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Immunization of proteins from *Toxascaris leonina* adult worm inhibits allergic specific Th2 response

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Abstract

Recently, the influence of parasitic infections on the incidence of allergic diseases has become the focus of increased attention. In order to ascertain whether parasite-derived proteins could inhibit the allergic specific Th2 response, we applied excretory–secretory protein (TI-ES) or total protein (TI-TP) of the adult worm *Toxascaris leonina* to asthma model mice prior to or simultaneously with OVA challenge, after which we assessed the OVA-specific Th2 responses. The group subjected to immunization with TI-ES and TI-TP (immunized group) evidenced a thinning of the bronchial epithelial and muscle layer, a disruption and shedding of epithelial cells, a reduction in the number of goblet cells, and a reduction in mucus production as compared to the group treated with TI-ES coupled with OVA challenge (challenge with OVA groups) and the OVA-induced asthma group. The administration of TI-ES and TI-TP, regardless of injection time, was shown to inhibit the recruitment of inflammatory cells into the airway, and in particular, macrophages, neutrophils, and lymphocytes were significantly reduced as the result of the parasite proteins. However, the total number of eosinophils was slightly reduced as the result of the administration of parasite proteins. Sensitization and OVA challenge was shown to accelerate the secretion of Th2 cytokines (IL-4 and IL-5) within the lung, but in the immunized groups, those levels were lower. The administration of TI-TP and OVA challenge group also evidenced a significant reduction in IL-4 levels as compared to the OVA-challenged group. The concentrations of Th2 cytokines in the TI-ES and OVA challenge group were more similar to those observed in the OVA-challenged group. The concentration of IL-10 and TGF- β in the lung was decreased substantially in the OVA-only challenge group, but the TI-TP immunized group exhibited significantly induced IL-10 cytokine. OVA-specific IgG2a, IgG1, and IgE levels in the immunized groups were significantly lower than those detected in the OVA-challenged group. In conclusion, parasite-derived protein is able to inhibit OVA-specific Th2 responses, and in particular, immunization with parasite proteins exerts a more profound protective effect than is seen with the treatment of allergic reactions. The results of our study are encouraging in terms of our further understanding of the molecular basis of immune evasion by nematodes.

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Keywords: Asthma model mouse; *Toxascaris leonina*; Immunization; Total proteins; Excretory–secretory proteins

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1. Introduction

In recent years, the incidence of immune-related diseases in developed nations has increased rapidly. In particular, the past four decades have seen a precipitous increase in atopy, hayfever, eczema, and asthma. With this has come increased research effort, and much is currently known, often in great detail, about the physiological and immunological mechanisms underlying these phenomena. In a landmark study of hygiene conducted in 1989, Strachan proposed that improved hygiene was the factor that best explained this rise (Strachan, 1989). The Hygiene Hypothesis suggests that parasites and microbes have been important in the shaping and tuning of the evolution of the human immune system (Dunne and Cooke, 2005).

The influence of parasitic infections on the incidence of allergic disease has been receiving an increased amount of attention of late, with studies conducted in Ethiopia and Gabon showing that parasitic infestation is associated with reduced atopic sensitization and dust-mite skin test sensitivity (Dagoye et al., 2003; Scrivener et al., 2001; van den Biggelaar et al., 2000). Children treated repeatedly for *Trichuris trichiura* and *Ascaris lumbricoides* evidenced increased dust-mite skin responses as compared with children that had not been treated for asymptomatic soil-associated helminthic infections (van den Biggelaar et al., 2004). Additionally, allergic diseases of pets, including asthma and atopic dermatitis, are common problems, as they are in humans (Maeda et al., 2008; Padrid, 2006).

Several molecules from helminth species induce pronounced Th2 responses in a manner similar to that observed in cases of full-blown parasitic infection. Excretory–secretory (ES) glycoproteins isolated from the rodent nematode, *Nippostrongylus brasiliensis*, have been shown to exhibit Th2-promoting activity on dendrite cells; however, the exact nature of the molecules involved in *N. brasiliensis* ES proteins remain to be clearly elucidated. This activity is both heat-labile and protease-sensitive, which suggests that the active component is proteinaceous in nature (Balic et al., 2004). Also, in schistosomiasis, the soluble extract of *Schistosoma mansoni* eggs (SEA) has been demonstrated to induce SEA-specific Th2 responses when injected into mice (MacDonald et al., 2001), and SEA was also demonstrated to condition human DCs to polarize Th response in the direction of Th2 *in vitro* (de Jong et al., 2002). When exposed to Th2 cytokines, these molecules can also activate host regulatory T cells (Treg) which subsequently release IL-10 and TGF- β , which may be functionally involved in the suppression

of the level of Th2 cytokines IL-4, IL-5, and IL-13. These parasites can establish a chronic infection; this highlights important issues (Allen and Maizels, 1996), in that the presence of these metabolically active pathogens indicates that the immune system is being relentlessly challenged with foreign antigens; this continuous immune reactivity, if uncontrolled, could eventuate severe pathology. In addition (Behnke et al., 1992), these pathogens may have developed evolutionary strategies by which they may evade the immune system for long-term survival in an immunocompetent host.

The majority of roundworms infects only one or two specific hosts, and live in the intestine of their definitive host; they are generally well adjusted to the immune system of their host. Although they can occasionally cause mild allergic responses in their hosts during the larval stage (migration period), after growing to the adult stage, these worms are not likely to provoke severe pathological effects in their definitive host's tissues. *Toxascaris leonina* is one of the best known intestinal parasites in dogs and cats. They have a very simple life cycle, which is distinct from that of other roundworms. After a dog or cat ingests infective eggs, the eggs hatch and the larvae mature into adult worms within the walls and lumen of the small intestine, without any tissue migration (Griffiths, 1978). In the intestine, roundworms absorb nutrients from the animal's food, interfere with digestion, and can damage the lining of the intestine. Dogs and cats with mild *T. leonina* infestations may not show any signs of disease (this is true in most cases). Animals with more severe infestations may be thin, have dull coats, and develop a pot-bellied appearance. Other animals, including humans, can function as paratenic hosts of *T. leonina*.

In order to ascertain, then, whether parasitic infections can reduce allergic reactions and whether their infective stage influences the host immune system, we have mimicked chronic infection conditions in our experiment via treatment with parasite-derived proteins (ES protein and total proteins) for one month prior to the administration of allergen treatment. We attempted to determine whether or not these parasite-derived proteins suppressed allergy-specific Th2 reactions.

2. Materials and methods

2.1. Parasite protein isolation

T. leonina was isolated from naturally infected dogs. The recovered worms were classified in accordance with the morphological features of the eggs and the

adult worms, all of which were maintained in our laboratory (Bellamy and Olexson, 2000; Griffiths, 1978). After collection, in order to prevent any contamination with the host material, the worms were thoroughly and carefully washed several times over a 3-h period in phosphate-buffered saline (PBS). They were then introduced into sterile flasks with RPMI 1640 medium supplemented with antibiotics (100 µg/ml Pen/Strep; Gibco). The culture was then maintained for 7 consecutive days at 37 °C in 5% CO₂. It was confirmed that, during this time, the majority of the adults remained alive and evidenced good mobility. Following centrifugation (12,000 × *g* for 30 min), the supernatants were concentrated by pressure applied in a concentrator (Amicon, Millipore Corporations, USA) with 3000 Da pore size membranes and dialyzed against PBS for 24 h with continuous agitation in a cold room in order to eliminate any antibiotic remnants. Finally, this excretory–secretory protein (TI-ES) was maintained until use in a freezer at –70 °C. The total somatic proteins (TI-TP) were prepared from *T. leonina* adult worms utilizing a modified version of Desowitz's method (Desowitz et al., 1985). In brief, *T. leonine* specimens were rinsed in PBS and homogenized in 50 mM Tris–HCl (pH 7.5) with a glass homogenizer (Cappello et al., 1993). The homogenates were briefly sonicated and then centrifuged for 30 min at 12,000 × *g* and 4 °C. The concentrations of both proteins (TI-ES and TI-TP) were determined using a bicinchoninic acid protein assay system (Pierce

Chemical Co., Rockford, IL.) with a bovine serum albumin standard curve.

2.2. Evaluation of OVA-specific Th2 response-inhibitory effects of parasite-derived proteins

Female BALB/cBY mice, 6 weeks of age and free of murine-specific pathogens, were acquired from Hyochang-Science (Daegu, Korea). The mice were housed throughout the experiments in a laminar flow cabinet, and were maintained in individual ventilation cages (Three Shine, Korea). In order to induce airway allergic reactions, the mice were intraperitoneally (I.P.) sensitized with 75 µg of ovalbumin (OVA, Sigma, Grade V) and 2 mg of aluminium hydroxide gel, on days 0 and 7. One week after the final sensitization, the mice were intra-nasally (I.N.) challenged with 50 µg of OVA on 4 consecutive days (days 13, 14, 19, and 20).

We mentioned the “Immunized group”, members of which were injected intraperitoneally with 100 µl of 10 µg/ml TI-ES and TI-TP, respectively, on 7 and 14 days prior to the airway allergic reaction procedure. We also mentioned the “Challenged with OVA” group, members of which were injected I.P. with 100 µl of 10 µg/ml of TI-ES and TI-TP respectively on the same day as the challenge in the airway allergic reaction procedure. The negative control group was challenged with PBS (I.N.) on the same day of the challenge in the airway allergic reaction procedure (Fig. 1). We utilized 4–6 mice per each group and repeated this experiment 3 times.

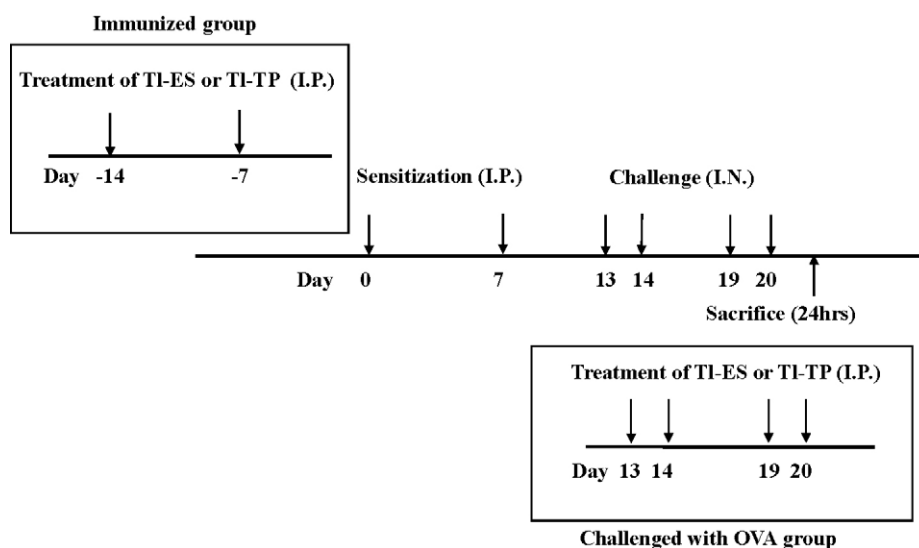


Fig. 1. Experimental design for treatment of mouse models of asthma. Mice were intraperitoneally sensitized 2 times with 75 µg of OVA, then treated 4 times intra-nasally with 50 µg of OVA. The OVA challenge group was treated with TI-ES and TI-TP simultaneously with OVA challenge on days 13, 14, 19, and 20. The immunized group was treated at –7. –14 days prior to the first OVA sensitization. Airway responsiveness was measured 24 h after the last airway OVA challenge, then sacrificed (*n* = 4–6 mice per group, 3 independent experiments).

2.3. Lung histopathology

Histological analyses were conducted as described previously (Trujillo-Vargas et al., 2005). In brief, the lung tissues were fixed with formaldehyde and embedded in paraffin. The thin sections of the embedding tissues were stained with hematoxylin–eosin. After staining, we examined the stained sections under microscopy.

2.4. Analysis of bronchoalveolar lavage fluid (BAL)

BAL was conducted 24 h after final challenge. At the time of lavage, the mice were sacrificed with 200 μ l of ketamine:lumpun:PBS (2:3:5). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was secured with ligatures. Pre-warmed PBS was slowly inflated into the lungs and withdrawn. The aliquots were then pooled and maintained at 4 °C. Part of each pool was then centrifuged, and the supernatants were maintained at –70 °C until use. The lungs were removed and the right lung tissues were fixed in 10% (volume/volume) neutral buffered formalin, and the left lung tissues were maintained in tissue-protective solution at –80 °C.

2.5. Differential cell counting

BAL was placed on ice and centrifuged for 5 min at $200 \times g$ at 4 °C. The supernatants were then decanted and frozen at –80 °C for subsequent use. The cell pellets were resuspended and washed twice in PBS. The total cell numbers were counted with a hemocytometer. BAL cell smears were prepared with a Cytospin. The smears were then stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico. Inc., Aguada, Puerto Rico) in order to determine the cell differentials, in accordance with conventional morphologic criteria. The results were calculated after 3 consecutive experiments.

2.6. Airway hyperresponsiveness measurements

The enhanced pause (PenH), a measure of bronchoconstriction, was evaluated at baseline and after treatment with increasing doses of aerosolized methacholine (2.5–50 mg/ml), via whole-body plethysmography for animals (Allmedicus, Korea). In the plethysmography procedure, the mice were permitted to acclimate for 3 min and then exposed for 4 min to nebulized saline, and subsequently treated with increasing concentrations (0, 2.5, 5, 10, 50 mg/ml) of nebulized methacholine (Sigma Chemical, USA) in

saline, using an ultrasonic nebulizer. After each nebulization, recordings were obtained for 3 min, and the PenH values measured during each 3 min period were averaged. Graphs of the PenH values were constructed in response to the increasing methacholine concentrations for each dose-matched group of mice.

2.7. ELISA

OVA-specific immunoglobulins (IgG1, IgG2a, IgE) in the serum and cytokine interleukin (IL)-4, IL-5, IL-10, tumor growth factor-beta (TGF- β) and interferon- γ (IFN- γ) levels in the BAL fluid were determined via ELISA in accordance with the manufacturer's instructions (ebioscience, R&D Systems, Korea). The absorbance of the final reactant was determined at 450 nm with an ELISA plate reader.

2.8. Statistical analysis

Normally distributed data were evaluated via the Student's *T* test. ANOVA and data that were not normally distributed were evaluated via nonparametric tests, where appropriate.

3. Results

3.1. TI-ES and TI-TP immunized and TI-TP with OVA challenge can inhibit airway inflammation in asthma-induced mice

After the induction of asthma, we noted influxes of inflammatory cells into the pulmonary parenchyma, and these influxes of inflammatory cells generated severe hemorrhages, pulmonary edema, and destruction of the alveolar wall (Fig. 2B). However, in the other groups (immunized and challenged with OVA in TI-ES and TI-TP groups), the cellular infiltration was minimal around the bronchial tree, with only a smattering of inflammatory cells (Fig. 2C–F). In particular, the immunized TI-ES and TI-TP groups evidenced a thinning of the bronchial epithelial and muscle layer, a disruption and shedding of the epithelial cells, a reduction in the number of goblet cells as compared to the OVA-challenged groups (Fig. 2C and D). The airway functions of these mice were monitored. After treatment, the mice were exposed to increasing doses of methacholine, and the airway responsiveness, expressed as PenH, was determined. The PenH values generated after methacholine treatment (from 2.5 to 25 mg/ml) were significantly higher in the OVA-inhalation mice than in the PBS group. The PenH values of the TI-TP

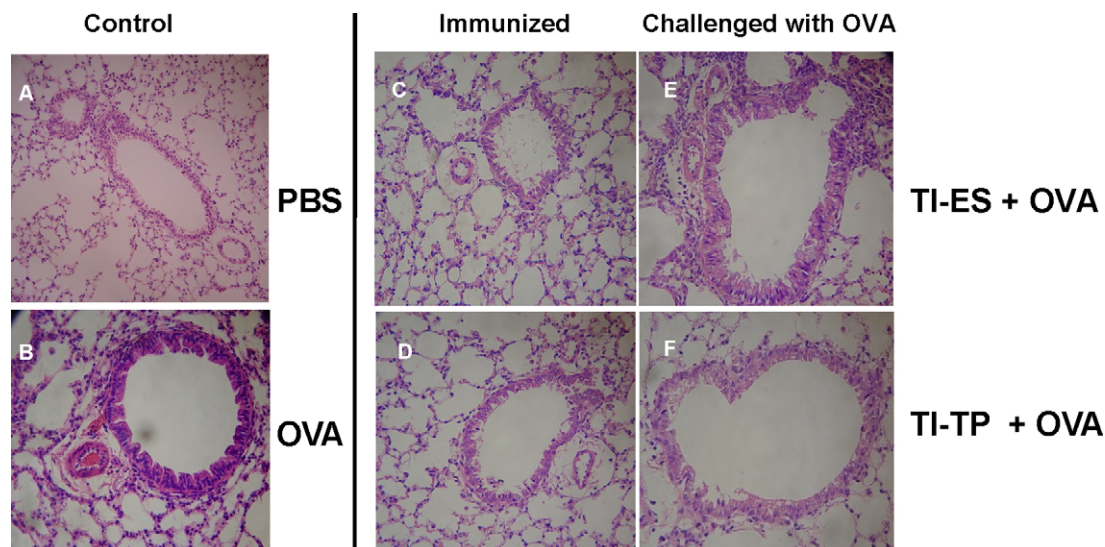


Fig. 2. Histological findings of airway inflammation in ova-challenged control mice and the effect of immunization and challenge with OVA groups. (A) PBS-treated, (B–F) OVA+ alum-treated (induced asthma), (C) immunized TI-ES protein, (D) immunized TI-TP protein, (E) TI-ES protein treatment with OVA challenge, and (F) TI-TP protein with OVA challenge.

immunized group were significantly suppressed as compared to the OVA challenge only group (Fig. 3A). The TI-ES and TI-TP challenged groups were quite similar to those in the OVA challenge group (data not shown).

3.2. Parasite-derived proteins could downregulate OVA-specific Th2 responses

The numbers of most inflammatory cells (macrophages, eosinophils, lymphocytes and neutrophils) in the BAL fluids were significantly increased in all of the OVA-challenged groups (Fig. 3B and C). The administration of TI-ES and TI-TP prior to asthma induction (immunized group) and the TI-ES and TI-TP with OVA challenge (challenged with OVA groups) evidenced inhibited recruitment of inflammatory cells into the airway (Fig. 3B and C). In particular, neutrophils and lymphocytes were significantly reduced by the parasite proteins at any administration time (p value < 0.05). The total number of eosinophils of the immunized and OVA-challenged group was slightly reduced; however, this reduction was not statistically significant (p value > 0.05).

In an effort to determine how parasite-derived proteins can influence the asthma response, we assessed the level of Th1 cytokines (IFN- γ) and Th2 cytokines (IL-4 and IL-5) in the BAL fluid, via ELISA. The cytokine levels in the BAL fluid were determined 48 h after the final airway challenge. Sensitization and challenge with OVA were demonstrated to accelerate

the secretion of Th2 cytokines (IL-4 and IL-5) within the lung (Fig. 4). However, the TI-ES and TI-TP immunized groups exhibited low Th2 cytokine levels in the BAL fluid as compared to the OVA challenge only group. The TI-TP and OVA challenge group also evidenced IL-4 and IL-5 levels significantly lower than those of the OVA challenge only group (Fig. 4). The concentrations of the Th2 cytokine levels in the TI-ES and OVA challenge group were similar to those of the OVA challenge only group.

The concentration of IL-10 was reduced slightly by OVA-challenge in the lung. All of the parasite protein-treated groups exhibited slightly higher concentrations of IL-10 cytokines than were observed in the OVA-only challenge group. The immunized TI-TP group exhibited significantly higher IL-10 cytokine levels than those of the OVA-only challenge group (Fig. 4). The level of TGF- β in the lung was also increased by parasite protein treatment, but this effect was not significant.

OVA-specific IgG2a, IgG1 and IgE levels in the BALB/c mice were measured in an attempt to evaluate the involvement of these immunoglobulins in allergic asthma. In the immunized groups, we noted a significant reduction in the serum levels of OVA-specific IgG2a as compared to the OVA-challenge group. Additionally, the IgE levels of immunized groups were reduced slightly, but this effect was not significant (Fig. 5). Otherwise, the serum levels of OVA-specific IgG1 in the OVA challenge groups were slightly reduced as compared to the OVA challenge group. However, the OVA-specific IgE levels in the TI-ES with OVA

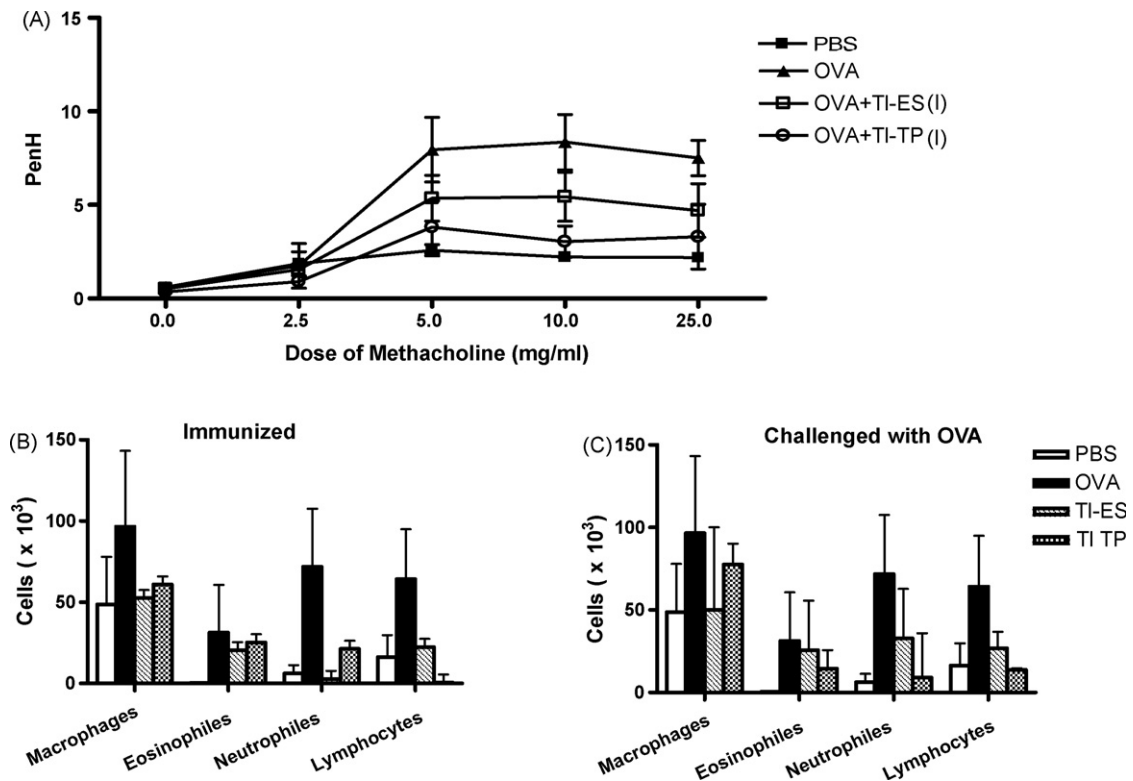


Fig. 3. Airway hyperresponsiveness measurements and comparison of differential cell counts obtained via airway inflammation in ova-sensitized PBS mice and the effects in immunized and OVA-challenged groups. Total protein of *T. leonina* immunization group [TI-TP (I)] has a lower PenH value than those of the OVA challenge group (A). The numbers of inflammatory cells were significantly lower in the parasite-derived protein-treated mice (B and C). The data were expressed as the means $\pm$ S.D. of individual mice.

challenge groups were similar to those observed in the OVA challenge control group.

4. Discussion

Long-lived parasites are highly accomplished practitioners of immune evasion and manipulation, utilizing strategies honed during their long co-evolutionary interaction with the mammalian immune system (Behnke et al., 1992; Maizels et al., 1993). How is the host affected by these parasitic strategies? Although many hypotheses have been advanced, until the present time there has been little evidence to support these theories. Th2 host response as the result of parasitic infection was apparent, although this is the result of parasitic strategies to escape from the host immune system or the result of a human protective system associated with parasitic infection. Additionally, we could readily detect Th2 responses in allergic patients. Thus, until now, there has been considerable controversy regarding the relationship between parasites and allergic reactions; particularly, whether parasites can evoke some allergic specific response or can reduce

allergen-specific responses (Demirturk et al., 2007; Sokol et al., 2007; van Riet et al., 2007).

In this study, we have attempted to determine whether parasite-derived proteins can accelerate or reduce asthma symptoms in accordance with the treatment period. Although some articles have previously asserted that nematodes can induce allergic reactions during their larval migration period (Baeza et al., 2004; Kim et al., 2005), many articles have reported that parasitic infections, particularly chronic parasitism, help to reduce host allergic reactions and to modulate host immune responses (Flohr et al., 2006; van Riet et al., 2007; Wang et al., 2001). We have determined that immunization with *T. leonina* adult worm ES and total proteins induces a downregulation of asthma-associated cytokines, including IL-4 and IL-5, in the BAL fluids (Fig. 4). However, these proteins did not significantly influence allergic airway inflammation response as the result of simultaneous OVA challenge, as compared to the immunization method. In particular, the TI-ES treatment with OVA challenge group exhibited more severe lung inflammation than was observed in the immunized group. We believe that

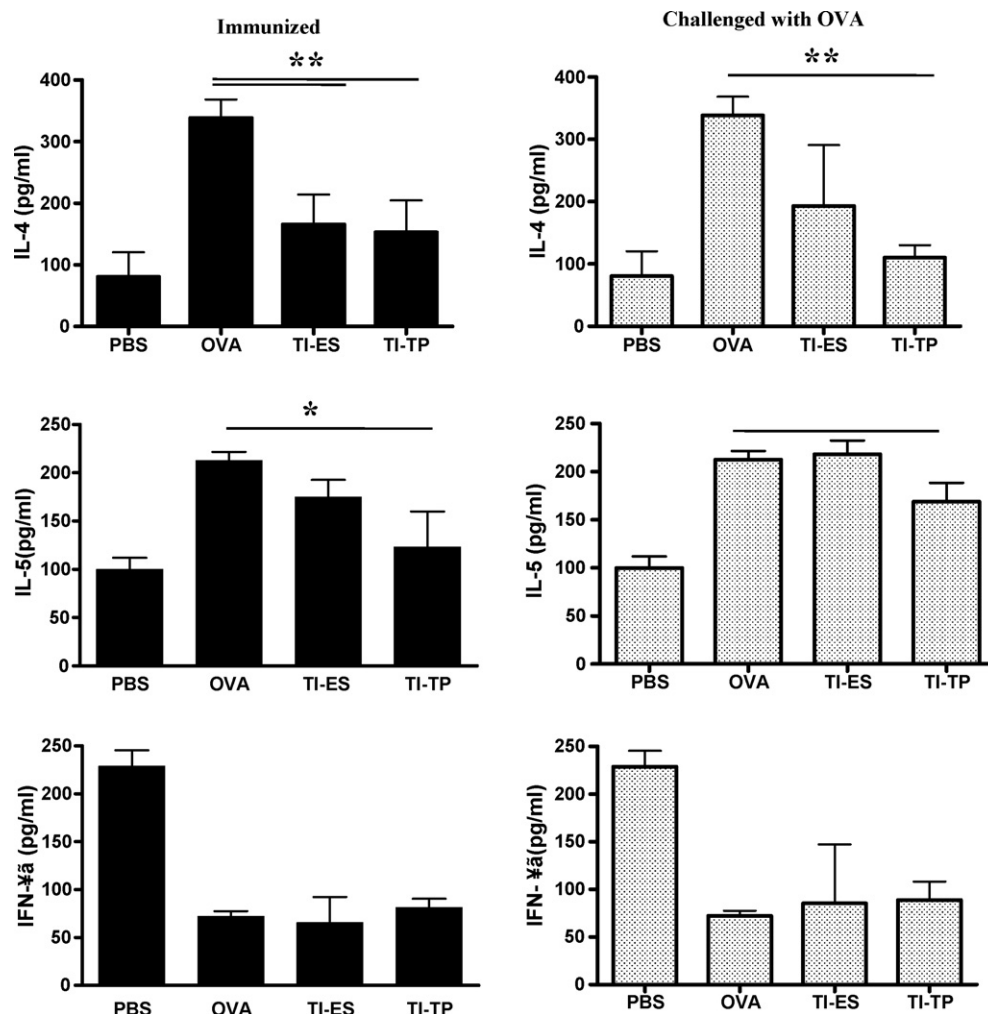


Fig. 4. Cytokine levels in the BAL fluids of TI-ES and TI-TP treated mice. The levels of all Th2 cytokines of the immunized group were contrasted with those of the OVA-challenged control mice. The data were expressed as the means $\pm$ S.D. of individual mice. (The underline groups were being compared to give a significant difference. * p value < 0.05 and ** p value < 0.01 .)

certain allergens or proteases might be included in the ES proteins, and parasitic proteases have also been identified as allergens (Furmonaviciene et al., 2000; Holt et al., 2003; Kobayashi et al., 2007; Shaw et al., 2003). Sokol et al. previously suggested that a host-derived sensor of proteolytic activity might involve cleavage via parasite or allergic proteases. This sensor, once cleaved, activates the cells of the innate immune system to induce a Th2 response (Sokol et al., 2007).

IL-4 has been demonstrated to regulate isotype class switching in B cells to IgE synthesis, and IL-5 stimulates eosinophil growth, activates these cells, and prolongs eosinophil survival. In this study, *T. leonina*-derived proteins could inhibit increases in the levels of IL-4 and IL-5 from OVA challenge. In particular, the level of IL-4 in the BAL fluid in the

immunized group was almost half of that observed in the OVA-only challenge group. This result was consistent with the results regarding the IgE concentration in the blood.

Although levels of the IL-5 cytokine of *T. leonina* ES and total protein-immunized mice were lower than those observed in asthma control mice, this effect was not remarkable. Also, the total number of eosinophils was not substantially reduced as the result of immunization. Eosinophils and IgE proved vitally important in allergy-induced Th2 response; additionally, the elevation of the numbers of these cells and IgE levels were identified as specific responses to parasite infection, and this response was shown to be elicited only by treatment with parasite total proteins (Ehigiator et al., 2000; Ishizaka et al., 1976).

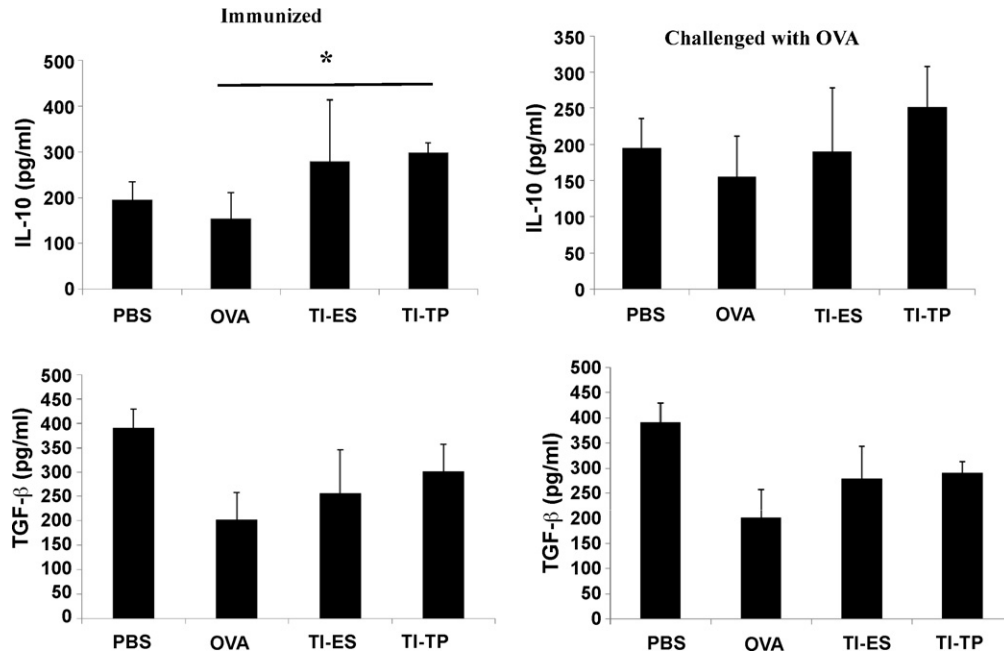


Fig. 4. (Continued).

How does helminth infection protect against allergy? Many hypotheses have been advanced thus far regarding this theme. One of these hypotheses is that nonspecific IgE generated as the result of helminthic infection may inhibit allergen-specific IgE binding sites

on mast cells or basophils. Although Jarrett suggested in 1980 that parasite-induced ‘nonspecific’ IgE does not protect against host allergic reactions (Jarrett et al., 1980). However, other scientists have suggested that nonspecific IgE can modulate host immune responses,

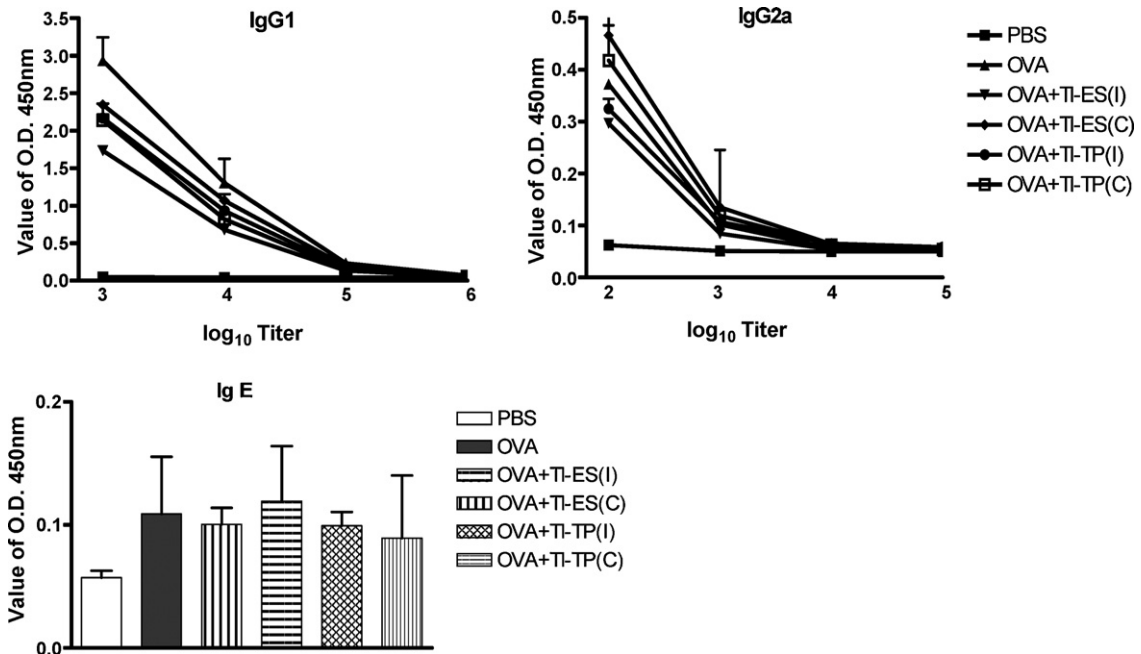


Fig. 5. OVA-specific immunoglobulin levels in the BAL fluids of TI-ES and TI-TP treated mice. IgE concentration was lower in the immunized groups (I) than in the OVA-challenged groups (C). The data were expressed as the means ± S.D. of individual mice.

as in the case of insulin-dependent diabetes, as well as Th2 response by allergens (Ehigiator et al., 2000; Imai et al., 2001a,b; Lynch et al., 1997). The other hypothesis states that helminth parasites stimulate the production of immunoregulatory mediators, which are likely to perform a function in the maintenance of the chronicity of infection, with no marked induction of pathology. In particular, elevated IL-10 levels have been associated previously with protection against allergic diseases in helminth-infected African children (van den Biggelaar et al., 2000). Helminth infections can induce CD4⁺CD25⁺ T cells (regulatory T cell, Treg cell) from hosts, and these cells secrete IL-10 and suppress the proliferation of other CD4⁺ T cells (Maloy et al., 2003; Mottet et al., 2003; Nagler-Anderson, 2006). In this study, parasite proteins could also induce the IL-10 cytokine, particularly in the TI-TP immunized group.

Finally, the data presented herein demonstrate that *T. leonina*-derived proteins may perform a crucial function in resistance against Th2 immune responses. We suggested that this inhibition might be related to the IL-10 cytokine, which was induced by parasite proteins. Further steps are currently being taken in an effort to gain a greater understanding of the molecular basis of immune evasion by nematodes. Thus, we are attempting to gain new insights into the immune regulation strategies of nematodes, and the growing number of new strategies employed by parasites to exert their marked downregulatory effects.

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