International Archives of Allergy and Immunology

Experimental Allergy – Research Article

Int Arch Allergy Immunol
DOI: 10.1159/000511532

Received: July 9, 2020
Accepted: September 11, 2020
Published online: November 11, 2020

Intralymphatic Administration of *Metagonimus yokogawai*-Extracted Protein Attenuates Experimental Murine Allergic Rhinitis Model

Hyung Chae Yang[a] Eun Jeong Won[b] Moon-Ju Kim[b] Chung Man Sung[a] Joon Haeng Rhee[c] Kwang Il Nam[d]

[a]Department of Otolaryngology-Head and Neck Surgery, Chonnam National University Medical School and Chonnam National University Hospital, Gwangju, South Korea; [b]Department of Parasitology and Tropical Medicine, Chonnam National University Medical School, Hwasun-gun, Jeollanam-do, South Korea; [c]Department of Microbiology, Chonnam National University Medical School, Hwasun-gun, Jeollanam-do, South Korea; [d]Department of Anatomy, Chonnam National University Medical School, Hwasun-gun, Jeollanam-do, South Korea

Keywords
Allergic rhinitis · Helminthic therapies · *Metagonimus yokogawai*

Abstract
Objectives: This study aimed to evaluate potential therapeutic effect of *Metagonimus yokogawai* on the OVA-induced allergic rhinitis model. ***Methods:*** OVA-sensitized mice were used to assess potential therapeutic effect of the extract protein of *M. yokogawai* (My-TP). My-TP was administrated via the intralymphatic route to cervical lymph nodes. The frequencies of sneezing or nasal rubbing were recorded. Histopathologic evaluation was performed for eosinophil infiltrations in the tissues of the nasal mucosa and skin. The mRNA relative expressions of the cytokine profiles including Th1, Th2, Th17, and Treg subsets in the nasal mucosa, cervical lymph nodes, and spleen were analyzed by quantitative real-time reverse-transcriptase polymerase chain reaction. The potential underlying mechanism was investigated by examining cytokine profiles including IL-4 and Treg subsets from lymphocytes of the spleen by flow cytometry. ***Results:*** Intralymphatic injection of My-TP reduced allergic symptoms and eosinophil infiltration in the nasal mucosa. My-TP-treated group showed markedly decreased levels of OVA-specific IgE and WBC counts in nasal lavage. My-TP-treated group showed the decreased expression levels of IL-4, while those of IL-10 were increased in both the nasal mucosa. The levels of IFN-γ and IL-17 were also decreased in the nasal mucosa and cervical lymph nodes. The immunological mechanism may involve the downregulation of Th2 response and upregulation of Tregs in the nasal mucosa and cervical lymph nodes. ***Conclusions:*** Our results provide the first evidence of potential therapeutic effect of *M. yokogawai* in OVA-sensitized allergic rhinitis mice, suggesting that a Treg/Th2 reorganization may play a role in clinical course of allergic rhinitis.
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Introduction

Allergic rhinitis (AR) is a common and chronic inflammatory disease which affects >400 million people worldwide and increases health-care cost [1, 2]. It is characterized by an influx of eosinophils and Th2 excessive activation and aggravates other conditions, such as sinusitis and asthma [3–5]. Several treatment options such as

Edited by: H.-U. Simon, Bern.

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Eun Jeong Won, Department of Parasitology and Tropical Medicine
Chonnam National University Medical School, Hwasun-gun or Kwang Il Nam
Department of Anatomy Chonnam National University Medical School Hwasun-gun
Jeollanam-do 58128 (South Korea)
Parasite.woni@jnu.ac.kr or atlas@jnu.ac.kr

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intranasal corticosteroids, antihistamines, decongestants, cromolyn, leukotriene receptor antagonists, nasal irrigation, subcutaneous or sublingual immunotherapy, and even surgical management have been suggested [6–8]. However, there is still lack of definitive treatment of AR.

Air pollution, climate change, and global warming are factors that underlie the increasing prevalence of allergic diseases, and most of all, urbanization or industrialization has been known to be associated with the higher prevalence of allergic diseases [9]. It is usually explained by the hygiene hypothesis [10, 11], which involves the loss of cellular and humoral immunoregulatory pathways as a result of the adoption of a Western lifestyle and the disappearance of chronic infectious diseases albeit clear mechanistic insights into the process are still unraveled. Based on this hypothesis, several attempts recently have been made to administer parasites, especially helminths, as new treatment modalities on treating allergic diseases by reversing the established Th2 response [12]. In terms of the parasite (trematodes), there was 1 study showing that *Clonorchis sinensis*-derived total protein can reduce OVA-induced airway inflammation using a murine asthma model [13]. Here, we attempt to find out an alternative therapeutic target for AR with less pathogenic parasites, instead of *C. sinensis* which is recognized as a biological carcinogen. We speculate that *Metagonimus yokogawai,* a prevalent intestinal trematode in Korea, also can possess the potential to the treatment of allergic diseases such as AR or asthma model. Thus, we aimed to assess the immunomodulatory effects of intralymphatic injection of *M. yokogawai*-induced proteins on the AR model, providing a basis for further clinical applications of *M. yokogawai* on treating allergic diseases.

Materials and Methods

Mice

Pathogen-free, female Balb/c mice, 6 weeks of age, were inbred and housed in a specific pathogen-free facility in individually ventilated and filtered cages according to institutional-approved guidelines on OVA-free diets and water ad libitum for all experiments. All experimental animal procedures used in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Ethics Review Committee for Animal Experimentation of the Chonnam National Medical School (CNU IACUC-H-2017-16).

M. yokogawai Parasites and Antigen Preparations

The metacercariae of *M. yokogawai* were collected from the muscle of the sweetfish, *Plecoglossus altivelis*, caught from Seomjin-gang (river), Jeollanamdo, a known endemic area in South Korea. For preparation of metacercariae, the mortar-ground fish flesh was treated with artificial digestive solution containing 0.6% pepsin (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) and 0.8% HCl at 37°C for 2 h. The resulting product containing free metacercariae was washed several times with normal physiological saline. The metacercariae were collected under a stereomicroscope and preserved at 4°C until use. The collected metacercariae were mixed with 1 mL of homogenation buffer (5 mM EDTA, 1% NP-40, and 0.2 mM PMSF) and were homogenized, vortexed for 5 min, and centrifuged under 13,000 rpm, 20 min at 4°C. After centrifuging, the supernatant was used for protein extraction using Pierce BCA Protein Assay Kit (Thermo Scientific Co., Rockford, IL, USA) according to the manufacturer's guideline. The extract protein (My-TP) was concentrated with 1.2–1.5 μg/10 μL of the working reagent.

Murine AR Model and Treatment

The sensitization and antigen challenge for the murine model of AR were induced as previously described with minor modification [14, 15]. In brief, under pathogen-free conditions, mice were sensitized using OVA (OVA; grade V; Sigma, St. Louis, MO, USA) as follows. OVA (40 μg/kg, about 25 ug) diluted in sterile phosphate-buffered saline (PBS) was administered along with 1-mg aluminum in a total volume of 200 μL PBS (alum adjuvant, 40 mg/kg) to mice 4 times by intraperitoneal injection on days 0, 7, and 14. This was followed by daily intranasal challenge with OVA diluted with sterile normal saline (20 μL of 25 mg/mL OVA per mouse) into each nostril from days 25 to 29. Twenty-four hours after the last OVA challenge, blood, nasal lavage fluids, spleen tissues, cervical lymph nodes, nasal mucosa, or nasal cavity tissues were collected from each mouse. For the collection of nasal lavage fluids, mice were held in the nose-down position, and a 22-gauge catheter was inserted into the tracheal opening in the direction of the posterior choanae [16]. About 200 μL of PBS was gently introduced into the nasal cavity over 30 s, and the fluid from the nostril was collected and centrifuged. After cytospin preparation, nasal lavage cells were stained with Wright's stain and were counted

Fig. 1. Immunomodulatory effects of intracervical lymphatic *M. yokogawai* total protein administration in our mouse model of AR. **a** Induction of experimental AR and allergic symptoms in each group. **b** Microscopic analyses revealed that PC mice exhibited typical characteristics of AR. PC and sham groups exhibited significant eosinophilic infiltrations in the nasal mucosa (within red dotted box). MY ICL administration reduced eosinophilic infiltration in the nasal mucosa (Sirius Red stain; magnification, ×600). **c** OVA-specific IgE levels were significantly lower in MY ICL-treated mice than in PC mice ($p < 0.001$). **d** MY ICL-treated mice had significantly lower IL-4 and GATA3 mRNA levels compared with PC or sham-treated mice ($p < 0.01$). IFN-γ, T-bet, IL-17, and RORt mRNA levels were also reduced in MY ICL-treated mice compared with PC or sham-treated mice, while IL-10 and TGFb mRNA levels were elevated ($p < 0.05$). AR, allergic rhinitis; NC, negative control; PC, positive control of ovalbumin-injected mice with AR; sham, sham-treated mice; MY ICL, mice that underwent intracervical lymphatic administration of *M. yokogawai* total protein.

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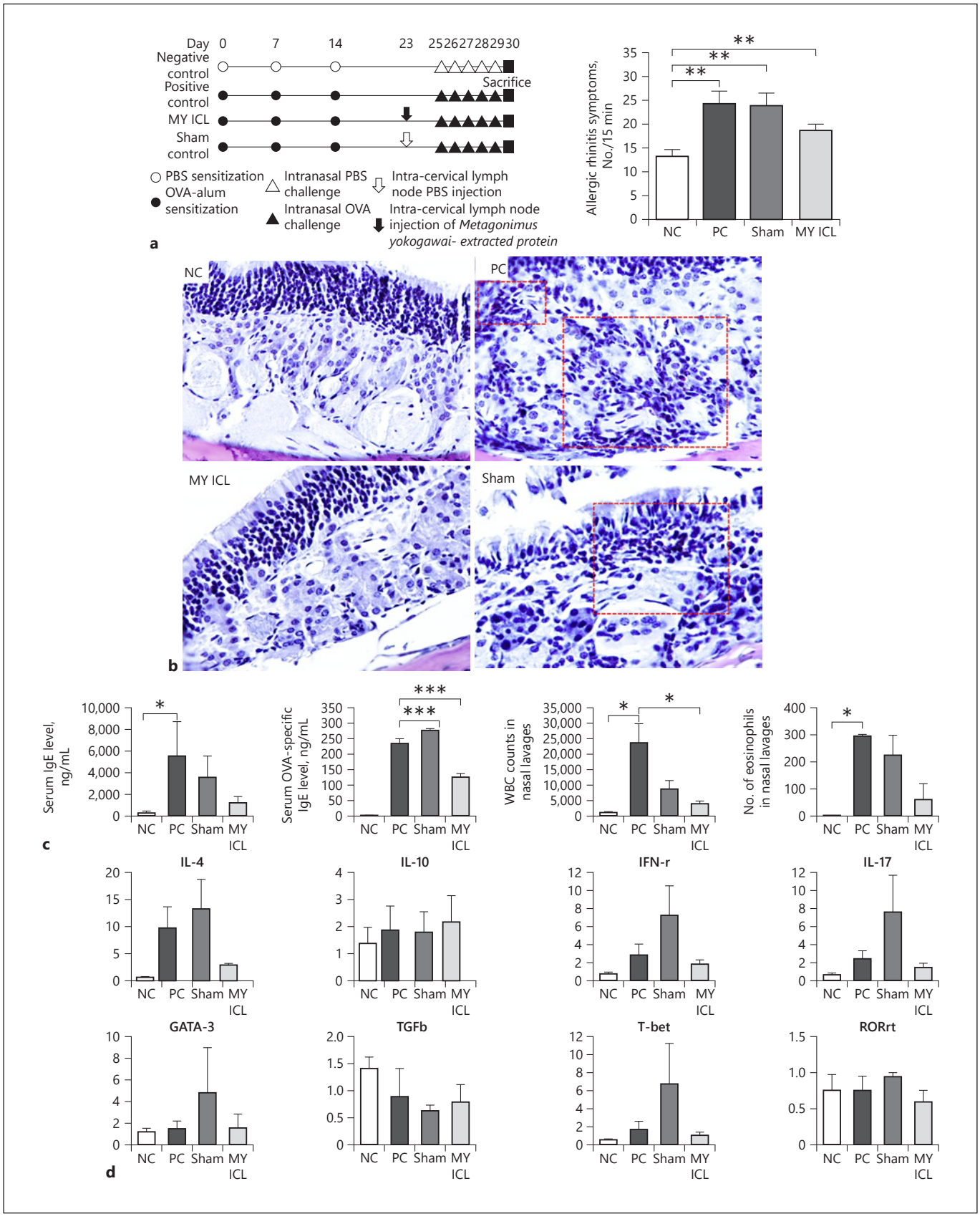

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manually. Mice were divided into 4 groups, with 8 mice in each group. For the negative control (NC) group, mice were sensitized and challenged with PBS without treatment. For the positive control (PC) group, mice were sensitized with OVA and alum and challenged with OVA without treatment. For the treatment group (MY ICL or sham), mice were sensitized with OVA and alum, and then intracervical lymphatic injection (My-TP or PBS) was done at day 23 and challenged with OVA, as previously described with minor modification [14]. In brief, mice were sedated with a subcutaneous injection of 2 mg of Zoletil and 0.2 mg of Rompun, and the cervical lymph node was located after skin incision around the neck. About 10 μL of My-TP or PBS were then injected into the lymph node for MY ICL and sham groups, respectively, and the skin was sutured.

Measurement of AR Symptoms

Fifteen minutes after the final nasal challenge with OVA, the frequencies of sneezing or nasal rubbing were recorded for each mouse by blinded observers, over a 15-min interval, following the methods of Kim et al. [15]. The mice were then killed 24 h after the last nasal challenge for further analyses [17].

Serum Levels of Total and OVA-Specific IgE

Serum samples collected from mice at the time of sacrifice were serially diluted. The levels of total IgE and OVA-specific IgE were measured using Mouse IgE ELISA Kit (MyBioSource, San Diego, CA, USA) and Mouse OVA-specific IgE ELISA Kit (MyBioSource, San Diego, CA, USA), respectively. The OD was recorded by a luminometer (iEMS Reader; Labsystems, Helsinki, Finland) set at 450 nm.

Nasal Histology

Twenty-four hours after the final OVA nasal challenge, nasal cavity tissues were removed from the mice, fixed in 4% paraformaldehyde, and embedded in paraffin. Samples were cut into 4-μm cross sections and were stained with Sirius Red stain, hematoxylin and eosin, Giemsa, and toluene blue stain for measuring mast cells and eosinophils. The numbers of eosinophils were counted in the nasal septal mucosa under a light microscope (600 magnification). The degree of inflammatory cell infiltration was scored in a double-blind screen by 2 independent investigators.

Real-Time RT-PCR for Th1/Th2/Th17/Treg Cytokines

Total RNA was extracted from the spleen, nasal mucosa, and cervical lymph nodes using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Complementary DNA (cDNA) was synthesized using PrimeScript™ first strand cDNA Synthesis Kit (Takara, Shiga, Japan). A quantitative analysis of the relative mRNA expression of different cytokines was performed to determine the balance between Th1, Th17, Th2, and Treg cells. The cDNA of IL-4, IL-17, IFN-γ, TGFb, IL-10, T-bet, RORrt, GATA, Foxp3, and GAPDH was amplified using PowerUp SYBR Green Master Mix (Applied Biosystems, Vilnius, Lithuania) and then quantified using StepOne Real-Time System (Applied Biosystems, Marsiling, Singapore). The forward and reverse primers were used according to the previous report [18]. The parameters for PCR amplification were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Each sample was experimented with duplication. GAPDH was used as an endogenous housekeeping gene to normalize the results (ΔCt), and then relative gene expression (ΔΔCt) was calculated as the fold change in expression of the group compared to the mean expression values of control mice without any procedures (set to 1.0). Final value of relative gene expression was demonstrated as $2^{-\Delta\Delta Ct}$.

Spleen Cell Culture and Flow Cytometry Analysis of Lymphocytes

The mouse spleen was isolated aseptically and then gently ground in the RPMI1640 medium. The cells were harvested after mashing through a 40-μm cell strainer and suspended in RPMI1640 supplemented with 10% bovine fetal serum, 2 mML-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were subjected to APC-labeled anti-mouse CD4 (BD Biosciences), followed by PerCP-Cy5.5-labeled anti-mouse IL-4 (BD Biosciences), for intracellular cytokine staining after using the Permeabilization Buffer (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions. For Tregs examination, cells were first stained with APC-labeled anti-mouse CD4 and FITC-labeled anti-mouse CD25 (BD Biosciences). After surface staining, the cells were stained with PE-labeled anti-mouse Foxp3 (eBiosciences) antibodies after incubation with Foxp3/Transcription (Invitrogen, San Diego, CA, USA) at 2–8°C for 30 min in protection from light. Cells were analyzed on a flow cytometer.

Statistical Analysis

Student *t* tests were used to compare continuous variables, and χ^2 or Fisher's exact test was performed to determine the distributions of categorical variables, respectively. All graphs show data of mean with standard error of mean. All statistical analyses were performed using the Graph Prism 5.0 software. A p value <0.05 was considered to indicate significance.

Results

AR was induced by OVA administration in mice; these mice exhibited typical allergic symptoms such as sneezing and rubbing, in addition to hair loss on the nose (Fig 1 and online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000511532). Cervical intralymphatic administration of *M. yokogawai*-derived proteins (MY ICL) mitigated nasal allergy symptoms compared with PC mice, suggesting that MY ICL suppressed OVA-induced AR. Histopathologic analysis revealed that the nasal mucosa of OVA-injected mice exhibited significant infiltrations with inflammatory cells, predominantly eosinophils (Sirius Red stain, ×600) (Fig. 1b). In contrast, MY ICL administration significantly reduced eosinophilic infiltration in the nasal mucosa. MY ICL administration also significantly reduced OVA-specific IgE levels, compared with PC mice ($p < 0.001$) (Fig. 1c).

To further investigate the immunomodulatory effects of MY ICL on T cells, we examined mRNA levels of var-

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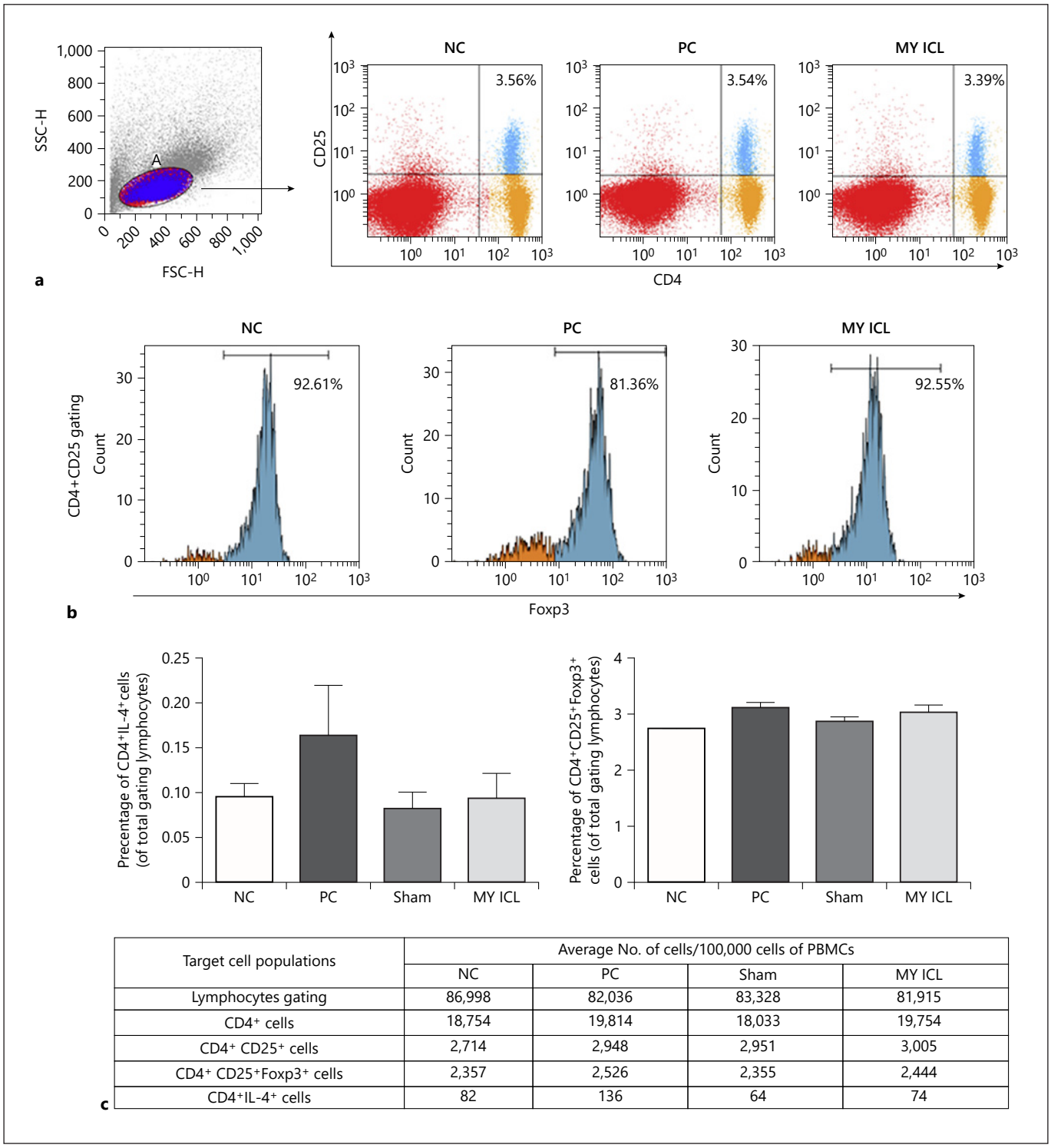

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Target cell populations	Average No. of cells/100,000 cells of PBMCs			
	NC	PC	Sham	MY ICL
Lymphocytes gating	86,998	82,036	83,328	81,915
$CD4^+$ cells	18,754	19,814	18,033	19,754
$CD4^+$ $CD25^+$ cells	2,714	2,948	2,951	3,005
$CD4^+$ $CD25^+Foxp3^+$ cells	2,357	2,526	2,355	2,444
$CD4^+IL\text{-}4^+$ cells	82	136	64	74

Fig. 2. Effects of intracervical lymphatic *M. yokogawai* total protein (MY ICL) administration on splenic T cells. **a**, **b** The proportion of $CD25^+Foxp3^+$ Tregs among splenic $CD4^+$ T cells was determined after staining with anti-CD4 (FITC), anti-CD25 (PE), and anti-FOXP3 (APC) and then analyzed by flow cytometry. **c** The proportion of IL-4-secreting $CD4^+$ T cells was elevated in mice with AR. MY ICL administration significantly reduced the proportion of IL-4-secreting $CD4^+$ T cells. The proportion of $CD4^+CD25^+$ $Foxp3^+$ cells did not differ significantly among the groups. The number of each target cell populations is listed as a subtable format. AR, allergic rhinitis; NC, negative control; PC, positive control.

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ious cytokines and transcription factors in the nasal mucosa (Fig. 1d). Specifically, we investigated the expression of Th1 markers (IFN-γ and T-bet), Th2 markers (IL-4 and GATA3), Th17 markers (IL-17 and RORt), and Treg markers (IL-10 and TGFb). MY ICL-treated mice had significantly lower *Il4* and *Gata3* levels, compared with PC or sham-treated mice ($p < 0.01$). Moreover, IFN-γ, T-bet, IL-17, and RORt mRNA levels were also reduced in MY ICL-treated mice. Conversely, IL-10 and TGFb mRNA levels were higher in MY ICL-treated mice than in PC or sham-treated mice ($p < 0.05$). These immunomodulatory effects of MY ICL administration were confirmed in T cells found in the spleen and adjacent lymph nodes (online suppl. Fig. 2, 3); however, these immunomodulatory effects were absent in cervical lymph nodes although elevated IL-4 levels and reduced IL-10 levels were found. Although the proportion of IL-4-secreting $CD4^+$ T cells was increased in PC mice, MY ICL administration reduced the proportion of this cell population (Fig. 2).

Discussion

Herein, we firstly suggest the potential of *M. yokogawai* as an immune-modulative therapeutic modality for AR controls. Previously, it had been proposed that helminth infection induced the adaptive production of regulatory T cells and cytokines, such as IL-10 and transforming growth factor-beta, indicating a therapeutic clue of *Trichuris suis* ova in TH2-mediated disease [13]. Additional studies performed in animals revealed adaptation of immune regulation by way of increasing regulatory T cells in airway hyperreactivity [19]. However, there is still controversy over the direct intake of *T. suis* eggs for the treatment of AR. In a placebo-controlled study treating grass pollen-induced AR with *T. suis* eggs [20], no statistically significant difference in AR symptoms was found, and further diarrhea due to helminth infection was an important side effect. The protective effects of *C. sinensis*-derived total protein were suggested on allergen-induced airway inflammation [13] although *C. sinensis* is limited as a biological carcinogen [21]. Instead of the direct application of parasites that is unlikely to be acceptable with risk of development of parasitic diseases, several researchers have attempted to use parasite-derived antigens instead of the parasite itself to obtain desirable outcomes. We also utilized *M. yokogawai*-derived proteins rather than direct infection because human immune response to helminth infections can be mimicking with allergic inflammatory responses. We found that intralymphatic injection of *M. yokogawai*-induced proteins ameliorated allergic nasal symptoms, including rubbing and sneezing, and reduced the numbers of eosinophils in the nasal mucosa. It could also reduce the circulating level of OVA-specific IgE and inflammatory cell infiltrations in the submucosa. Th2 cytokine levels were declined according to the intralymphatic injection of *M. yokogawai*-induced proteins. The protective effect on AR was found even with small amount of *M. yokogawai*-induced proteins through the intralymphatic administration. Our data supported that the intralymphatic treatment might be effective by enhancing immunogenicity in AR. Collectively, our data supported that downregulation of the excessive activation of Th2 can be induced by intralymphatic injection of *M. yokogawai*-induced proteins and may be a feasible way to control AR.

The current study showed that *M. yokogawai*-induced proteins also decreased Th2 cytokine levels but increased regulatory cytokine levels such as IL-10 and TGFb and $CD25^+Foxp3^+$ Treg population. Previously, IL-10 has been known to suppress immune responses by inhibiting the priming of naïve T cells and inflammatory cytokine production by dendritic cells and also suppressed cytokine release from Th2 cells and affects allergic responses by mast cells and eosinophils [22]. However, although the authors proposed that Tregs mediated the protective effect, the role of TGFb-secreting macrophages was not explored. Therefore, notwithstanding the fact that Tregs can afford protection from autoimmune disease, as has been demonstrated in a multitude of adoptive transfer studies, we suggest that by the intralymphatic injection of *M. yokogawai*-induced proteins, the modulation of macrophage function into a phenotype that acts to regulate immune responses (the expansion of Tregs being one such regulatory mechanism) may be the critical first step in the protection of AR. Comprisingly, our data showed that IL-10 and Treg cell recruitments are the most important factors in amelioration effects of airway inflammation by *M. yokogawai*-induced proteins.

There are some limitations in this study. First, we could not confirm the exact distribution of intranasal particles after challenge [21]. Albeit, AR symptom score and histological findings support that our AR models were made successfully. Second, we could not stain with anti-CD45 and anti-CD3 beads due to limitation of fluorescent color of our flowcytometry; however, we focused on $CD4^+CD25^+Foxp3^+$ cells and IL-4^+ T cells instead. Collectively, our data highlight that cervical intralymphatic injection of *M. yokogawai*-derived proteins sup-

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presses Th2 immune responses and mitigates AR in a Treg-dependent manner. Hence, cervical intralymphatic injection of *M. yokogawai*-derived proteins may serve as a promising approach to treat AR.

Statement of Ethics

All experimental animal procedures used in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Ethics Review Committee for Animal Experimentation of the Chonnam National Medical School (CNU IACUC-H-2017-16).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017M3A9E802302; NRF-2016R1C1B1009746; NRF-2019R1C1C1004605; NRF-2019M3E5D1A02067953) and a grant (BCRI-20071) of the Chonnam National University Hospital Biomedical Research Institute. The funders had no role in the study design, data collection, and analysis decision to publish or in preparation of the manuscript.

Author Contributions

H.C.Y. and E.J.W. designed this study. H.C.Y. contributed in the data collection and the writing and critical revision of this paper. M.J.K. and C.M.S. contributed in the interpretation of the results and reviewed the related articles. J.H.R. also contributed in data collection and data analysis. K.I.N. and E.J.W. equally contributed to this study as corresponding authors and supervised this study, conceptualized much of this study, designed the measures analyzed, reviewed and revised the initial version of this paper, and approved the final version.

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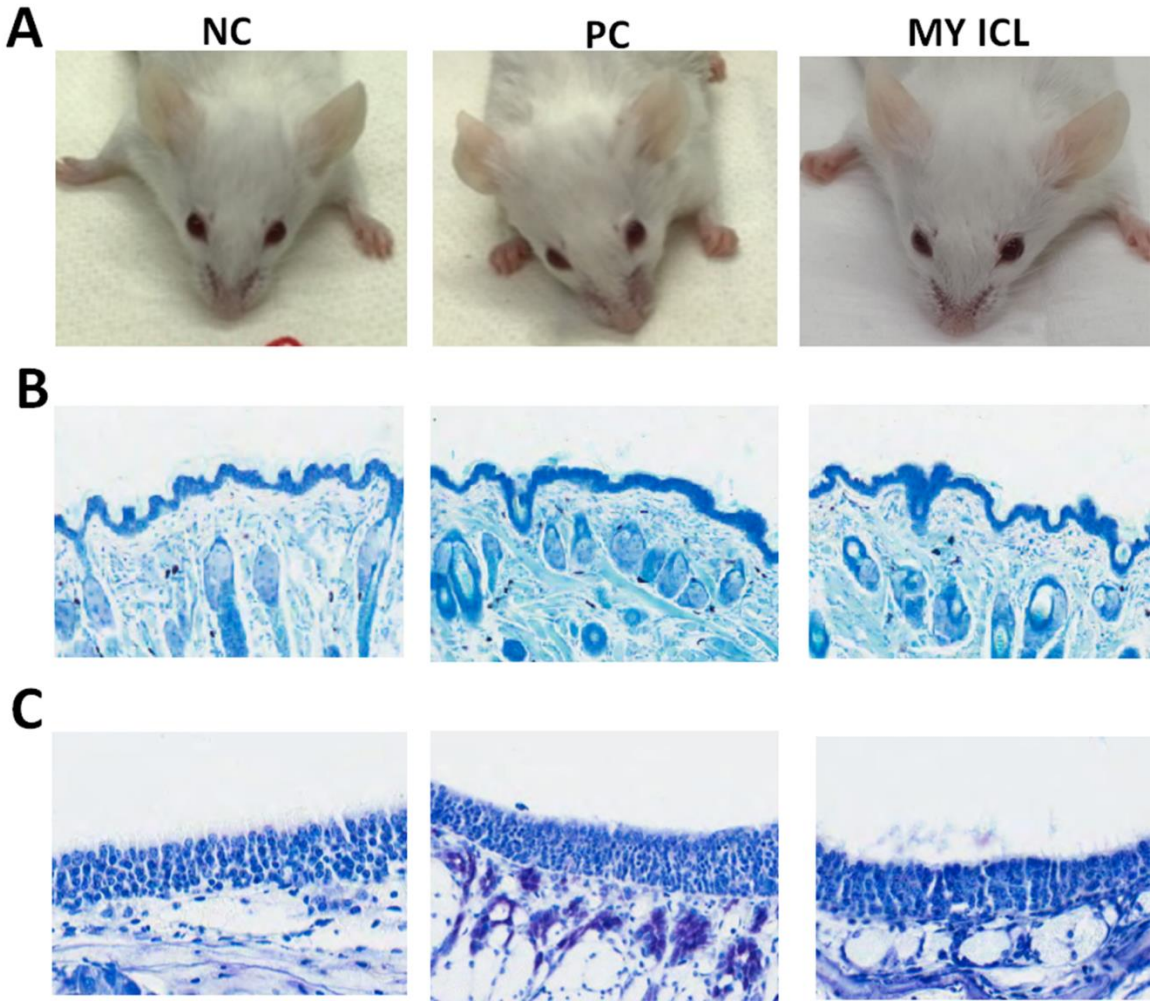
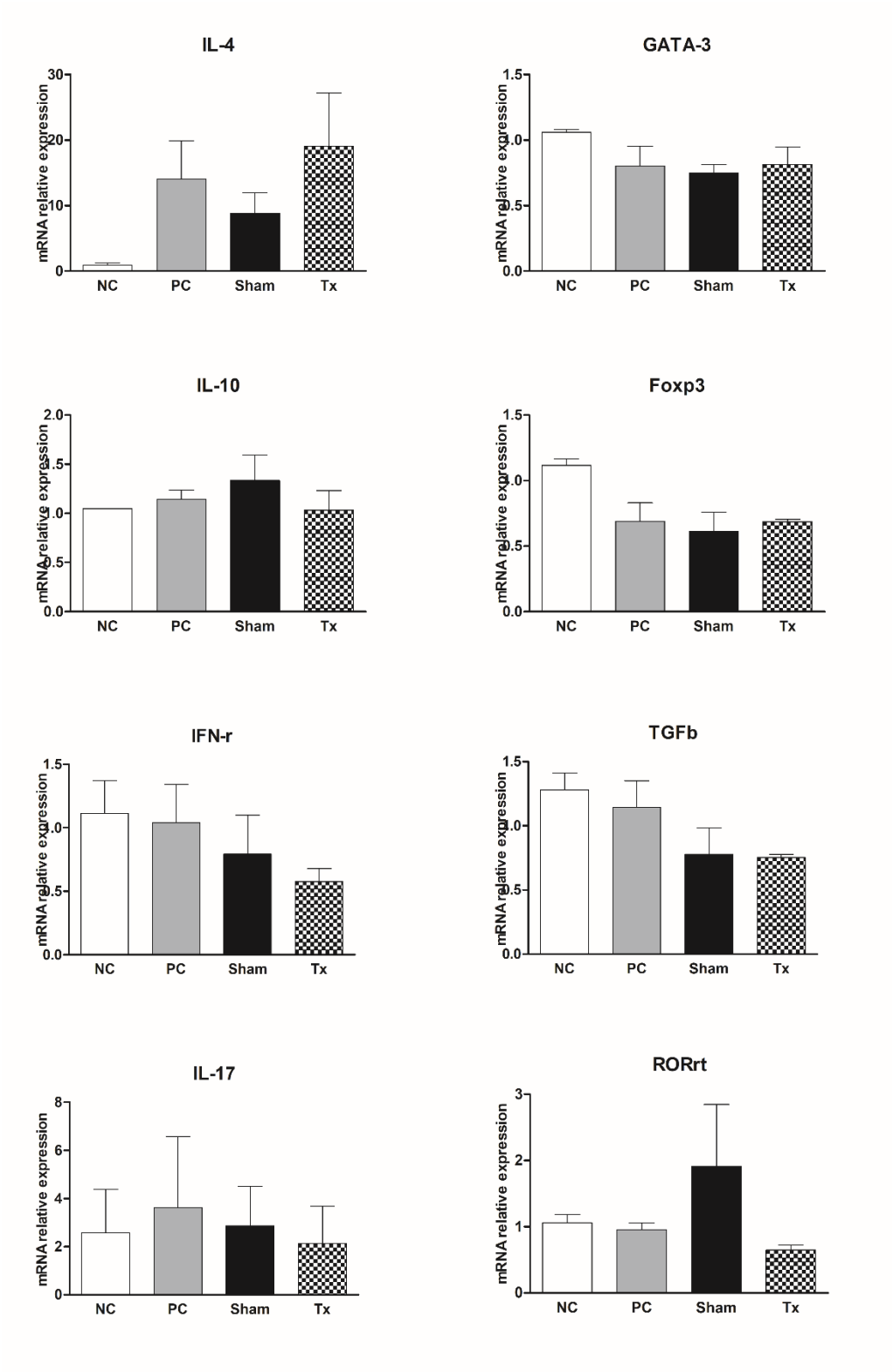
Supplementary Data

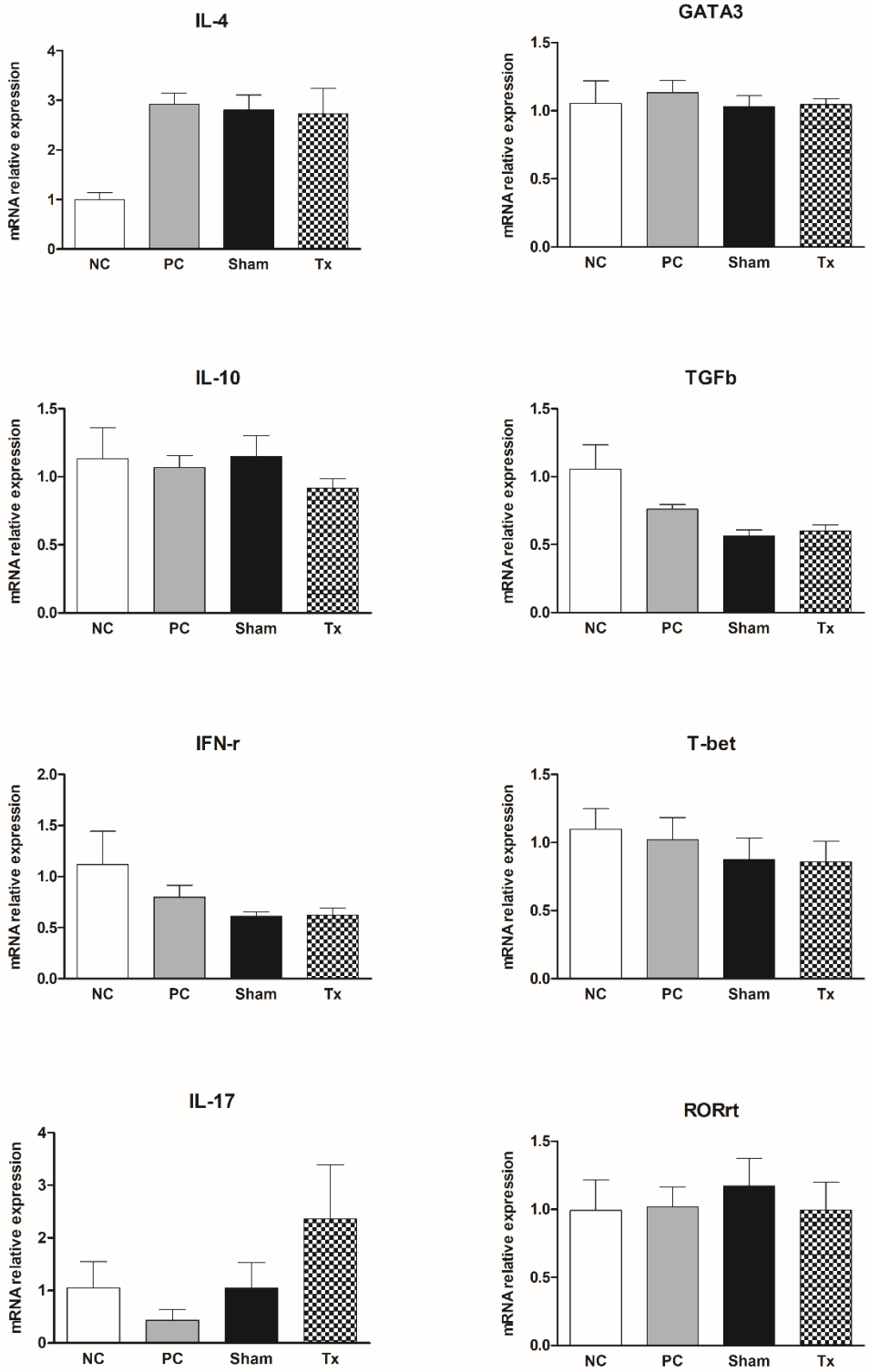


Supplementary Figure 1. Macroscopic and microscopic findings in each group (NC, negative control; PC, positive control of ovalbumin-injected mice with allergic rhinitis; MY ICL, mice that underwent intral cervical lymphatic administration of *Metagonimus yokogawai* total protein). (A) Significant hair loss on nose was found in PC group compared to NC or MY ICL groups. (B and C) Toluene blue and Giemsa stain were performed for measuring Mast cells and eosinophils. Infiltration of numerous mast cells in the submucosa of nasal skin and eosinophils in the nasal mucosa were reduced in MY ICL group.



Supplementary Figure 2. Analysis of the effects of intra-cervical lymph nodal administrations of *Metagonimus yokogawai*-extracted protein on the cervical lymph nodal expression of mRNAs encoding Th1, Th2 and Th17 cell-related cytokines and of Th1, Th2, Th17, and Treg

cell-specific transcription factors using real-time PCR. The relative mRNA expression levels of IL-4 and GATA-3, IL-10 and Foxp3, IFN-γ and T-bet, IL-17 and RORγt were demonstrated according to the study groups.



Supplementary Figure 3. Analysis of the effects of intra-cervical lymph nodal administrations of *Metagonimus yokogawai*-extracted protein on the splenic expression of mRNAs encoding Th1, Th2 and Th17 cell-related cytokines and of Th1, Th2, Th17, and Treg cell-specific

transcription factors using real-time PCR. The relative mRNA expression levels of IL-4 and GATA-3, IL-10 and Foxp3, IFN-γ and T-bet, IL-17 and RORγt were demonstrated according to the study groups.