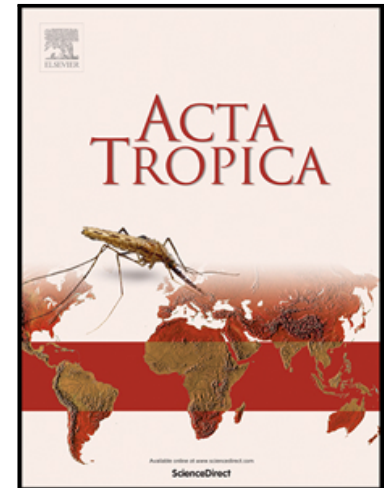


Immunoprotective inference of experimental chronic *Trichinella spiralis* infection on house dust mites induced allergic airway remodeling

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Immunoprotective inference of experimental chronic <i>Trichinella spiralis</i> infection on house dust mites	1
induced allergic airway remodeling	2
Dalia A. Elmehy^{a*}, Dina I. Abdelhai^b, Rasha A. Elkholy^b, Maram M. Elkelany^c, Dina M. Tahooun^{d1}, Reem A.	3
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Highlights	14
• Chronic <i>T. spiralis</i> infection recruited and induced Treg cells.	15
• Induced Treg cells effectively suppressed HDM-induced AAR.	16
• Treg cells cytokines, particularly IL-10, ameliorated hypersensitivity responses.	17
• IL-17 promoted inflammation and exaggerated the airway hyper-responsiveness.	18
	19
ABSTRACT	20
Allergic bronchial asthma is characterized by chronic inflammation of the respiratory airways mediated by T-helper 2 (Th2), Th17 and their cytokines. Although most asthmatic patients suffer from allergic airway remodeling (AAR), aggressive anti-allergic treatment failed to reverse it. The hygiene hypothesis illuminated the counter relationship between allergy and helminthic infections. The immune system is modulated by <i>Trichinella spiralis</i> (<i>T. spiralis</i>) infection to maintain homeostasis. Therefore, this work aimed to investigate the impact of chronic <i>T. spiralis</i> infection on induced AAR in C57BL/6 mice sensitized by house dust mites (HDM) allergens. Forty mice were divided into 3 groups: I (10 healthy mice), II (15 HDM sensitized mice), and III (15 <i>T. spiralis</i> chronically infected mice and sensitized with HDM allergens). The assessment aimed to evaluate the effects of regulatory CD4 ⁺ CD25 ⁺ FOXP3 ⁺ cells (Tregs) and their cytokines comparative to hypersensitivity mediated cytokines. Chronic <i>T. spiralis</i> infection effectively prevented the host's AAR. This result was evidenced by upregulated Tregs in blood by flow cytometric analysis and increased interleukin-10 (IL-10) levels in bronchoalveolar lavage (BAL) by Enzyme linked immunosorbent assay (ELISA) as well as improved lung histopathological changes. Also, serum HDM specific immunoglobulin E (IgE), BAL eosinophils, BAL IL-5 levels, and IL-17 gene expression in lung tissues were significantly reduced in <i>T. spiralis</i> chronically infected mice. In conclusion, the immune response in chronic <i>T. spiralis</i> infection could provide a promising mechanistic tool for protection against AAR, which paves the way for innovative preventive measures of other immunological disorders.	21
Keywords: <i>Trichinella spiralis</i> ; house dust mites; bronchoalveolar lavage; allergic airway remodeling; CD4 ⁺ CD25 ⁺ FOXP3 ⁺ T cells; IgE.	22
	23
	24
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	31
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	33
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1. Introduction

Currently, more than 300 million people around the world are suffering from allergic asthma; a chronic inflammatory disorder affecting the respiratory tract (Stern et al., 2020). The pathophysiology of asthma comprises airway infiltration with inflammatory cells especially eosinophils, increased smooth muscle contractibility, and hyperplasia in addition to hyperplasia of the glands hence the increase of mucus production (Voskamp et al., 2020). Chest distress, wheezing, short breath, and occasionally productive cough are distinctive symptoms of asthma (Voskamp et al., 2020). Environmental factors, genetic background, and some allergens like dust mites and pollens may cause and exacerbate asthma (Akar et al., 2020). Over the next two decades, the predictable increase in the number of asthmatics may reach up to 400 million especially in the urbanized communities (Agache et al., 2021). Moreover, the occurrence of asthma in rural areas in developing countries has been underestimated for several years (Voskamp et al., 2020).

Although β_2 -agonists and inhaled corticosteroids are effective in the long-term control of asthma, occasionally some patients may show resistance to these drugs (Hanania et al., 2020). Besides, the long-term severe side effects of steroids still hamper patients from their usage (Cooper et al., 2015). To date, no therapy has been shown to reverse chronic allergic airway remodeling (AAR) (Tripple et al., 2020). Owing to its severity, indistinct causal factors, and the lack of curable drugs or vaccines in many countries, AAR should be a priority for the governments' health strategies (Pizzichini et al., 2020). Therefore, future studies should fill the gaps in our knowledge about airway remodeling to improve the specific target therapy and the long-lasting follow-up for asthmatic patients.

As soon as the hygiene hypothesis was suggested in 1989 by Strachan, the counter relationship between allergic diseases and infection exposure has been elucidated in epidemiological research (Strachan, 1989). This hypothesis paves that the immune system can be shaped by the helminthic infection to maintain homeostasis (Fernandes et al., 2019). Several experimental studies had supported this hypothesis and confirmed the immunomodulatory properties of some helminths (Wilson et al., 2005). Subsequently, the effect of helminthic therapy on rhinitis and allergic asthma have been investigated in clinical trials with promising therapeutic efficacy (Maizels, 2019).

Both helminthic infections and allergy are analogous in the induction of Th2 polarized immune responses with high levels of total IgE, eosinophilia, mast cell degranulation, and up-regulation of IL-4, IL-5, and IL-13. This may be due to immunological similarities between parasitic antigens and allergens' components (Maizels, 2019). Unexpectedly, epidemiological and experimental evidence showed that Th2 allergic immune response is

damped with chronic helminthic co-infection (Feary et al., 2010). The characteristic feature of the immune response against helminthic infection is the mobilization of regulatory immune cells and mediators, such as forkhead transcription factor P3 (FOXP3) of Tregs in an IL-10 and transforming growth factor-beta (TGF- β)-dependent manner. This regulatory response can dampen both Th1 and Th2 responses, hence clarifies the hypothesis, which proposes that helminthic infection can also control Th2-dominated response in allergy and atopy (Dittrich et al., 2008). Lately, it became unmistakable that Tregs and Th17 cells are extensively tangled in the pathogenesis of severe asthma (Ramakrishnan et al., 2019).

Trichinella spiralis (*T. spiralis*) is an intracellular tissue-dwelling parasite. It establishes an enduring infection in the host striated muscles through a modulation of the host's immune response to avoid destruction, hence ensuring its survival. This strategy makes it one of the most successful parasitic symbionts. The presence of guardian Tregs was observed during chronic muscular infection by *T. spiralis* encysted larvae to maintain homeostasis (Sun et al., 2019). Therefore, the progression of infection is the main factor that determines the relative ratio between effector and regulatory cells. It can be noticed that *T. spiralis* can readjust the host immune response not only to itself but also to other party antigens. This elucidates why helminths are masters of immunoregulation that could alleviate the manifestations of allergic asthma (Aranzamendi et al., 2013).

Till now, there is no solid confirmation of the counter relationship between helminthic infections and chronic allergic diseases. Therefore, in the current study, we induced an experimental model of chronic allergic asthma with prominent AAR using HDM antigens to explore mechanism of the probable protective potential of chronic trichinellosis. Such research will deliver a new modality for the prevention of severe chronic asthma.

2. Materials and methods

2.1. Type and duration of the study

This animal study was conducted at Medical Parasitology, Clinical Pathology, Pharmacology, Medical Biochemistry, and Histology Departments, Faculty of Medicine, Tanta University between June 2019 and February 2020.

2.2. Ethical consideration

The research work was approved by the Research Ethics Committee, Faculty of Medicine, Tanta University, Egypt (approval code: 33567/12/19).

2.3. Experimental animals

Forty laboratory-bred wild-type C57BL/6 mice, 6-7 weeks-old and weighing nearly 25 grams were purchased from Theodore Bilharz Research Institute (Giza, Egypt). All mice were housed under sterile conditions following

the institutional and national guidelines. The animal House was maintained at 22-24 °C with a 12-hour light/dark cycle. The mice were acclimatized for 4 days and were fed a standard pellet diet and water ad libitum. The health status of the animals was checked daily, and none of the mice died or became ill before the research endpoint.

2.4. Chronic *T. spiralis* infection

The original *T. spiralis* species (code ISS6158) was used in the current research as it is thoroughly maintained by repetitive passages in rats and mice in the Medical Parasitology Department Laboratory, Faculty of Medicine, Tanta University. Infective inoculums were prepared by digestion of muscles of *T. spiralis* chronically infected mice in 1% pepsin and 1% concentrated HCL for 2 hours with continuous electric stirring. The subsequent fluid was sieved. The collected larvae were left to sediment to be counted. The infective inoculum was adjusted to 300 living larvae/ 0.25 ml/ mouse (Dunn and Wright, 1985). Mice were starved for at least 10 hours then infected with the inoculum by intra-esophageal gavage.

2.5. Experimental HDM-induced AAR model

House dust mites AAR model was induced using a lyophilized, freeze-dried, crude antigen consisting of equal parts of *Dermatophagoides (D.) pteronyssinus*, and *D. farina* (Catalog Number: 22840-0033-8, Greer Laboratories, Lenoir, NC, USA). HDM protein was adsorbed to aluminum hydroxide [Al (OH)₃] alum gel as an adjuvant sensitizer (Catalog Number: 21645-51-2, Sigma-Aldrich, St. Louis, MO, USA). The HDM antigen was then dissolved and reconstituted in phosphate-buffered saline (PBS) and passed through a 0.45 µm filter then a 0.02 µm filter to facilitate micro sprayer use. Mice were sensitized with 10 µg of filtered HDM extract through subcutaneous (SC) injections that were applied at the base of the tail on days 0, 7, 14 with a booster dose on day 21 (Aun et al., 2015). Mice were then challenged daily for 7 consecutive days from day 28 to day 35 with an intra-nasal aerosol solution containing 5 µg of HDM in vaporous PBS using a micro sprayer. Models of AAR usually need more than 4 weeks to develop rather than acute allergic airways inflammation that is typically induced in less than 3 weeks (Woo et al., 2018).

2.6. Experimental design

Mice were classified into three groups:

Group I (negative control group): 10 normal healthy mice.

Group II (positive control group): 15 mice sensitized with HDM allergen.

Group III (experimental infected group): 15 *T. spiralis* chronically infected mice, which were sensitized with HDM allergens 4 weeks post-infection.

Two days after the last HDM challenges, mice were euthanized by cervical dislocation. Samples from the	128
diaphragm, blood, bronchoalveolar lavage (BAL) fluids, and lungs were collected for further investigations.	129
<i>2.7. Parasitological parameters (mean number of larvae/ diaphragm)</i>	130
To ensure the establishment of chronic <i>T. spiralis</i> infection, the diaphragm of each mouse in group III was	131
artificially digested by 1% pepsin and 1% HCl in distilled water at 37 °C for 2 hours via the same process of	132
preparing the infective muscle larvae. The diaphragms larval counts were determined according to the method	133
of Dunn and Wrigh (1985).	134
<i>2.8. Blood sampling technique</i>	135
After euthanization, blood was withdrawn by exsanguination through cardiac puncture. A blood sample	136
from each mouse was divided into two tubes; VACUETTE® blood collection tubes (Greiner Bio-One,	137
Kremsmünster, Austria) containing K2EDTA for flow cytometric analysis of Tregs and clot activator/sep tubes	138
for measurement of serum HDM specific IgE.	139
<i>2.8.1. Expression of CD4+CD25+FOXP3+ Tregs by flow cytometry (FCM)</i>	140
To evaluate the expression of Tregs in blood samples, Mouse Regulatory T Cell Staining Kit (FlowXTM	141
Mouse Regulatory T Cell Multi-Color Flow Cytometry Kit, Catalog Number: FMC022, R&D Systems, Inc.	142
Minneapolis, St. Paul.) was used following the manufacturer's recommendations. The leucocyte count was	143
adjusted to 10 ⁶ cells/tube and dispensed into two tubes then 10 µL of CD4- fluorescein isothiocyanate (FITC)	144
and 10 µL of CD25- allophycocyanin (APC) antibodies were dispensed into the appropriately labeled tubes. The	145
mixture was vortexed and incubated for 30 minutes at 2-8 °C. For intracellular staining of FOXP3, the cells were	146
resuspended in 0.5 ml fresh 1X FOXP3/transcription factor fixation buffer and incubated at 2-8 °C for 30	147
minutes. Samples were analyzed by four-color FCM using the Becton Dickinson FACS Calibur instrument	148
(Becton Dickinson, San Diego, California, USA). A total of (10000 events were acquired and analyzed using	149
Cell Quest software; CELL QUEST SOFTWARE (Becton Dickinson, version 3, verify software House	150
Topsham, ME, USA). Lymphocytes were selectively gated for immunophenotyping analysis using forward and	151
side scattering (FSC and SSC) and then Tregs were identified by their expression of CD4/CD25/FOXP3	152
(Aranzamendi et al., 2013).	153
<i>2.8.2. Measurement of serum HDM specific IgE</i>	154
Blood samples were left to clot for 5-10 minutes at room temperature then they were centrifuged at 3,000	155
rpm for 5 minutes. The serum specific IgE titers to HDM were determined using the mouse IgE ELISA kit	156
(Mouse Serum Anti-HDM IgE Antibody Assay Kit, Catalog number: 3037, Chondrex, Inc., Fisher Scientific,	157

USA). ELISA technique was done according to the manufacturer's protocol and read on a microplate reader (Stat Fax[®] 2100, Fisher Bio block Scientific, France) at 450 nm with the correction wavelength set at 570 nm.

2.9. BAL sampling technique

The BAL fluid was collected for assessment of the inflammatory responses in the lungs. Momentarily after euthanization, the trachea was exposed and cannulated allowing slow infusion and withdrawal of 500 µl ice-cold sterile PBS. Lavage was gently repeated for a further two consecutive times (1.5 ml total). The BAL was kept in ice throughout the lavage process then it was centrifuged at 3,000 rpm for 5 minutes at 4 °C and the cell-free supernatant was collected and stored at -20 °C for cytokines analysis (Southam et al., 2007). All biochemical parameters were measured in triplicates.

2.9.1. BAL eosinophils analysis

After BAL centrifugation, the cell pellet was treated with red blood cell lysing buffer (Sigma) and resuspended in 1 ml of fresh PBS. Then, total cells were counted and calculated by hemocytometer. Cells were then stained for differential immune cell counts using Wright-Giemsa stain (Choi et al., 2014). Cells were identified and counted based on staining and standard morphological criteria. Eosinophils have been counted for each mouse over three different slides in a blinded manner. Based on international guidelines, eosinophils normally not present in bronchial secretions but less than 3% were within a normal upper value (Balbi et al., 2007).

2.9.2. BAL cytokines ELISA analysis

Levels of IL-5 and IL-10 were detected in cell-free BAL supernatant. Their levels were detected according to the manufacturer instructions using RayBio[®] Mouse ELISA Kits of IL-10 and for IL-5, Catalog numbers: ELM-IL10 and ELM-IL5, respectively, Los Altos, California, USA.

2.10. Lung tissue sampling

Lungs were removed *en bloc* after BAL and washed in sterile PBS. The right lobes were fixed with 10% formaldehyde solution for histopathological analysis. The left lobes were obtained for molecular analysis.

2.10.1. Histopathological Hematoxylin and eosin (H&E) plus Masson's trichrome staining of lung tissues

Lung specimens were fixed in 10% neutral-buffered formalin for 24 h. The lung tissues were dehydrated in ascending grades of ethanol and cleared in xylol and then embedded in pure soft paraffin blocks. Paraffin sections were taken of 5 µm thickness by microtome for further histopathological staining (Bancroft et al., 2019). Sections were stained with H&E to visualize the lung tissue structure and to examine the inflammation around the bronchial and vascular areas. Three pathologists graded the visual fields on each slide simultaneously via a

multiheaded microscope where they were blinded to the animals' study groups. More than seven sections of the bronchus of each animal were randomly selected and examined (Qiu et al., 2017). Semiquantitative scaling was attained using the higher power magnification ($\times 400$) as follow: 0 represents no inflammation, 1 signifies mild inflammation, 2 indicates moderate inflammation, and 3 implies severe inflammation (Hopfenspirger and Agrawal, 2002). Staining of lung sections by Masson's trichrome was performed to determine collagen content that reflects the process of airway fibrosis and remodeling (Woo et al., 2018).

2.10.2. RNA extraction, cDNA synthesis, and real-time polymerase chain reactions (RT-PCR) for IL-17 mRNA expression

The left lobes of the lungs were processed, and total RNA was extracted using the RNeasy Mini Kit, Catalog number: 74104, Qiagen, Valencia, CA according to the manufacturer's instructions. A High-Capacity cDNA Reverse Transcriptase Kit, Catalog number: 4368814, Applied Biosystems, Foster City, CA, USA was used to synthesize cDNA from extracted RNA according to the manufacturer's recommendations and its concentration was measured by photometry. Quantitative RT-PCR was performed with a standard protocol using the QuantiTect SYBR Green PCR kit, Catalog number: 204243, Qiagen, Valencia, CA. Gene-specific primer pairs used for PCR sequences for IL-17 (NM_010552.3) were (forward: 5' AAG GCA GCA GCG ATC ATC CC 3' and reverse 5' GGG TCT TCA TTG CGG TGG AG 3') and for GAPDH (NM_001289726.1) were (forward: 5' CAA GGT CAT CCA TGA CAA CTT TG 3' and reverse 5' GTC CAC CAC CCT GTT GCT GTA G 3') [25]. The primers were designed using Primer 3 plus software (Alnahas et al., 2017). The thermal cycling process started with an initial denaturation at 95 °C for 10 min then, 40–45 cycles of DNA denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec and finally extension at 72 °C for 30 sec. All reactions were run on step one® Real-Time PCR System (Applied Biosystems). The cycle threshold (Ct) values were calculated for target genes and the housekeeping gene (GAPDH). Relative gene expression was automatically calculated from the cross point values of the target and the reference gene by Rotor- Gene Q 6plex and its specific software (Qiagen, Valencia, CA, USA).

2.11. Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences software (IBM SPSS Statistics for Windows, IBM Corp, Version 22.0. Armonk, NY, USA). All values were expressed as mean $\pm$ standard deviation. Differences were regarded as significant if probability value $P < 0.05$ and highly significant if $P < 0.001$. Three biological replicates were done for each sample and the mean was recorded.

3. Results

3.1. Parasitological results (Mean number of larvae/ diaphragm)

At the endpoint of the experiment, the larval counts in the diaphragms of *T. spiralis* infected mice (group III) were generally high, indicating the establishment of chronic infection in all mice of group III with mean $\pm$ SD (3,932 $\pm$ 457).

3.2. Expression of CD4+CD25+FOXP3+ Tregs by FCM

In the present study, CD4+CD25+FOXP3+Tregs accounted for 4.92 ± 0.19 , 3.07 ± 0.13 , and $23.67 \pm 3.27\%$ of CD4+CD25+ T cells in groups I, II, and III, respectively. Both group I and group II had a significantly ($P < 0.001$) lower percentage of CD4+CD25+FOXP3+Tregs than those of group III (Fig. 1-A) indicating the expansion of these cells upon chronic *T. spiralis* infection (Fig. 2).

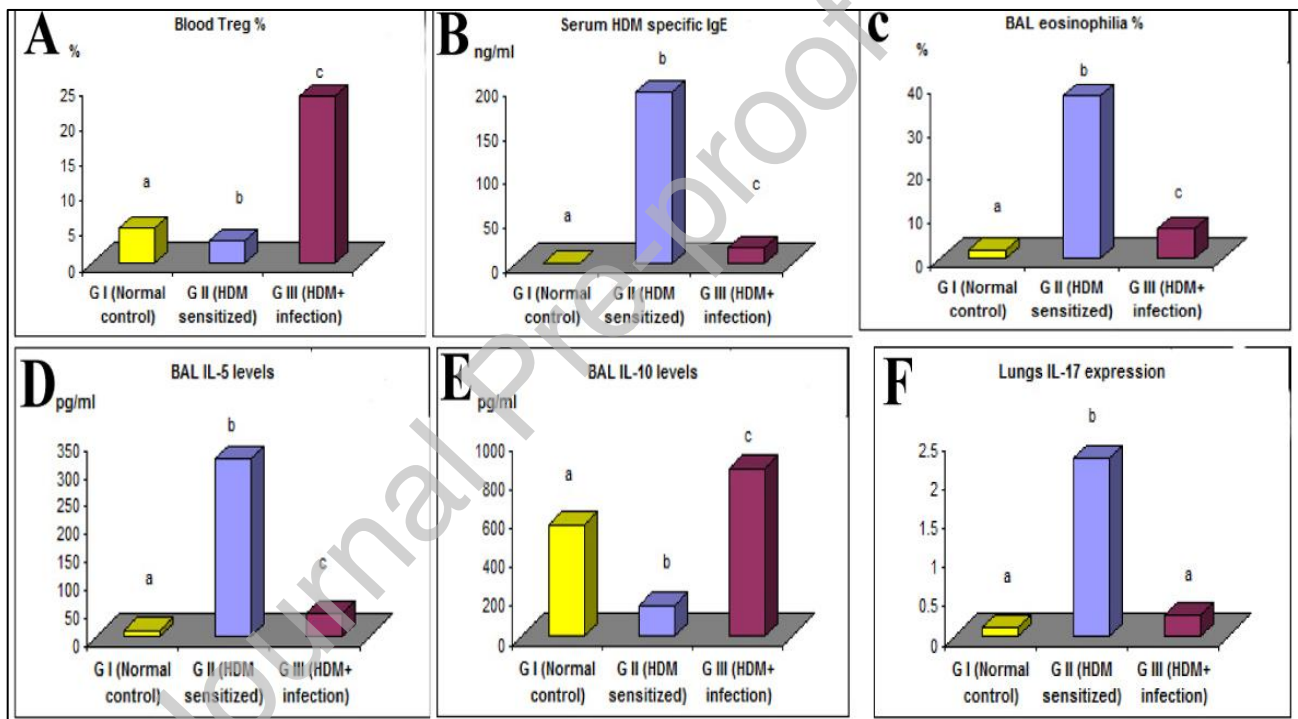


Fig. 1. Bar chart showing statistical analysis of data. **A)** Tregs percentage in blood by flow cytometry, **B)** HDM specific IgE serum levels by ELISA, **C)** BAL eosinophils count by hemocytometer, **D)** BAL IL-5 levels by ELISA, **E)** BAL IL-10 levels by ELISA, and **F)** IL-17 mRNA expression in the lung tissues by real-time PCR. Statistical analysis of data was represented by letters (a, b and c). Similar letters: non statistical significance; Different letters: statistical significance. GI (n=10) is the normal control group; GII (n=15) is the HDM sensitized group; GIII (n=15) is the HDM sensitized and *T.spiralis* chronically infected group. Tregs: CD4+CD25+FOXP3+ T regulatory cells; IL-10: Interleukin-10; IL-5: Interleukin-5; IL-17: Interleukin-17; BAL: Bronchialveolar lavage; IgE: Immunoglobulin E; HDM: House dust mite.

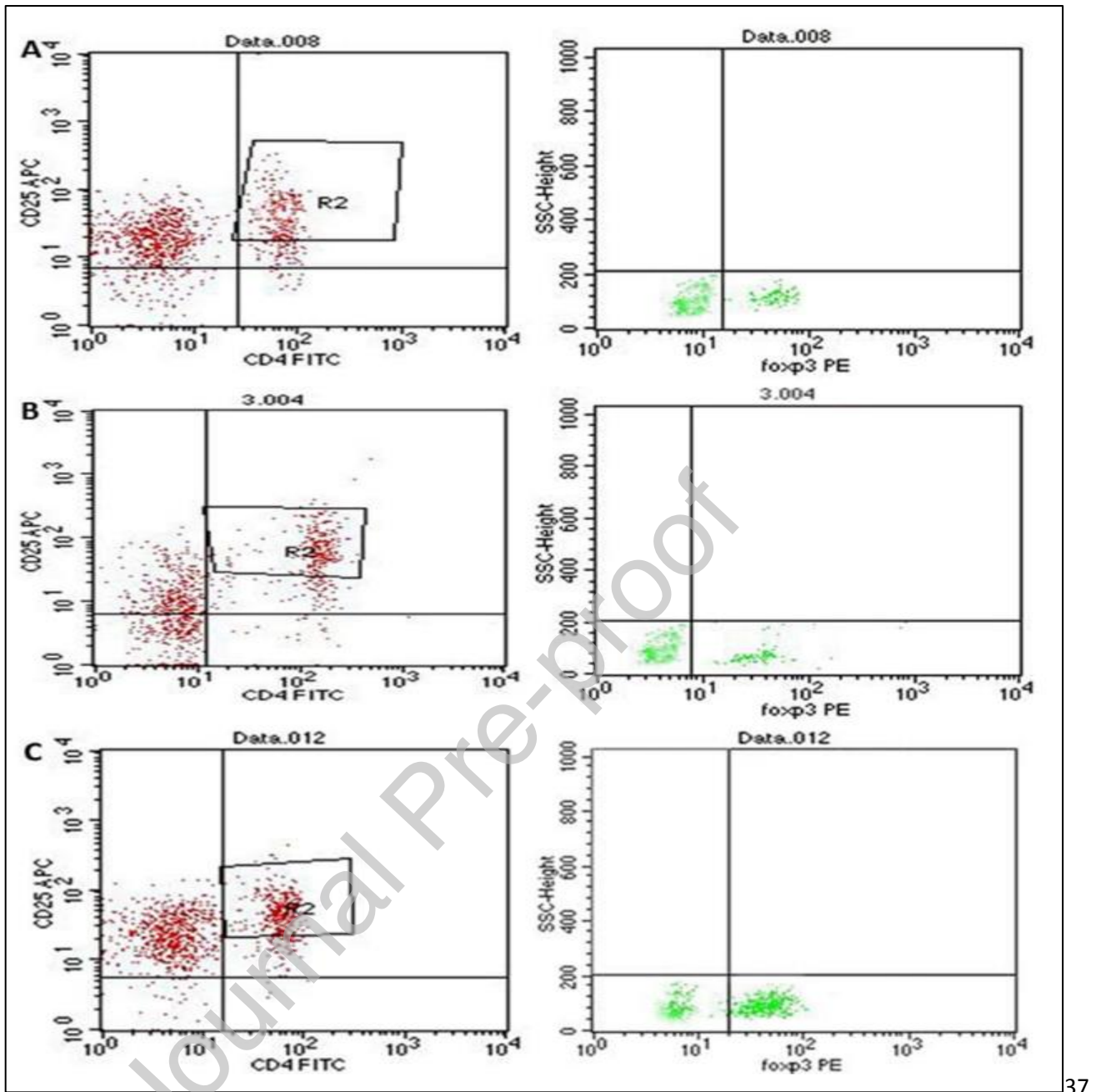


Fig. 2. Gating of the flow cytometric analysis of mouse CD4+CD25+Foxp3+ Tregs in representative dot plots. Lymphocytes were selected by first gating via their FSC-SSC properties. Tregs were characterized by CD4 (FITC) and CD25 (APC) surface expression and intracellular Foxp3 (PE) staining. **(A)** The dot plot gating of CD4+CD25+ (R2) cells with normal Foxp3+ expression (15%) in normal healthy control group I. **(B)** Decreased Foxp3+ expression (10%) in group II sensitized with HDM allergen. **(C)** Increased Foxp3+ expression (60%) in (group III) *T. spiralis* chronically infected mice.

3.3. Results of serum HDM specific IgE

As regards serum levels of HDM specific IgE in the study groups, there was a statistically significant difference between them $P < 0.001$. A significantly higher HDM specific IgE serum levels were found in group

II (194.67 ± 29.73) compared to group III (18.27 ± 2.37) with $P < 0.001$ (Fig. 1-B) indicating that serum levels of HDM specific IgE were significantly reduced by chronic *T. spiralis* infection.

3.4. Results of BAL eosinophils analysis

Differential counting of BAL cells recovered high cellular infiltration with eosinophils in group II (37.60 ± 4.10). However, a significant decrease in the proportions of eosinophils was greatly observed in *T. spiralis* chronically infected group III (6.87 ± 1.30) ($P < 0.001$) (Fig. 1-C).

3.5. Results of BAL cytokine analysis

The Th2 effector IL-5 concentrations were elevated in the BAL fluid of group II (318.53 ± 47.62). However, their levels were significantly diminished in chronic *T. spiralis* infected group III (40.93 ± 4.20) with a statistically significant difference ($P < 0.001$) (Fig. 1-D). Whereas, the regulatory IL-10 cytokine levels were greatly increased in *T. spiralis* chronically infected group III (861.33 ± 25.60) mice in comparison to uninfected group II (157.33 ± 24.92) with $P < 0.001$ (Fig. 1-E).

3.6. Histopathological staining of lung tissues

3.6.1. Results of H&E staining

Staining by H&E of the lung sections of group II showed disrupted bronchioles with sloughing of its epithelial lining and cellular debris. Dense inflammatory cellular infiltration in the peribronchial spaces was also observed with vascular congestion as well as discontinuous blood vessel wall. These inflammatory cells led to the destruction of the alveolar wall with epithelial shedding (Fig. 3-B). On the other hand, all these inflammatory changes in the peribronchial areas were greatly reduced in *T. spiralis* chronically infected mice of group III (Fig. 3-C).

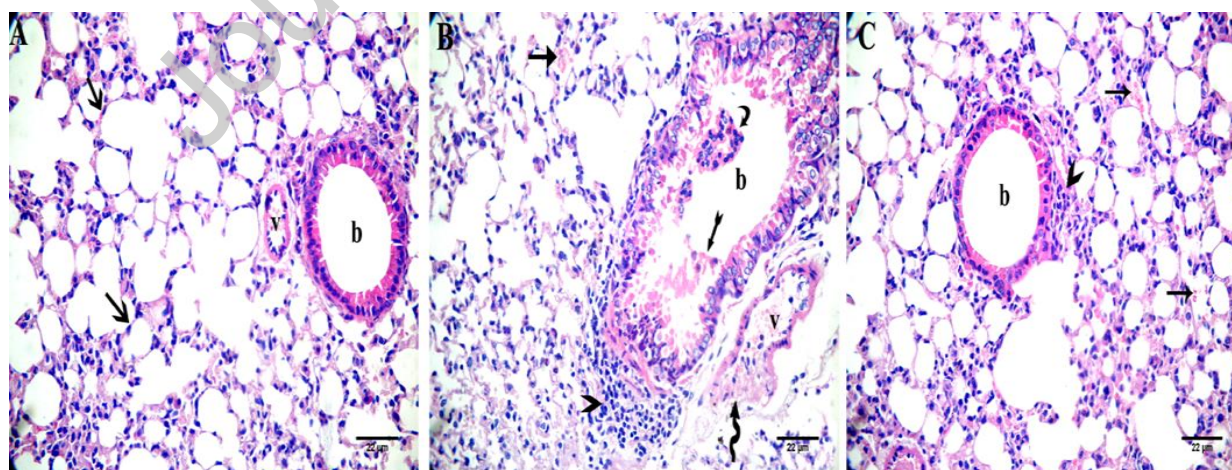


Fig. 3. A) Photomicrograph of a section in the lung from healthy control group I showing grade 0 with no inflammation, normal bronchial lining (b), normal alveolar lining (arrow), and normal blood vessel (V). B)

Photomicrograph of a section in the lung from group II (sensitized with HDM allergen) showing grade 3 that implies severe inflammation with disrupted bronchiole (b) with sloughing of its epithelial lining (bifid arrow) and cellular debris (curved arrow). Severe inflammatory cellular infiltration is observed with vascular congestion (V), discontinuous blood vessel wall (zigzag arrow), and leakage of RBCs into alveoli (arrow). **C)** Photomicrograph of a section in the lung from *T. spiralis* chronically infected group III showing more or less normal bronchial lining (b) with grade 1 mild inflammatory cellular infiltration (arrowhead) and leakage of RBCs into alveoli (arrow). **(H&E, Mic. Mag. ×400). The scale bar is 22µm.**

3.6.2. Results of Masson's trichrome staining

Masson's trichrome staining of lung sections in group II showed disarrangement of collagen fibers with abnormal deposition in the interstitial tissue of the lung and perivascular space (Fig. 4-B). Whereas, lung sections from group III showed nearly normal collagen arrangement in its interstitial tissue (Fig. 4-C). Therefore, chronic *T. spiralis* infection showed a protective effect against AAR.

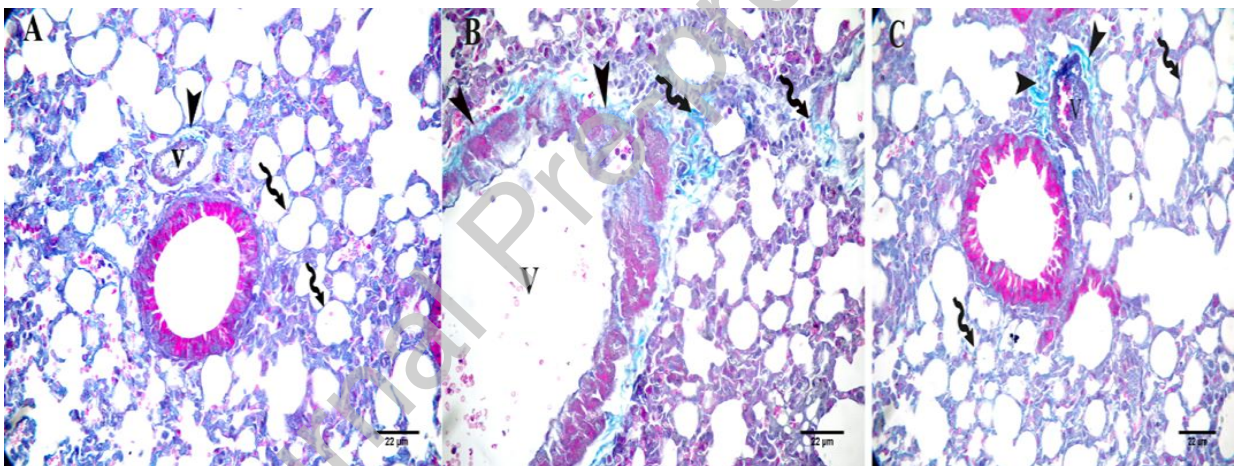


Fig. 4. A) Photomicrograph of a section in the lung from negative healthy control group I showing normal collagen arrangement in the interstitial tissue of the lung (zigzag arrow) and perivascular space (V) (arrowhead). **B)** Photomicrograph of a section in the lung from group II (sensitized with HDM allergen) showing disarrangement of collagen fibers with abnormal deposition in the interstitial tissue of the lung (zigzag arrow) and perivascular space (V) (arrowhead). **C)** Photomicrograph of a section in the lung from *T. spiralis* chronically infected group III showing more or less normal collagen arrangement in the interstitial tissue of the lung (zigzag arrow) with abnormal deposition in the perivascular space (V) (arrowhead). **(Masson trichrome stain, Mic. Mag. ×400). The scale bar is 22µm.**

3.7. Results of IL17 gene expression in lung tissues

The IL-17 mRNA expression levels in the lung tissues of HDM induced mice (2.28 ± 0.92) were significantly higher than the normal level in group I (0.11 ± 0.07) ($P < 0.001$). However, the IL-17 mRNA expression levels were near the baseline in *T. spiralis* chronically infected mice (0.26 ± 0.11) which is significantly lower than group II ($P < 0.001$) (Fig. 1-F).

4. Discussion

Currently, the immunomodulatory capability of parasitic infection to alleviate allergic disorders has become a prevailing research topic. Moreover, the hygiene hypothesis has inspired the development of novel remedies against allergy (Maizels, 2019; Zhu et al., 2020). Asthma is supposed to elicit chronic airway inflammation, which is eosinophils and Th2 cells dominant. Up till now, there is no effective treatment that can effectively reverse airway remodeling (Voskamp et al., 2020; Akar et al., 2020). That is why researchers are doing their best to understand the causal mechanisms of airway remodeling to reduce collagen deposition and improve asthma control (Akar et al., 2020). Since it is well recognized that HDM sensitization is one of the main risk factors of airway allergy; current researches employ HDM experimentally for the trustful mirroring of such clinical disease (Wang et al., 2020).

Nowadays, most studies on asthma focus on preventive and curative treatment during the sensitization phase rather than the challenge phase. Therefore, in the current study, we developed a murine chronic AAR model with collagen deposition mimicking severe chronic asthma by subcutaneous sensitization and intranasal challenge with HDM allergens for more than 4 weeks (Piyadasa et al., 2016; Woo et al., 2018).

One of the possible factors affecting the interaction between helminthic infection and allergic diseases is the timing of infection relative to allergen exposure. Therefore, the current study investigated the effect of chronic *T. spiralis* infection rather than acute one depending earlier observations of Aranzamendi et al. (2013) that showed a protective effect of chronic experimental helminthic infection against ova albumin (OVA)-induced airway responsiveness. These results can provide valuable insights about the time needed by chronic helminthic infection to induce a powerful immunoregulatory response that simultaneously allows the parasite survival and helps efficient immunoregulation of allergic hyper-responsiveness (Kang et al., 2014).

This research aimed to investigate whether chronic *T. spiralis* infection can induce expression of Tregs and find the correlation between this expression and alleviation of AAR. In the current study, Foxp3⁺ Tregs were upregulated upon chronic *T. spiralis* infection and ameliorated AAR. Fortunately, the immune system can preserve homeostasis against pathogens as well as allergens by the normally expressed CD4⁺CD25⁺FOXP3⁺ Tregs in the peripheral blood and spleen (Zhu et al., 2020). Allergic patients usually have defective Tregs as evidenced by successful immunotherapy of HDM allergy via Tregs induction (Wei et al., 2010). Consequently, the induction of

these guardian cells may develop unique immunomodulatory implications in cases of allergic hyper-responsiveness. 323
 The expression of FOXP3 can be promoted by several enhancers with helminthic infections being in the foreground 324
 through numerous regulators including the regulatory IL-10 (Qiu et al., 2017). 325

In our study, the percentages of CD4⁺CD25⁺FOXP3⁺ Tregs in the peripheral blood were significantly lower 326
 in HDM sensitized group II than in healthy control group I, which reflects a great imbalance of the Tregs in AAR in 327
 our model. The central role of Tregs in maintaining peripheral immune tolerance and controlling allergic diseases, 328
 such as asthma was confirmed by Agarwal et al. (2014) and Yun et al. (2014). The results herein also showed that 329
 chronic *T. spiralis* infection upregulated the recruitment of Tregs to a greater extent in the infected group III mice 330
 than in the uninfected group II mice, and it strongly suppressed HDM specific cytokine production. Standing with 331
 our results, Aranzamendi et al. (2013) had demonstrated that chronic infection with *T. spiralis* reduced OVA- 332
 induced allergic airway inflammation in mice with increased numbers of suppressive CD4⁺CD25⁺FOXP3⁺ Tregs. 333
 Moreover, the administration of induced Tregs by *T. spiralis* infection before sensitization effectively prevented 334
 allergic airway inflammation compared to the group receiving CD4⁺CD25⁺FOXP3⁺ Tregs from uninfected mice 335
 (Aranzamendi et al., 2013). Similar suppression of OVA-induced allergic airway inflammation was demonstrated 336
 by *T. spiralis*, *Heligmosomoides polygyrus* (*H. polygyrus*) (Wilson et al., 2005; Park, et al., 2011) and congruently, 337
Schistosoma mansoni (*S. mansoni*) infections (Amu et al., 2010). Studies had interestingly shown that Tregs can 338
 migrate into inflammatory sites and suppress effector T cell function and activity (Wei et al., 2006). 339

Humoral specific IgE to HDM is an important mediator of allergic airway inflammation as it activates mast 340
 cells and basophils through its binding to Fc receptors (Akar et al., 2020). The results of the current work showed 341
 that chronic *T. spiralis* infection was potent in the reduction of HDM specific IgE. Therefore, the reduced serum 342
 level of HDM specific IgE greatly mirrors the impaired function of Th2 effector cytokines as IgE isotype switching 343
 is preferentially induced by IL-4 (Rzepecka et al., 2013). Results herein were supported by Aranzamendi et al. 344
 (2013) who reported suppressed OVA-specific IgE in *T. spiralis* infected mice and Larson et al. (2012) who 345
 reported that chronic intestinal helminthic infection with high total IgE levels blocked basophils and mast cells 346
 sensitization by allergen-specific IgE in the clinical fields. These results can be convincing according to the “IgE 347
 saturation hypothesis” advocating that high titers of helminth-induced polyclonal IgE may saturate high-affinity IgE 348
 receptors on basophils and mast cells hence, alleviating allergen-specific hyper-responsiveness (Eberle et al., 2019). 349
 This hypothesis was reinforced in our model and has been proposed as one of the protective mechanisms against 350
 HDM-induced AAR. 351

Conversely, Buijs et al. (1997) showed that allergic sensitization with higher serum IgE levels was noticed in *Toxocara canis* seropositive children more than seronegative ones. Running in the same way, Mitre et al. (2005) suggested that saturation of IgE binding sites was not crucial in helminth-induced allergy protection where high polyclonal IgE in patients infected with *Brugia malayi* did not decrease specific IgE titers to HDM allergens. Furthermore, a positive association between *Ascaris suum* antibodies and allergic sensitization were described among children (Pinelli et al., 2009). Also, *Ascaris*-specific IgE titers have been powerfully allied with HDM airway allergy, which may result from the IgE cross-reactivity of HDM with *Ascaris* (Levin et al., 2012). These discrepancies may refer to the intensity and duration of infection, which necessitate further research on the mechanistic effect of helminths on basophils and mast cells concerning protection against allergic diseases (Cooper et al., 2003).

It is well established that eosinophils participate to a great extent in the pathogenesis of asthma as they play a key role in persistence of the inflammatory process in the airways. After degranulation of mast cells by allergen-specific IgE, eosinophils migrate to the site of inflammation, causing epithelial damage (Akar et al., 2020). In the present study, the decreased BAL eosinophilia in group III relative to group II indicates that chronic *T. spiralis* infection deviated the immune response towards tolerance. In accordance with the results herein, Kitagaki et al. (2006) observed suppression of OVA-induced airway eosinophilia and bronchial hyperreactivity by *H. polygyrus*. Similarly, *S. mansoni* and *S. japonicum* chronically infected mice were protected against OVA-induced airway allergy as indicated by the reduction of eosinophils in BAL, Th2 cytokine levels in lungs, and mucus production (Pacífico et al., 2009; Liu et al., 2010). More confirmation was gained from Park et al. 2009 who reported amelioration of OVA-induced asthma during the challenge phase and suppression of eosinophilia by *Anisakis simplex* infection via recruitment of CD4⁺CD25⁺FOXP3⁺ Tregs (Park et al., 2009).

It is worth to be noted that IL-5 is crucial in the pathogenesis of allergic airway inflammation as it induces eosinophils to produce IL-4, which is crucial in IgE isotype class-switching and differentiation of mast cells as well as eosinophil migration (Rzepecka et al., 2013; Dileepan et al., 2020). In the current study, BAL fluid was selected as a keynote sample for precise immunological determination of the Th2-mediated airway hyper-responsiveness in which IL-5 was significantly increased in group II. However, IL-5 level reduction in BAL of *T. spiralis* chronically infected mice provided a mechanistic explanation for the dramatically reduced airway eosinophilia. These observations are comparable to those reported by Piyadasa et al. (2016). Likewise, the decreased IL-5 in *T. spiralis* chronically infected group was consistent with the results of Kang et al. (2014) who reported that suppression of IL-5 levels by *T. spiralis* infection was crucial in the prevention of OVA-induced asthma. In line with our results, *H.*

polygyrus, *Litomosoides sigmodontis*, and *S. mansoni* infections induced an IL-5 suppression in murine asthma 382
models (Kitagaki et al., 2006; Dittrich et al., 2008; Liu et al., 2010). 383

Interestingly, helminth products can accomplish the same immunomodulant effect proved by the ability of 384
filarial and *Angiostrongylus cantonensis* cystatins to suppress the IL-4, IL-5, and IgE production in OVA-induced 385
airway allergy (Schnoeller et al., 2008; Ji et al., 2014). In the same way, *Ascaris lumbricoides*, *Angiostrongylus* 386
costaricensis and *S. japonicum* extract also significantly decreased IL-5 and airway inflammation in OVA-induced 387
models (Pitrez et al., 2015; Ren et al., 2016). Our results can be explained by the ability of chronic *T. spiralis* 388
infection to suppress Th2 response to protect itself from the host defense and promote its survival. However, 389
contrary to our results, Th2 response biasing occurred in *Toxocara canis* infected mice accounting for the 390
exacerbation of experimental allergic airway inflammation (Pinelli et al., 2008). These conflicting results may be 391
explained in light of the differences in host/parasite relationships as well as the parasites' life cycles (Ferreira et al., 392
2006). 393

IL-10 is one of the key anti-inflammatory cytokines that has been assumed to downregulate airway 394
inflammation (Chen et al., 2020). Therefore, researches on the helminths and allergy relationships cannot 395
marginalize its measurements. IL-10 is produced by several cell types including regulatory T cells and B cells 396
playing an important role in the helminth-induced regulation. The results of BAL IL-10 levels in the current work 397
are consistent with those of van den Biggelaar et al. (2000) and Flohr et al., (2009) who reported that *S.* 398
haematobium and *Necator americanus* specific IL-10 levels were inversely associated with allergen skin 399
sensitization in infected patients and infected children, respectively. Moreover, these results were confirmed by 400
decreased IL-10 levels with *Schistosoma* helminthic therapy (van der Vlugt et al., 2012). Similarly, the protective 401
effect of *H. polygyrus* against allergen-induced atopic asthma was proved to be IL-10 dependent through induction 402
of CD4⁺CD25⁺FOXP3⁺ Tregs (Kitagaki et al., 2006). The results herein can be interpreted according to Amu et al. 403
(2010) who described that the protective effect of IL-10 against OVA-induced airway inflammation in *S. mansoni* 404
infected mice is closely related to Tregs, as IL-10 can be released by CD4⁺CD25⁺FOXP3⁺ Tregs and 405
simultaneously can induce them. 406

Nevertheless, other studies verified that the Tregs preventive effect against asthma may be IL-10 407
independent as proved by Pacífico et al. (2009) who reported that *S. mansoni* infection alleviated OVA-induced 408
asthma via Tregs but did not support the modulatory role of the helminth-induced IL-10. Also, *H. polygyrus* 409
infection was shown to suppress the OVA or HDM induced airway inflammation model via Tregs in a TGF- β 410
dependent manner rather than IL-10 (Wilson et al., 2005). The inconsistency between these results can be 411

elucidated by the whole annulment of the cytokine in the IL-10^{-/-} mice and the deficient blocking of IL-10 412
response (Kropp et al., 2020). 413

In the present study, the airway remodeling model with collagen deposition was induced to mimic severe 414
chronic asthma (Woo et al., 2018). As regards the remodeling of the airways in our model, the findings in the HDM 415
sensitized mice were similar to previous studies that proved that major features of airway remodeling were induced 416
mainly by chronic exposure to allergen rather than acute exposure (Woo et al., 2018). Similarly, chronically 417
infected BALB/c mice with *S. mansoni* were protected against OVA-induced airway allergy as indicated by reduced 418
inflammatory cells in their lungs (Pacífico et al., 2009). Also, chronic infection with *Litomosoides sigmodontis* 419
suppressed all pathological features in the OVA-induced allergic model (Dittrich et al., 2008). In our study, we 420
suggested that chronic *T. spiralis* infection mediated its inhibitory effect on AAR through altered immune responses 421
of various T helper subsets mainly Th2 and Tregs even with sustained allergen exposure. 422

One of the cytokines that does not accept negligence in the pathogenesis of asthma and airway 423
hyperreactivity is IL-17. It largely promotes Th2 polarization, eosinophil and neutrophil recruitment, and mucus 424
hypersecretion in the upper airway, contributing to a severe, steroid-resistant asthma (Ramakrishnan et al., 2019). In 425
agreement with our findings, *Angiostrongylus cantonensis* cystatins and the filarial nematode; *Acanthocheilonema* 426
viteae glycoproteins ameliorated the OVA-induced allergic airway hyper-responsiveness accompanied by reduction 427
of IL-17 in experimental models (Schnoeller et al., 2008; Rzepecka et al., 2013). Also, a study conducted by Qiu et 428
al. (2017) reported downregulated HDM-induced airway inflammation in mice by *S. japonicum* infection with 429
inhibited IL-17 secretion in BAL caused by activation of Tregs in the airways. From these results, we suggest that 430
the magnitude of the preventive effect of chronic *T. spiralis* infection is probably regulated by secretory 431
components of *T. spiralis* encysted larvae. Therefore, further studies are mandatory to recognize the helminths 432
components that can regulate the cross-talk between the effector Th17 and Tregs. 433

Despite the powerful immunoprotective potential of chronic *T. spiralis* infection against HDM-induced 434
airway remodeling in our study, it is worth mentioning that not all parasites protect against asthma. A strikingly 435
increased possibility of *Ascaris lumbricoides* infection associated with asthma was reported in a systematic review 436
and meta-analysis of more than 30 epidemiological studies (Leonardi-Bee et al., 2006). *Toxocara* may even 437
enhance asthma as morbidity of asthma was positively associated with *Toxocara* seropositive children in cross- 438
sectional studies in the Netherlands and Acre State, northwestern Brazil (Buijs et al., 1997; Ferreira et al., 2007). 439
Toxocara canis has also been positively associated with an increased atopy and asthma-like symptoms in humans 440
(Fernando et al., 2009). However, a study in Austria revealed no association between bronchial asthma and 441

Toxocara seropositivity (Zacharasiewicz et al., 2000). These conflicting observations may be due to differences in the immunological status, age of the host, inadequate helminth eradication, nature of the parasite, infectious dose, stage of infection, or presence of co-infections. For example, a low infectious dose may neither be able to create an adequate immune response nor activate Tregs proliferation. Also, it should be kept in mind that inhaled corticosteroids may suppress Tregs number and activity (Leonardi-Bee et al., 2006).

5. Conclusion

Taken together, along with all the immunological parameters measured in our study, it could be concluded that the immune response in chronic *T. spiralis* infection deviates the cytokine network towards the recruitment and activation of Tregs, which effectively suppressed HDM-induced AAR. Therefore, analysis of the expression of Tregs in intestinal, muscle, spleen, and lung tissues will be very helpful for a more comprehensive understanding of the protective effect of *T. spiralis* infection. However, despite the promising results of helminthic therapy, the concept of worm infection won't be acceptable by many asthmatics. Therefore, more in-depth investigations should explore the helminthic molecules that induce such mechanistic regulation to develop innovative preventive strategies for chronic, severe asthma. In order to jump to clinical trials as well, further investigations are needed to determine the minimal dose of infection that gives the best influence.

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Declaration of interest

The authors declare that no competing interests exist.

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