

Therapeutic effects of *Echinococcus granulosus* cystic fluid on allergic airway inflammation

Hye-Jin Kim^{a,b}, Shin-Ae Kang^{a,b}, Tai-Soon Yong^c, Myeong-Heon Shin^c, Kyu-Jae Lee^d, Gab-Man Park^e, Uktamjon Suvonkulov^f, Hak Sun Yu^{a,b,*}

^a Department of Parasitology, School of Medicine, Pusan National University, Yangsan-si, Gyeongsangnam-do, 626-870, Republic of Korea

^b Immunoregulatory Therapeutics Group in Brain Busan 21 Project, Republic of Korea

^c Department of Environmental Medical Biology, Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul, 03722, Republic of Korea

^d Department of Environmental Medical Biology, Yonsei University Wonju College of Medicine, Wonju, 26426, Republic of Korea

^e Department of Environmental Medical Biology, Catholic Kwandong University College of Medicine, Gangneung, 25601, Republic of Korea

^f Isaev Research Institute of Medical Parasitology, Ministry of Health, Republic of Uzbekistan

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ABSTRACT

Previous studies showed that *Echinococcus granulosus* infection reduces allergic airway inflammation in experimentally infected hosts and the cystic fluid of *E. granulosus* is known to activate regulatory T (CD4⁺CD25⁺Foxp3⁺T, Treg) cells. To evaluate the effects of cystic fluid of *E. granulosus* on allergic airway inflammation, we investigated the regulation of the inflammatory reaction by cystic fluid using an allergic airway inflammation animal model. Cystic fluid was administered to C57BL/6 mice seven times every other day, after which allergic airway inflammation was induced using ovalbumin and aluminum. The airway resistance, number of eosinophils and other immune cells in the bronchoalveolar lavage fluid, and levels of Th2 and Th17-related cytokines were significantly reduced by cystic fluid pre-treatment in allergic airway inflammation-induced mice. The number IL-4⁺CD4⁺ T cells decreased, the number of Treg cells increased in the lung-draining lymph nodes and spleen of cystic fluid pre-treated mice. In conclusion, *E. granulosus*-derived cystic fluid may alleviate the Th2 allergic airway inflammatory response via Treg cells. Further studies of the immune regulation of cystic fluid may lead to the development of therapeutic agents for immune disorders.

1. Introduction

Parasite infection stimulates a powerful localized T helper (Th)2 response as part of the protective immunity of the host immune system (Fallon and Mangan, 2007). Therefore, regulation of the strong Th2 response is thought to be important for parasite survival against the actions of the host immune system. After infection, parasites promote the induction of regulatory responses, such as increased numbers of interleukin (IL)-10-releasing lymphocytes, which has been linked to immune modulation in many disease models (Wang et al., 2014). Additionally, helminths cause an increase in the number of CD4⁺CD25⁺forkhead box P3⁺ (Foxp3⁺) T cells (regulatory T cells; Treg cells), which can elevate the production of interleukin (IL)-10 and transforming growth factor-β (TGF-β).

Echinococcosis is a zoonotic disease distributed worldwide and caused by infection with *Echinococcus granulosus* (Eckert and Deplazes,

2004). The disease is vastly endemic in livestock-farming countries and a large number of people suffer from echinococcosis. During the pathogen life cycle, an *E. granulosus* cyst can develop in various organs, particularly in the liver and lungs (Moro and Schantz, 2009). The host immune system typically becomes activated during parasite infection at the parasite-host interface or infected tissues. *Echinococcus granulosus* infection and extracts of the *E. granulosus* laminated layer also promote immune regulation by the host to modulate inflammatory responses (Mondragon-de-la-Pena et al., 2002; Soufli et al., 2015). The number of peripheral Treg cells, cytokine levels of IL-10 and TGF-β, and levels of Foxp3 in patients who suffer from echinococcosis are significantly increased, while the levels of Th17-related cytokines are decreased compared to those without the infection (Tuxun et al., 2012). *In vivo* experiments showed that infection with *E. granulosus* ameliorates the colitis and allergic airway inflammatory response, and extracts of the hydatid laminated layer alleviated colitis symptoms in mice (Khelifi

* Corresponding author. Department of Parasitology, School of Medicine, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do, 626-870, Republic of Korea.

E-mail address: hsyu@pusan.ac.kr (H.S. Yu).

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et al., 2017; Soufli et al., 2015; Wang et al., 2014).

Immunomodulatory molecules (IMs) from helminths promote immune modulatory cells, such as regulatory-like dendritic cells (DCs) and alternatively activated macrophages (AAM, M2) to regulate the balance between Th1/Th17 cells which mediate autoimmune diseases and Th2 cells which mediate allergy or asthma (Finlay et al., 2014; Khan and Fallon, 2013). In *Echinococcus* infection, the hydatid cystic fluid may contain numerous IMs that ameliorate host immune disorders. However, no studies have examined the amelioration abilities of hydatid cystic fluid in an allergic and inflammation mouse experimental model.

To determine whether *E. granulosus* cystic fluid plays an immunological role in allergic airway inflammation, we evaluated its immune regulatory effects in an ovalbumin (OVA)-induced allergic airway inflammation model.

2. Materials and methods

2.1. Cystic fluid sampling

Hydatid cystic fluids were collected from *E. granulosus*-infected lungs and livers of sheep from a sheep slaughterhouse. Cystic fluid was centrifuged at $500 \times g$ at 4°C for 5 min to remove the protoscoleces and stored at -20°C until analysis.

2.2. Experimental design for inducing allergic airway inflammation

Six-week-old female C57BL/6 mice were purchased from Orient-bio Co. (Gyeonggi-do, Korea) and maintained in a specific pathogen-free facility at the Institute for Laboratory Animals of Pusan National University during experimentation. To induce allergic airway inflammation, an OVA-Alum immunization model was used (Fig. 1A). Briefly, cystic fluid was administered orally to C57BL/6 mice 7 times every other day at a dose of 1 mg. Next, the mice were sensitized with $75\ \mu\text{g}$ of OVA (Sigma-Aldrich, St. Louis, MO, USA) adsorbed onto 2 mg of aluminum hydroxide (Alum; Sigma-Aldrich) in $200\ \mu\text{L}$ of phosphate-buffered saline (PBS) by intra-peritoneal injection on days 14, 15, 21, and 22. The mice were challenged with $50\ \mu\text{g}$ of OVA by intra-nasally

(I.N.) administration on days 28, 29, 35, and 36. On day 37, airway hyperresponsiveness (AHR) was assessed. On day 38, the mice were sacrificed. We divided three mice groups for this experiment, control group (PBS I.P. injection and PBS I.N. treatment), OVA group (OVA and alum I.P. injection and OVA I.N. treatment), and cystic fluid + OVA group (7 times pretreatment of cystic fluid before OVA and alum I.P. injection and OVA I.N. treatment), each group was composed 5 mice. We got the representative results from three independent experiments.

2.3. Analysis of bronchoalveolar lavage fluid (BALF) and differential cell counting

The tracheas of mice were exposed and cut just below the larynx to obtain BALF from the mice. A polyurethane flexible tube (outer diameter: $0.4\ \text{mm}$, length: $4\ \text{cm}$; BD Biosciences) attached to a blunt 24-gauge needle (Boin Medical Co., Seoul, Korea) was placed in the trachea, and then the lungs were lavaged once with $800\ \mu\text{L}$ of cold PBS. The BALF was centrifuged at $1500\ \text{rpm}$ at 4°C for 5 min. The supernatant was transferred to new microcentrifuge tubes and frozen at -20°C until ELISA. The remaining cell pellet was suspended in $100\ \mu\text{L}$ of cold PBS. After total cell numbers were counted using a hemocytometer, the same number of cells in the BALF were centrifuged on each slide at $500\ \text{rpm}$ for 5 min using a Cytospin apparatus (Micro 12™, Hanil Co., Seoul, Korea) to determine the differential cells counts. After drying, each slide was stained with Diff-Quik solution (Sysmex Co., Kobe, Japan). At least 500 cells/slide were counted to evaluate differential leukocyte counts.

2.4. Measurement of AHR

After I.N. administration of OVA, changes in AHR were monitored by whole-body plethysmography for animals (Allmedicus, Anyang, Korea) as a response to aerosolized methacholine (Sigma) (Takeda et al., 1997). To measure bronchoconstriction, the enhanced pause (PenH) was calculated at baseline (PBS) and with increasing doses of aerosolized methacholine from 0 to $50\ \text{mg/mL}$. During nebulization, the mice were exposed to aerosolized PBS or methacholine for 10 min

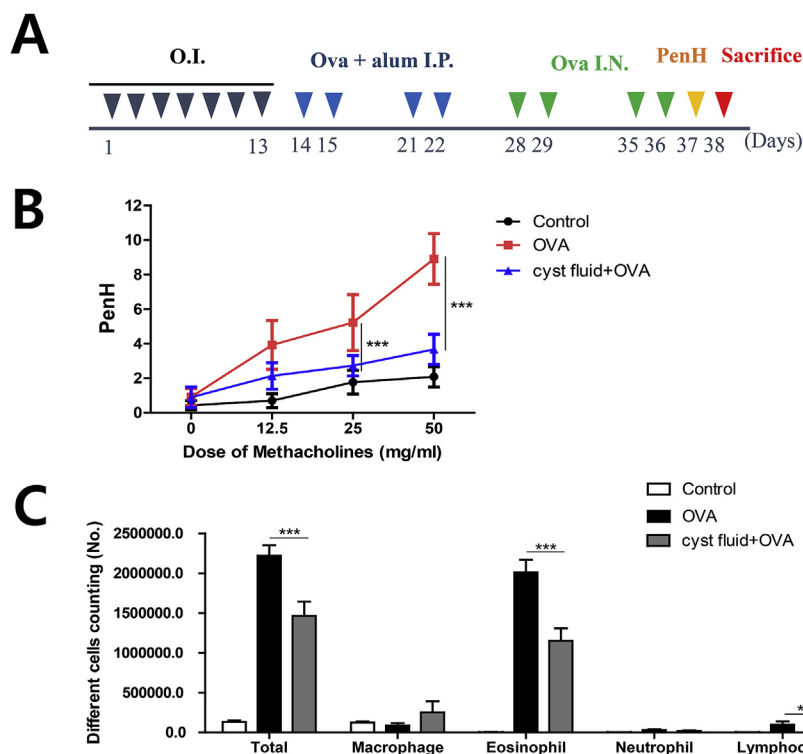


Fig. 1. Effect of *E. granulosus* cystic fluid treatment on OVA-induced allergic airway inflammation. Schedule of an allergic airway inflammation mouse model (A). Cystic fluid was administered orally to C57BL/6 mice 7 times every other day at a dose of 1 mg. (O.I.; Oral injection, I.P.; Intra-peritoneal, I.N.; Intra-nasal.) The value of PenH was assessed at baseline and after inhalation of aerosolized methacholine with increasing doses (0, 12.5, 25, and $50\ \text{mg/mL}$) (B). After Diff-Quik staining of the cells in the BALF, the number of differential immune cells was counted (C). (Control; PBS treatment in normal mouse, OVA; OVA + alum induced asthma group, cyst fluid + OVA; cyst fluid treated group before OVA + alum induced asthma, *; $p < 0.05$, ***; $p < 0.001$, the value of cyst fluid treated in OVA induced asthma group was compared with those of OVA induced asthma group. $n = 5$ mice/group, representative results of three independent experiments).

using an ultrasonic nebulizer (Omron, Kyoto, Japan). After each nebulization, the average values of PenH were measured for 3 min.

2.5. Isolation of lymphocytes from spleen and lung-draining lymph node (LLN)

Lymphocytes from the spleen and LLN were isolated from mice to analyze cytokine production and examine the specific cytokines secreted by lymphocytes after the mice were sacrificed. The LLN in normal mouse (PBS treated mouse) is too small to get enough number of lymphocytes for ELISA experiment, therefore we used only LLN of OVA and cystic fluid + OVA group for ELISA experiment. The spleen and LLN were ground using a MONOJECT (Covidien, Dublin, Ireland) and then incubated with ammonium chloride potassium hypotonic lysis solution (Sigma) for 2 min at 24 °C to lyse the red blood cells. After cell lysis, splenocytes and lymphocytes were filtered through 100- μ m meshes (Small Parts, Inc., Miramar, FL, USA) and washed with medium. The cells were counted with a hemocytometer and cultured in 24-well cell culture plates with 0.5 μ g/mL anti-CD3 antibody (BD Biosciences, Franklin Lakes, NJ, USA) for CD4⁺ T cell activation at 1×10^6 cells/well in RPMI 1640 with 10% fetal bovine serum and penicillin/streptomycin for 72 h at 37 °C in 5% CO₂. After incubation, the culture supernatants were collected and stored at –20 °C until the enzyme-linked immunosorbent assay (ELISA).

2.6. Measurement of cytokine production

The levels of cytokines including IL-4, IL-5, IL-13, IL-17A, TGF- β , IL-10, and interferon- γ were measured in the culture supernatants of splenocytes and lymphocytes, and BALF supernatant using an ELISA kit (eBioscience, San Diego, CA, USA). The assay was carried out according to the manufacturer's instructions. Briefly, 96-well immunoplates were coated with the capture antibody and incubated at 4 °C overnight. The immunoplates were blocked with 1% bovine serum albumin (Bioworld, Atlanta, Georgia, USA) at 37 °C for 1 h. The standards and samples were added to each well and incubated at 4 °C overnight, after which the immunoplates were washed 6 times with 200 μ L of PBS containing 0.05% Tween-20 (PBS-T), and 100 μ L of biotin-conjugated anti-mouse detection antibodies were added into individual wells and incubated at 24 °C in the dark for 1 h. The immunoplates were washed as described above with PBS-T. Next, avidin-conjugated horseradish peroxidase was added to each well and incubated at room temperature in the dark for 1 h. For blue color development, 100 μ L of tetra-methyl-benzidine was used as a substrate. The reaction was stopped by adding 100 μ L of diluted H₂SO₄ (Merck, Darmstadt, Germany) as a stop solution and then the blue color changed to yellow. The absorbance value was measured at 450 nm on a multimode microplate reader (SpectraMAX M2e, Molecular Devices, Sunnyvale, CA, USA).

2.7. Flow cytometry

To determine the proportion of IL-4-secreting cells and T_{reg} cells from the spleen and LLN in allergic airway inflammation-induced mice, isolated cells from the spleen and LLN were subjected to CD4, CD25, IL-4, and Foxp3 staining with anti-CD4-FITC, anti-CD25-APC, anti-IL-4-PE, anti-Foxp3-PE-Cy7 (eBioscience) antibodies according to the manufacturer's instructions. Flow cytometry was performed using a FACS Canto II cytometer (BD Biosciences) equipped with Canto software. All experimental data were analyzed with Single Cell Analysis Software V10, FLOWJO (Ashland, OR, USA).

2.8. Statistical analysis

All experiments were performed three times for confirmation of statistical significance. The mean $\pm$ SD was calculated using data collected from individual experiments. For all experiments, data are

shown as the mean $\pm$ SD ($n = 3$ –5). Student's *t*-test and analysis of variance were used to determine significant differences. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was represented as follows; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.9. Ethics statement

The study was performed with approval from the Pusan National University Animal Care and Use Committee (IACUC protocol approval; PNU-2018-1905), in compliance with “The Act for the Care and Use of Laboratory Animals” of the Ministry of Food and Drug Safety, Korea. All animal procedures were conducted in a specific pathogen-free facility at the Institute for Laboratory Animals of Pusan National University.

3. Results

3.1. Treatment with *E. granulosus* cystic fluid alleviated airway inflammation

We evaluated whether *E. granulosus* cystic fluid inhibits airway inflammation in an OVA-Alum model. The mice were orally administered cystic fluid and airway inflammation responses were examined after OVA-Alum induction. The experimental design is shown in Fig. 1A. The induction of allergic airway inflammation using OVA generates Th2-mediated airway inflammation, leading to AHR. To assess lung function, AHR was evaluated after inhalation of aerosolized methacholine with increasing doses from 0 to 50 mg/mL. After methacholine inhalation, the value of PenH was increased in mice challenged with OVA-Alum in a dose-dependent manner as compared to the control mice. The value of PenH, in contrast, was decreased in mice pre-treated with *E. granulosus* cystic fluid as compared to the mice treated with OVA alone (Fig. 1B). Differential cell counting of cells in the BALF indicated that mice pre-treated with *E. granulosus* cystic fluid before induction of allergic airway inflammation had significantly decreased numbers of total cells and eosinophils in the BALF as compared to the OVA-treated only mice (Fig. 1C).

3.2. Cytokines related to Th2 and Treg changed in the BALF, spleen, and LLN

To determine whether treatment with *E. granulosus* cystic fluid impacts the immune response of systemic immunity, the production of cytokines including IL-4, IL-5, IL-13, IL-17A, IL-10, and TGF- β were measured in the BALF, LLN, and spleen by ELISA. IL-4, IL-5, and IL-13, known as Th2-associated cytokines, were excessively secreted in the BALF, LLN, and spleen from OVA-treated only mice as compared to the control mice. Additionally, a significantly increased level of IL-17A in OVA-treated only mice was observed in the BALF, LLN, and spleen (Figs. 2–4). In contrast, cytokine production was altered in the BALF, LLN, and spleen of cystic fluid pre-treated mice. In the BALF, the concentrations of IL-4, IL-5, IL-13, and IL-17A in pre-treated mice were decreased, while the levels of IL-10 and TGF- β were increased as compared to the OVA-treated only mice (Fig. 2). In the LLN, the level of IL-4, but not IL-5, IL-13, and IL-17A, was significantly decreased in pre-treated mice. Furthermore, IL-10 and TGF- β production was significantly increased as compared to the OVA-treated only mice (Fig. 3). In the spleen, the concentrations of IL-4, IL-5, IL-13, and IL-17A in pre-treated mice were also decreased, while the level of TGF- β but not IL-10, was increased as compared to the OVA-treated only mice (Fig. 4).

3.3. *Echinococcus granulosus* cystic fluid provoked changes in immune cell populations in the spleen and LLN

To examine whether *E. granulosus* cystic fluid promotes changes in immune cell populations, we investigated the proportion of IL-4-

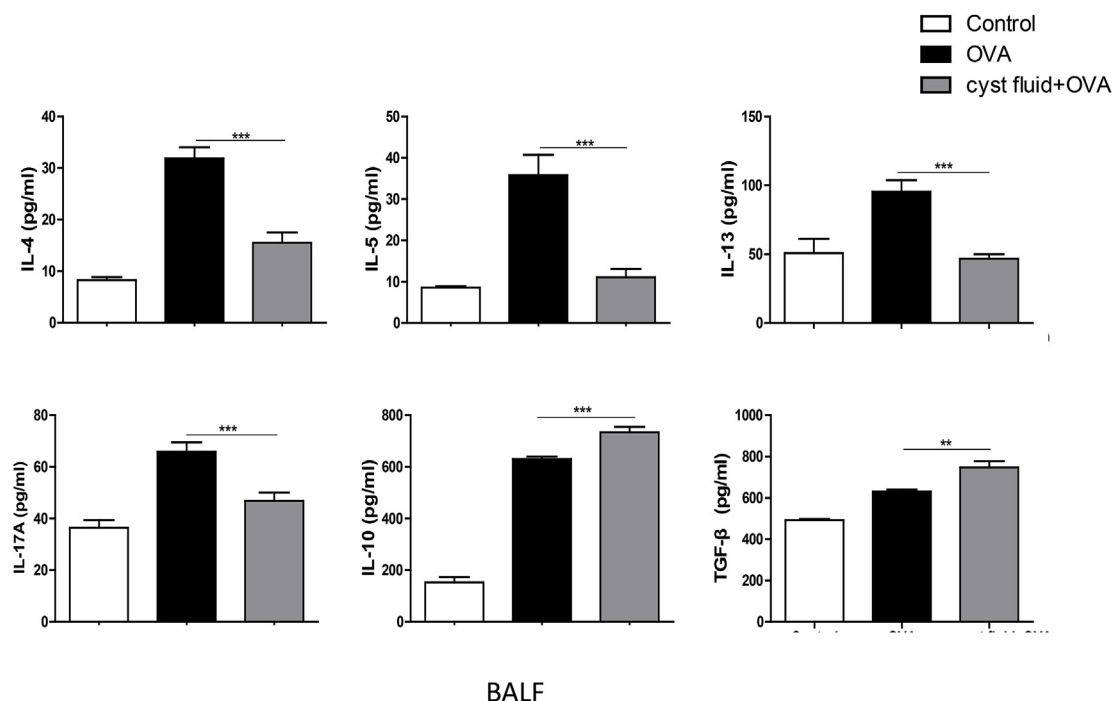


Fig. 2. Cytokine concentration in BALF after cystic fluid administration. To determine the effects of cystic fluid administration on cytokine levels in the BALF, the levels of IL-4, IL-5, IL-13, IL-17A, IL-10, and TGF-β were measured. (**, $p < 0.01$, ***, $p < 0.001$, $n = 5$ mice/group, representative results of three independent experiments).

secreting CD4⁺T cells (IL4⁺CD4⁺T cells) and Foxp3⁺ CD4⁺T cells in the spleen and LLN by fluorescence-activated cell sorting (FACS). The lymphocytes were first gated based on forward scatter (FCS) versus side scatter (SSC), and then live lymphocytes were gated with CD4 and the percentage of CD4⁺IL-4⁺ cells calculated using FACS analysis. For Treg cells, the live lymphocytes were firstly gated with CD4 and the percentage of CD25⁺Foxp3⁺ cells calculated using FACS analysis.

IL4⁺CD4⁺T cells in the spleen and LLN from cystic fluid pre-treated mice were markedly reduced as compared to the OVA-treated only mice (Fig. 5A and B). Although, Foxp3⁺CD4⁺T cells in the spleen of cystic fluid pretreated mice were not increased (Fig. 6A), the proportion of Foxp3⁺ CD4⁺T cells in the LLN from pre-treated mice was significantly increased as compared to the OVA-treated only mice (Fig. 6B).

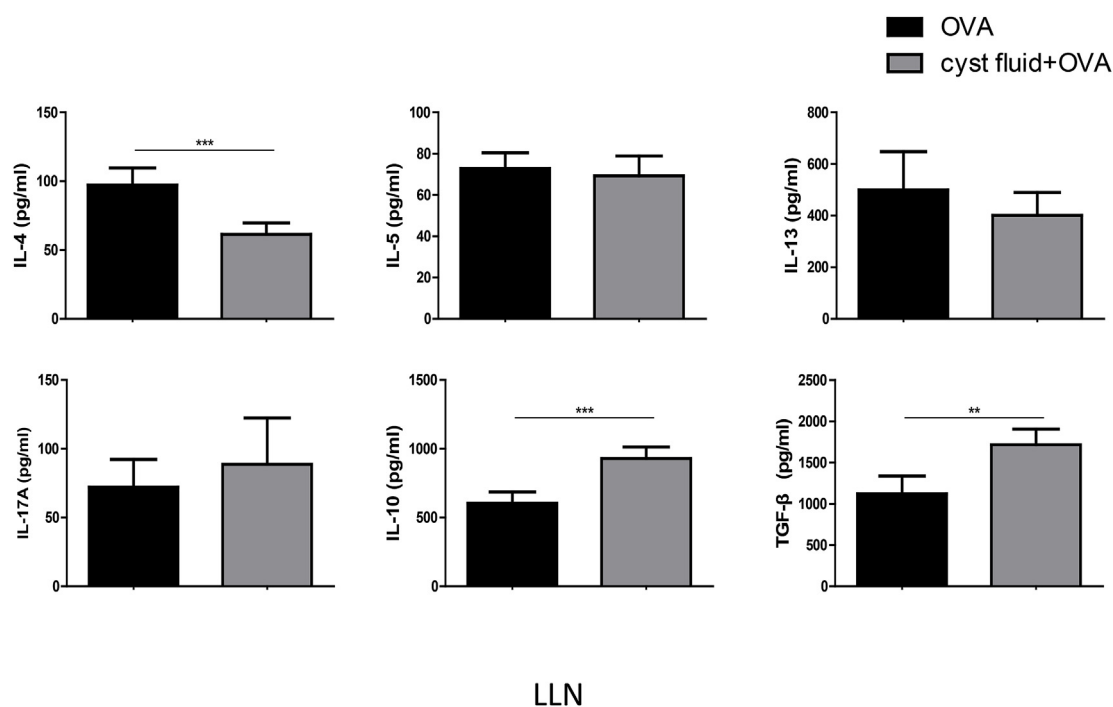


Fig. 3. Cytokine concentration in LLN after cystic fluid administration. To determine the effects of cystic fluid administration on cytokine production of lymphocytes in the LLN, the levels of IL-4, IL-5, IL-13, IL-17A, IL-10, and TGF-β in the culture supernatant of anti-CD3 antibody (0.5 μg/mL)-stimulated lymphocytes from LLN were measured. (**, $p < 0.01$, ***, $p < 0.001$, $n = 5$ mice/group, representative results of three independent experiments).

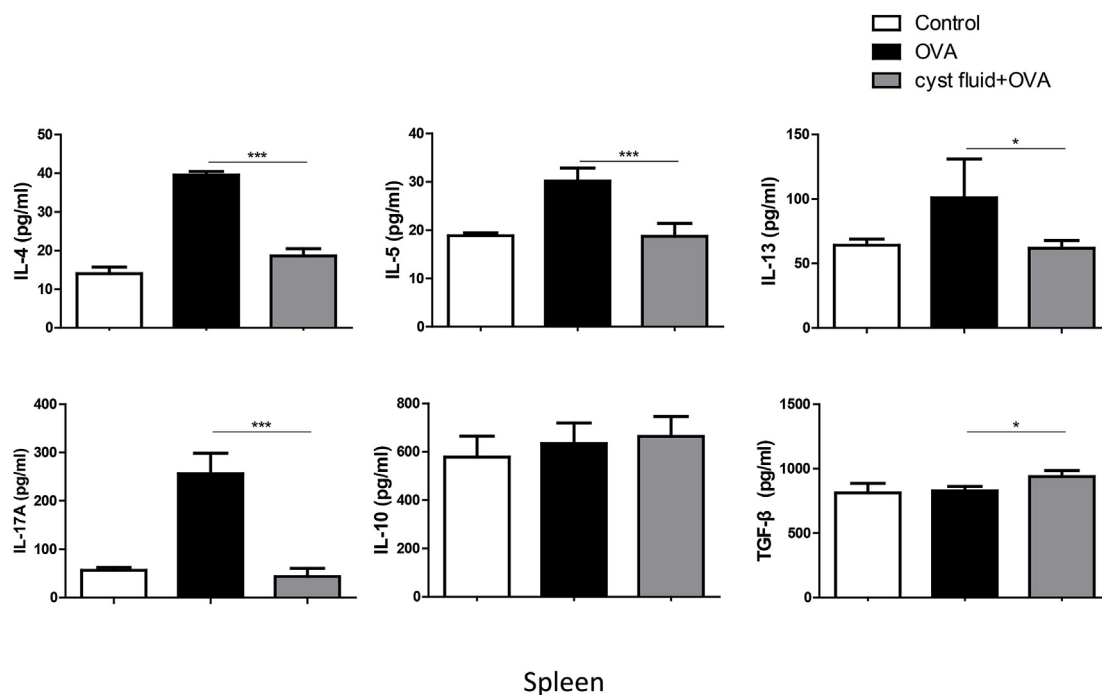


Fig. 4. Cytokine concentration in the spleen after cystic fluid administration. To determine the effects of cystic fluid administration on cytokine production of splenocytes, the levels of IL-4, IL-5, IL-13, IL-17A, IL-10, and TGF-β in the culture supernatant of CD3 (0.5 μg/mL)-stimulated splenocytes were measured. (*; $p < 0.05$, ***; $p < 0.001$, $n = 5$ mice/group, representative results of three independent experiments).

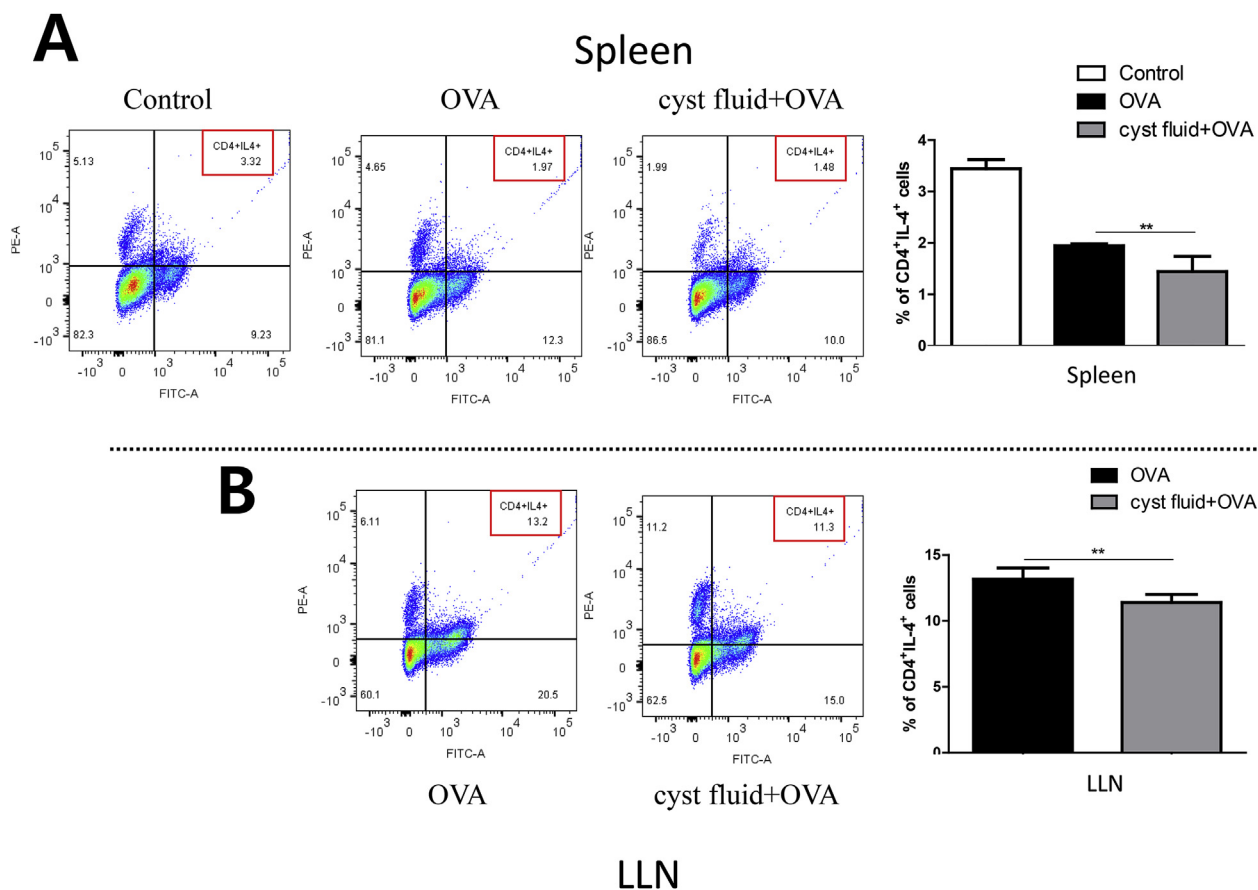


Fig. 5. Cell population of IL-4⁺CD4⁺ T cells in the spleen and LLN. The populations of CD4⁺IL4⁺ cells in the spleen (A) and LLN (B) were analyzed by FACS. Lymphocytes isolated from the spleen and LLN were stained with anti-CD4-FITC and anti-IL-4-PE. CD4⁺ cells were gated firstly and then the proportions of IL-4⁺ cells were analyzed. All experimental data were analyzed with Single Cell Analysis Software V10, FLOWJO (**; $p < 0.01$, $n = 5$ mice/group, representative results of three independent experiments).

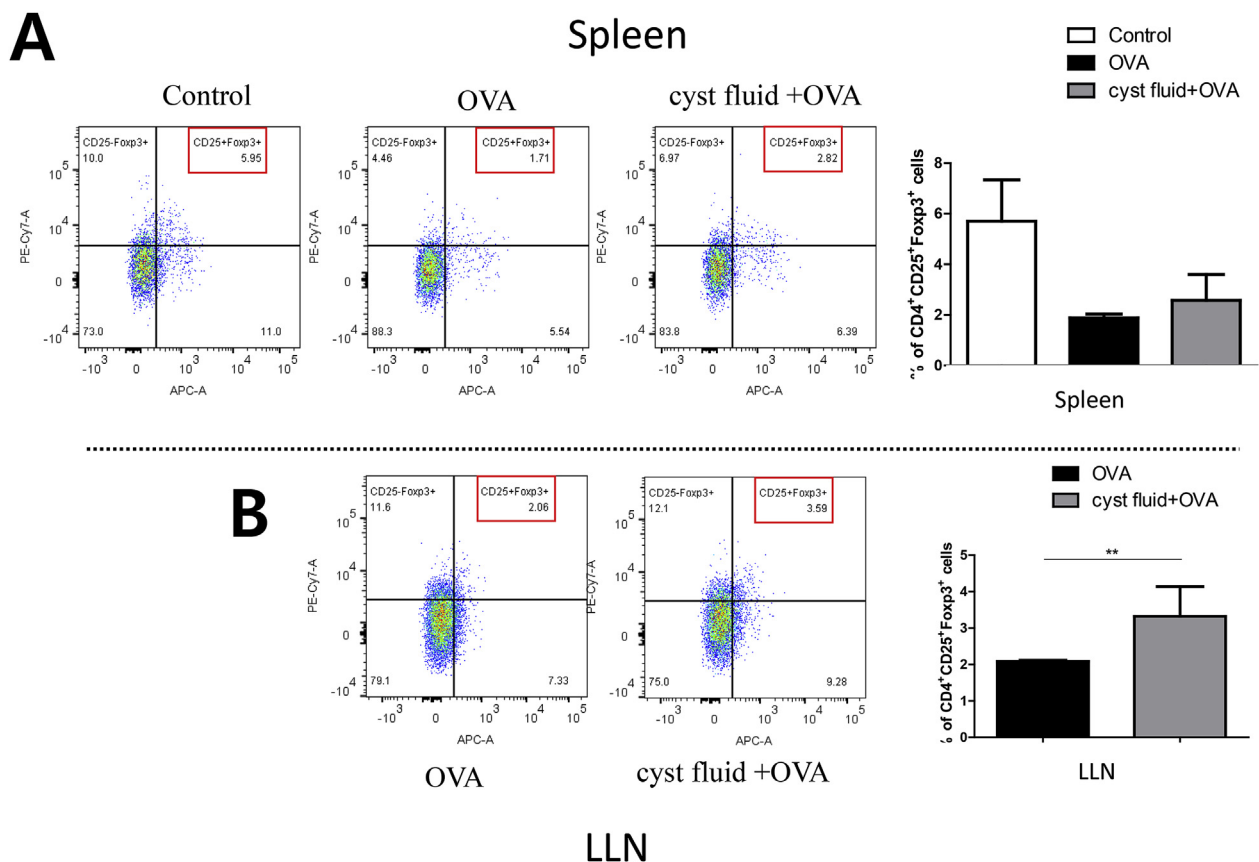


Fig. 6. Cell population of CD4⁺CD25⁺Foxp3⁺T cells in the spleen and LLN. The populations of CD4⁺CD25⁺Foxp3⁺T cells in the spleen (A) and LLN (B) were analyzed by FACS. Lymphocytes isolated from the spleen and LLN were stained with anti-CD4-FITC, anti-CD25-APC, and anti-Foxp3-PE-Cy7. CD4⁺ cells were gated firstly and then the proportions of CD25⁺Foxp3⁺ cells were analyzed. All experimental data were analyzed with Single Cell Analysis Software V10, FLOWJO (**; $p < 0.01$, $n = 5$ mice/group, representative results of three independent experiments).

4. Discussion

In this study, we demonstrated that oral administration of *E. granulosus* cystic fluid ameliorated allergic airway inflammation via regulatory T cell recruitment in the lung and inhibition of Th2 cell activation, demonstrating that cystic fluid may contain IMs that are useful therapeutic agents for immune disorder treatment, including asthma.

Asthma is a common allergic airway inflammatory disease among immune disorders and is related to up-regulation of AHR leading to respiratory symptoms (Ismail et al., 2015; Saadeh et al., 2015). Typical features of asthma include increased production of Th2 cytokines such as IL-4, IL-5, and IL-13, eosinophilia, mucus hypersecretion, pulmonary inflammation, and AHR (Paul and Zhu, 2010). Additionally, IL-17 is greatly elevated in the lung tissues of patients with allergic asthma, which is known to play a very important role in the development and progress of asthma. (Cosmi et al., 2011; Hasegawa et al., 2017). Previous studies showed that parasites or their secreted proteins directly regulate immune responses (Maizels et al., 2004; Wilson and Maizels, 2004), and infection with parasites has been consistently linked to the regulation of asthma (Leonardi-Bee et al., 2006; Park et al., 2011). Particularly, some mechanisms of host immune down-regulation by *Echinococcus* infection are well-known and closely related to the inhibition of DC maturation, regulatory T cell activation via IL-10 and TGF- β elevation, and inhibition of IL-17 and IL-5 (Kanan and Chain, 2006; Tuxun et al., 2012; Wang et al., 2014). Although numerous studies have shown that *E. granulosus* infection induces inhibitory mechanisms that attenuate allergic diseases or autoimmune diseases, the exact mechanism of immune responses regulated by *E. granulosus* cystic fluid from humans is unknown and preclinical studies using animal

models are insufficient.

There are three main mechanisms of host immune downregulation by cystic fluid treatment in asthma and immune disorder. First, excretory-secretory products and cystic fluid derived from *E. granulosus* may affect the maturation and activation of DCs (Kanan and Chain, 2006; Rigano et al., 2007; Wang et al., 2015a,b). Wang et al. suggested that although the ES proteins of *E. granulosus* treatment increased the expression levels of surface markers such as CD40, MHC class II, CD86, and CD80 on bone-marrow-derived DCs, the expression level changes of surface markers were not significant. Other reports suggested that hydatid fluid failed to induce surface marker expression and stimulate T cell proliferation; the fluid appears to induce host immunosuppression to ensure parasite survival by inhibiting DC maturation (Wang et al., 2015a,b). However, ES protein treatment of bone marrow-derived DCs co-cultured with CD4⁺T cells induced the generation of Treg cells (Wang et al., 2015a,b).

Remain two mechanisms of immune down regulation by cystic fluid treatment in asthma were enhancing of IL-10 secretion (Treg cell activation) and down-regulating IL-5 and IL-17A. The current results suggest that *E. granulosus* cystic fluid administration can significantly reduce the severity of OVA-induced airway inflammation including reducing eosinophil cell infiltration and mucus production and ameliorating AHR (Fig. 1). Additionally, the IL-17A concentration in the BALF of cystic fluid administration mice was significantly lower than that in OVA induced asthma mice (Fig. 2). Additionally, secretion of this cytokine was reduced in splenocytes in mice administered cystic fluid (Fig. 4). Wang et al. also demonstrated that *E. granulosus* infection significantly reduced the severity of allergic airway inflammation by enhancing IL-10 and down-regulating IL-5 and IL-17A in *E. granulosus*-

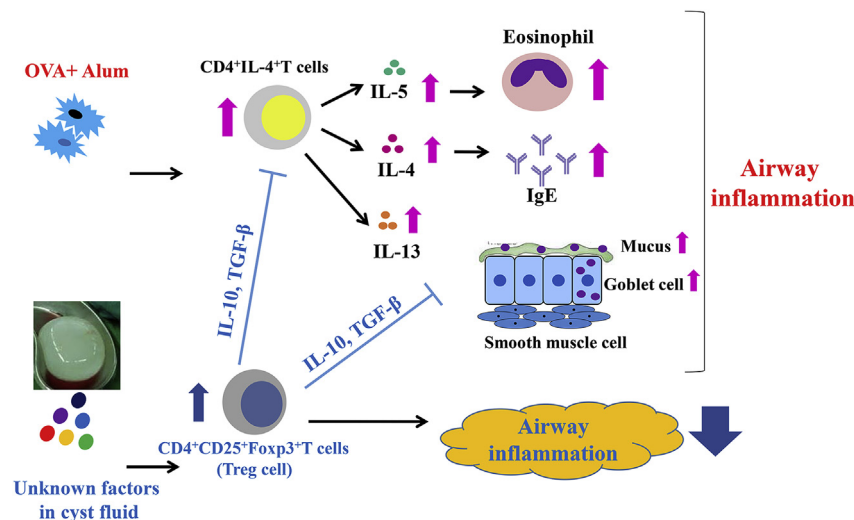


Fig. 7. Schematic drawing about mechanisms of down regulation in allergic airway inflammation by cystic fluid of *Echinococcus*.

infected mice (Wang et al., 2014). These results demonstrate that *E. granulosus* infection, as well as cystic fluid, can ameliorate lung inflammation by inhibiting IL-17 production in the lungs and immune organs. After *E. granulosus* infection, local immunosuppression is promoted through the regulation of IL-10 and TGF- β , resulting in decreases of inflammatory cytokines (Mondragon-de-la-Pena et al., 2002). In this study, we found elevated IL-10 and TGF- β levels in the BALF, while IL-10- and TGF- β -secreting lymphocytes were increased in the LLN and splenocytes of the cystic fluid administration group (Figs. 2–4). Additionally, Treg cells in the LLN were significantly increased and CD4⁺IL-4⁺T cells in the spleen and LLN were significantly decreased in the cystic fluid administration group compared to in the control group (Figs. 5 and 6). IL-10 is an immune suppressive cytokine that plays a critical role in parasitic infection-induced immune regulation responses. Most echinococcosis patients show increased IL-10 levels compared to the levels in healthy people (Tuxun et al., 2012). Additionally, several parasite infections and IMs isolated from parasites may regulate the asthma reactions, which is closely related to elevated IL-10 levels (Lee et al., 2008; Marinho et al., 2016; Pacifico et al., 2009; Pitrez et al., 2015). Hydatid cystic fluid is a complex of *E. granulosus* antigens that are thought to contribute to immunomodulatory activities in immune disorders, including the activation of cellular responses (Rigano et al., 2007). Thus, not only *E. granulosus* infection but also cystic fluid administration may decrease the severity of allergic asthma by enhancing IL-10 production and Treg cell recruitment and down-regulating IL-17A production. Although IL-4 have been known as anti-inflammatory agent on some situation like as Th1 and Th17 related immune response included auto-immune disease. However, IL-4 also is inflammatory agent in Th2 responses (especially enhancing of IgE secretion) like as allergic response and parasite infection (Fig. 7).

In this study, *E. granulosus* cystic fluid obtained from sheep was used to determine whether cystic fluid alters the functions of immune cells. We revealed hydatid cystic fluid altered the cytokine balance and immune cell activation (Fig. 7). Thus, *E. granulosus* may be useful for controlling the host immune system to achieve therapeutic effects, which will contribute to the development of new therapeutic agents for immune disorders. However, key molecules in the cystic fluid responsible for these effects remain unknown and thus additional studies are needed. We will next analyze the compositions of the cystic fluid and identify the molecules that act as regulatory factors.

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Appendix A. Supplementary data

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