egg antigen which showed it can suppress production of TLRs ligand -induced (IL-

12p40 and IL-12p70) in Dendritic cells (DCs) and the increase of IL-10 (24).

In addition, our results demonstrated a significant upregulation of IL-10, TGF- β and IFN γ in lung tissues of SE group mice which is partly in line with the study on mouse model of *Toxoplasma gondii* infection that showed high levels of IFN γ in acute phase of the disease. That is due to the decrease of Th2 associated cytokines and a noticeable increase in Th1 responses (25). The crude antigens of *Paragonimus westermani* and *Clonorchis sinensis* caused an increase in gene expression of IL-10 and TGF- β in mouse model (24). Each molecule on the cell surface or cell extract in the parasite has either a stimulatory or suppressor function. The summation of these molecules can direct their behavior to be inflammatory or anti-inflammatory, to induce cytokine network. The immune system is believed to be capable of communicating either by cells or molecules to induce responses which in turn prompt a Th1 or Th2 response.

Conclusion

SE of *T. canis* is able to alter the immunoregulatory cytokines, resulting in suppression of airway inflammations. More comprehensive investigations are needed to precisely indicate the anti-inflammatory mechanism of *T. canis* on airway inflammations.

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Conflict of interest

The authors declare that there is no conflict of interest.

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