

MISS MI-JIN JEONG (Orcid ID : 0000-0001-9392-0428)

MISS SHIN AE KANG (Orcid ID : 0000-0001-9934-9787)

Article type : Original Article

Title: Extracellular vesicles of *Echinococcus granulosus* have therapeutic effects in allergic airway inflammation.

Authors: Mi Jin Jeong¹, Shin Ae Kang¹, Jun Ho Choi¹, Da in Lee¹, Hak Sun Yu^{1,2*}

Institutional affiliations:

¹*Department of Parasitology and Tropical Medicine, School of Medicine, Pusan National University, Yangsan, 50612, Republic of Korea.* ²*Research Institute for Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital, Yangsan, 50612, Republic of Korea.*

***Corresponding author: Hak Sun YU, PhD,**

Department of Parasitology and Tropical Medicine, School of Medicine, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do, 626-870, Republic of Korea. Tel: +82+51-510-8022, Fax: +82+51-980-0872, E-mail: hsyu@pusan.ac.kr

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/PIM.12872](#)

This article is protected by copyright. All rights reserved

Abstract

Background

Previous studies have shown that *Echinococcus granulosus* cystic fluid can alleviate Th2 allergic airway inflammatory responses by increasing the number of CD4⁺CD25⁺ Foxp3⁺T (regulatory T; Treg) cells. Parasite-derived extracellular vesicles (EV) are known to not only promote parasite infection by communicating between parasites but also regulate the inflammatory response by acting as an immunomodulatory agent in the host.

Methods

To evaluate the effect of EV extracted from the cystic fluid of *E. granulosus* on allergic airway inflammation, gene expression was investigated after administering EV to mouse lung epithelial cells (MLE-12) following 2 h of pretreatment with *Aspergillus* proteins. An allergic airway inflammation animal model was used to investigate the regulation of the inflammatory response by EV and induced with ovalbumin.

Results

EV treatment significantly reduced airway resistance and the number of eosinophils and other immune cells in the bronchoalveolar lavage fluid and Th2- and Th17-related cytokine levels. EV pretreatment decreased the number of IL-4⁺CD4⁺T cells and increased the number of Treg cells in the lung-draining lymph nodes and spleen.

Conclusions

E. granulosus cystic fluid derived EV ameliorated Th2 allergic airway inflammatory through Treg cells, similar to whole cystic fluid treatment. Thus, EV may be important immunomodulatory molecules in cystic fluid.

1. Introduction

Echinococcus species (spp.) is a parasite of the Cestoda and belongs to the Platyhelminthes family ¹. Echinococcosis, caused by infection with genus *Echinococcus*, is an animal-derived disease that occurs globally. Echinococcosis occurs in four forms, among which is hydatid disease caused by *Echinococcus granulosus* ². This disease is a cystic echinococcosis known as hydatidosis. According to the WHO, the human incidence of cystic echinococcosis can reach more than 50 per 100,000 person-years, and prevalence levels as high as 5~10% may occur in parts of Argentina, Peru, East Africa, Central Asia and China. Adult *E. granulosus* mainly inhabit dogs, but hydatid cysts are found in a variety of mammals, including humans ³.

The early stages of *E. granulosus* infection involve activation of cell-mediated immunity, including cellular inflammatory responses and pathological changes. Early infection with *E. granulosus* causes infiltration of neutrophils and macrophages and causes leukocytosis, resulting in an increase number of myeloid cells such as eosinophils, lymphocytes and macrophage ⁴. In chronic infection, cellular infiltration of eosinophils and fibrocytes as well as neutrophils and macrophage occurs, causing fibrosis/necrosis and obstruction of bile ducts and blood vessels ⁵. Also, early infection with *E. granulosus* does not induce an immune response. However, chronic infection with *E. granulosus* causes a Th2 response and increases IFN- γ , interleukin (IL)-4 and IL-10 ⁶. Increased expression of IL-10 and IL-4 caused by *E. granulosus* regulates the host immune response, including T helper (Th)1 type cytokines ⁷. In addition, in patients with cystic echinococcosis, the levels of IL-10 and TGF- β and transcription factor (Foxp3) are significantly increased relative to the number of peripheral CD4⁺CD25⁺Foxp3⁺ T (regulatory T; Treg)-cells and the levels of IL-17 and IL-23 and ROR γ t, a transcription factor, decreased relative to the number of Th17 cells ⁸.

Parasite-derived substances have various mechanisms that interfere with the host response to ensure their own survival and to reduce the immune response and suppress allergic reactions to protect the host from excessive inflammation ⁹. Thus, parasite-derived substances can modulate host immune responses and have therapeutic effects on various immune diseases associated with allergies and inflammation ^{9 10}. Infection with the parasitic nematode *Heligmosomoides polygyrus* has been shown to protect against allergic asthma, and EVs derived from this parasite have been shown to act like vaccines by regulating immune response ^{11 12}. In several studies, infection with *E. granulosus* has been shown to alleviate the incidence of dextran sulfate sodium-induced colitis ¹³. Hydatid cyst, a substance derived from *E. granulosus*, Th1 and Th2 cytokine secretion patterns

are stably induced¹⁴. Th2 cells express IL-4, IL-5 and IL-6 and are related to disease susceptibility, and Th1 cells produce IL-2 and IFN- γ and act as protective immunity¹⁵.

Cell-to-cell communication occurs through direct contact or the delivery of secreted molecules. Extracellular vesicles (EV) play a role in cell-to-cell communication, including cell-to-cell migration¹⁶. EV consist of several subtypes, and exosomes are small EV (< 150 nm) formed from multivesicular bodies in the endosome pathway. Vesicles also form EV in the 200–500 nm range that are called microvesicles or ectosomes. However, smaller vesicles (~100 nm) are also known to form in the plasma membrane and can be separated along with exosomes. In cancer cells, larger EV (1–10 μ m), called large tumor bodies, can act as EV in the cell membrane¹⁷. Unlike the monolayer high-density and low-density lipoproteins found in body fluids, the EV membrane is composed of a lipid bilayer similar to the cellular plasma membrane. EV contain enzymes involved in lipid metabolism, including phospholipases D and A2, whose activity is triggered by the addition of GTP¹⁸. In addition, EV can contribute to the apoptosis of recipient cells because cholesterol and sphingomyelin are present in a high ratio. EV carry a variety of cellular components, including proteins, DNA, and miRNA, a non-coding RNA class that is approximately 22 nucleotides in length¹⁹.

Parasite-derived EV derived can control infection via cell-to-cell communication between parasites. Coakley and Maizels found that microscopic vesicles migrate between red blood cells infected with *Plasmodium falciparum* and promote parasitic transmission to surrounding cells and that the genetic material of the parasite is also involved in this transmission of material²⁰. Furthermore, parasite-derived EV play a role in alleviating the inflammatory response by regulating the host immune response. Twu, de Miguel et al. found that EV derived from *Trichomonas vaginalis* induce regulation of IL-6 and IL-8 secretion, thereby regulating inflammation of the genitourinary tract²¹. EV derived from *Leishmania* have been shown to cause overproduction of several pro-inflammatory cytokines and chemokines, including IL-8, an inflammatory cytokine that recruits other inflammatory immune cells such as neutrophils via signaling with the host²². In addition, *Schistosoma*-derived EV decreases the levels of pro-inflammatory cytokines such as IL-17a, IFN- γ , IL-22, IL-12 and TNF- α , and increases the level of anti-inflammatory cytokine. It has been shown to be useful in attenuating and inhibiting the severity of mouse inflammatory bowel disease (IBD)²³. In recent studies, a recombinant protein modeled based on the parasite's EV protein has been used to attenuate clinical symptoms of asthma²⁴.

In a previous study, hydatid cystic fluid collected from the lungs and liver of sheep infected with *E. granulosus* was demonstrated to change the balance of cytokines and immune cell activation in a mouse model of ovalbumin (OVA) allergic airway inflammation and played an important role in regulating the host immune system ²⁵. We hypothesized that EV, extracted from cystic fluid, would also alleviate allergic airway inflammatory reactions by acting as an immunomodulator and prompting a host immune response. To clarify the role of EV extracted from *E. granulosus* cystic fluid, we evaluated the immunomodulatory effect in an allergic airway model.

2. Materials and Methods

2.1. Isolation of EV

EV were extracted from hydatid cyst fluid collected surgically from *E. granulosus* infected sheep in Uzbekistan. The supernatant of cystic fluid was concentrated using a Quixsand filter (GE Healthcare, Little Chalfont, UK) and filtered through a 0.22 µm filter. The filtrate was pelleted by ultracentrifugation in a 45Ti rotor (Beckman Coulter, Fullerton, CA, USA) at 100,000 g for 2 h at 4 °C. The final pellets were resuspended in PBS and stored at – 80 °C. EV were placed on 300-mesh copper grids and stained with 2% uranyl acetate. The diameters of EVs were measured using a Zetasizer Nono ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633-nm laser line at a scattering intensity of 10x30s ²⁶. The EV was identified with Transmission electron microscopy (TEM).

2.2. Cell culture

Mouse lung epithelial cells (MLE12) were obtained from ATCC (Manassas, VA, USA). MLE12 were cultured in 24-well cell culture plates 4 ×10³ cells /well in 1 mL DMEM-F12 (ATCC, 30-2006) with 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 nM sodium selenite, 10 mM hydrocortisone, 10 nM β-estradiol, and 2 mM L-glutamine. MLE12 cells were stimulated with EV (0.5, 1, and 2 µg/mL) for 2 h and *Aspergillus* protease antigen (ASP; 200 ng/mL) for 2 h. All conditions were performed for 24 h at 37 °C in 5% CO₂.

2.3. RNA extraction and cDNA synthesis

The MLE-12 culture medium was removed, and cells were treated with 1 mL of QIAzol (Qiagen science, USA) in each well. To extract RNA, 200 µL of chloroform was added and the mixture vortexed. The supernatant was obtained by centrifugation 12000 rpm for 15 min at 4 °C, and RNA

precipitation was induced by 500 μ L isopropanol. After incubation for 10 min at room temperature (24 °C), the samples were centrifuged for 10 min at 4 °C, and the supernatant was removed and dried. Extracted RNA was measured for concentration and diluted to 2 μ g in diethyl pyrocarbonate. RNA was transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA). RNA levels were determined via real-time PCR using the iCycler iQ™ (Bio-Rad Laboratories, Inc., USA) real-time PCR machine. Primer sequences are listed in Table 1. PCR amplification was conducted in a 20 μ L volume containing 2 μ L cDNA, 0.2 μ M primers, and SYBR premix Ex Taq™ (Takara Bio Inc., Japan). Genes were amplified using the following conditions: 5 min hot start at 95 °C, followed by denaturation at 95 °C for 10 s, primer annealing at 55 °C for 20 s, and elongation at 72 °C for 30 s for 40 cycles. The relative expression was calculated as the ratio to a housekeeping gene (*Gapdh*) using the Gene-x program (Bio-Rad Laboratories, Inc., USA).

2.4. Mice and experimental design for 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 (IRB Approval No. PNU-2019-2287). EV were orally administered to C57BL/6 mice seven times at a dose of 1 mg every other day. Then, EV were administered on days 14, 15, 21, and 22 through intraperitoneal injection with 75 μ g of OVA (Sigma-Aldrich, St. Louis, MO, USA) adsorbed with 2 mg of aluminum hydroxide (Alum; Sigma-Aldrich) in 200 μ L of phosphate-buffered saline (PBS). Mice were administered 50 μ g of OVA intra-nasally on days 28, 29, 35, and 36. Airway hyperresponsiveness (AHR) was assessed on day 37, and mice were euthanized on day 38. Our experiment was divided into four mouse groups, and each group consisted of five mice.

2.5. Measurement of AHR

AHR is a measure of bronchoconstriction, which was assessed in mice after administration with PBS and increasing doses of aerosolized methacholine (12.5, 50, and 50 mg/mL) for whole body plethysmography (Allmedicus, Anyang, Korea). Mice were exposed to methacholine in PBS for 10 min using an ultrasonic nebulizer (Omron, Kyoto, Japan). The average PenH values were measured for 3 min.

2.6. Immune cell counting in bronchoalveolar lavage fluid (BALF)

The mice were anesthetized, and their trachea was isolated by dissection. As shown in the previous paper, a catheter was inserted into the lungs, which were washed twice with PBS (0.8 mL). The BALF was centrifuged at 1500 g at 4 °C for 5 min. The supernatant was maintained at –20 °C until use for enzyme-linked immunosorbent assay (ELISA), and pellets were suspended in 100 µL of cold PBS. BALF cells were smeared on a slide at 500 rpm for 5 min using a Cytospin apparatus (Hanil Co., Korea). The slides were stained with Diff-Quik solution (Sysmex Co., Kobe, Japan) to determine the number of different cells (300–500).

2.7. Lymphocyte isolation

Spleen and lung draining lymph nodes (LLNs) were collected from euthanized mice. The spleen was treated with red blood cell lysis buffer to dissolve red blood cells and filtered through a 100 µm mesh (Small Parts, Inc., Miramar, USA). Cells were plated in 24-well cell culture plates at 1×10^6 cells/mL in RPMI 1640 with 10% fetal bovine serum and penicillin/streptomycin. For CD3 stimulation, 0.5 µg/mL of CD3 antibody (BD bioscience, USA) was added for CD4⁺ T cell activation and incubated for 72 h at 37 °C in an atmosphere of 5% CO₂. After incubation, the culture medium was harvested and stored at –20 °C for ELISA analysis.

2.8. ELISA

Levels of cytokines, including IL-4, IL-5, IL-13, IL-17A, TGF-β, and IL-10, were measured in culture supernatants of splenocytes, lymphocytes, and BALF by ELISAs (eBioscience, USA). The experimental design followed those described in a previous study ²⁵.

2.9. Flow cytometry

To determine the proportion of IL-4 secreting cells and regulatory T cells from the spleen and LLN of allergic airway inflammation-induced mice, were cultured on anti-CD3-coated plates for 6 hours. Isolated cells from the spleen and LLN were stained with anti-CD4-FITC, anti-CD25-APC (eBioscience, San Diego, CA, USA). After surface staining, the cells were permeabilized. After permeabilization, the cells were stained anti-IL-4-PE, anti-Foxp3-PE-Cy7 (eBioscience). Fluorescence measurements were measured using a FACS Canto II cytometer (BD Biosciences) cytometer equipped with Canto software (BD biosciences).

2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad

Software Inc., La Jolla, CA, USA). All experiments were conducted in triplicate, with similar results. Data are expressed as mean $\pm$ SD (n = 3–5) from each experiment. Statistical significance is represented as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

3. Results

3.1. Characterization of EV

The cyst fluid of *E. granulosus* was collected from the lungs and liver of sheep at a sheep slaughterhouse in Uzbekistan. Cystic fluid was centrifuged at 500 xg to remove protoscoleces for 5 min at 4 °C and analyzed. Transmission electron microscopy (TEM) of EV obtained from concentrated pellets showed that EV derived from *E. granulosus* cystic fluid were spherical and had a lipid bilayer (Fig. 1A). The total protein concentration of EV by Bradford protein analysis was 608.46 μ g/mL. The average size of EV measured using dynamic light scattering was 33.93 $\pm$ 27.87 nm in diameter (Fig. 1B).

3.2. Treatment of EV induced Th2 chemokine gene expression in mouse lung epithelial cells

To determine whether EV can increase chemokine gene expression, EV extracted from cyst fluid after pretreatment with ASP were administered to MLE-12 cells (Fig. 2). ASP treatment significantly increased the expression of thymic stromal lymphopoietin (*Tslp*) and *Il-25* genes in MLE-12 cells. The addition of 2 μ g EV to ASP-treated cells significantly reduced the expression of *Tslp* and *Il-25* genes. In addition, eotaxin (CCL11) gene expression was significantly increased when EV was treated with 0.5 μ g compared with that in the group treated with ASP only. The expression of *Il-4* mRNA, which encodes a cytokine related to Th2 cells, increased after pretreatment with ASP and decreased significantly with administration of 0.5 μ g EV. The expression of *Il-10* mRNA, which encodes a Treg cell-related cytokine, was significantly increased with ASP pretreatment and 1 μ g EV treatment. Additionally, the expression of *Il-35* mRNA, which encodes an anti-inflammatory cytokine in the IL-12 family, was significantly higher with ASP pretreatment and with 0.5 μ g EV than that in the group treated with ASP only. There was no significant difference in mRNA expression of *Il-33*, a member of the IL-1 family.

3.3. Treatment with EV to inhibit airway inflammation

In a previous study, we confirmed that *E. granulosus* cystic fluid can suppress airway inflammation in an OVA-alum induced model. Here, we conducted an experiment to determine

whether EV isolated from *E. granulosus* cyst fluid can also play the same role. After oral administration of EV to mice and OVA-alum induction, the airway inflammatory responses were investigated and histological changes were confirmed (S.1). The experimental design is shown in figure 3A. AHR for methacholine was evaluated after OVA induction (Fig. 3B). After inhalation of methacholine, PenH values were increased in mice challenged with OVA-alum compared with those in the control group. In contrast, PenH values in mice pretreated with EV were significantly lower than those in mice treated with OVA alone. After induction of allergic airway inflammation, immune cells, especially eosinophils, were recruited into the BLAF (Fig. 3C). However, the number of eosinophils in the lungs of mice pretreated with EV was significantly reduced, and the total number of cells was also decreased.

3.4. Th2 and Treg-related cytokine changes in spleen, LLN, and BALF

When we examined *E. granulosus* cystic fluid to determine its effect on the systemic immune response, we found that the levels of Th2-related cytokines were decreased and that those of cytokines related to regulating T cells were significantly increased compared to the control. We measured cytokine production, including IL-4, IL-5, IL-13, IL-17A, TGF- β , and IL-10, by ELISA to determine whether EV derived from cystic fluid affect the immune response of systemic immunity in the spleen (Fig. 4A), LLN (Fig. 4B), and BALF (Fig. 4C). Levels of IL-4, IL-5, and IL-13, known as Th2-related cytokines, were increased in OVA-treated only mice compared to those in control mice; in addition, IL-17A levels were increased in OVA-treated mice only. However, cytokine expression levels in EV-treated mice were altered in the spleen (Fig. 4A), LLN (Fig. 4B), and BALF (Fig. 4C). The levels of IL-4, IL-5, IL-13, and IL-17A in EV-treated mice after OVA treatment decreased in the spleen, while those of IL-10 and TGF- β were increased compared with those in OVA-treated mice (Fig. 4A). The levels of IL-4, IL-5, and IL-17A were decreased in EV-treated mice compared control mice after OVA treatment in LLN, whereas the levels of IL-10 and TGF- β were increased compared with those in OVA-treated mice (Fig. 4B). In BALF, the levels of IL-5 and IL-13, but not those of IL-4 and IL-17A, were significantly reduced in EV-treated mice compared control mice after OVA treatment, and the levels of IL-10 and TGF- β were increased compared with those in OVA-treated mice (Fig. 4C). Interestingly, when EV were used as the sole treatment, the levels of IL-10 were significantly increased in the spleen and BALF compared with those in the control group.

3.5. EV change the population of immune cells in the spleen and LLN

E. granulosus cyst fluid promoted changes in the immune cell populations in the spleen and LLN. We investigated whether EV extracted from *E. granulosus* cyst fluid using fluorescence-activated cell sorting (FACS) altered the population of IL-4-secreting cells and Foxp3⁺ T cells. Lymphocytes were gated with forward scatter (FCS) versus side scatter (SSC), and live lymphocytes were gated with CD4; the number of CD4⁺IL-4⁺ T cells and CD4⁺CD25⁺Foxp3⁺ T cells were calculated through FACS analysis. As a result, as in cyst fluid, the number of IL-4-secreting cells in the spleen (Fig. 5A) and LLN (Fig. 6A) decreased in EV-pretreated mice compared with that in OVA-treated mice. However, the population of CD4⁺CD25⁺Foxp3⁺ T cells in the spleen (Fig. 5B) and LLN (Fig. 6B) of EV-pretreated mice was significantly increased compared with that of OVA-only treated mice. Interestingly, the population of CD4⁺CD25⁺Foxp3⁺ T cells in mice treated only with EV was increased compared with their level in the control group, although the difference was not significant.

4. Discussion

Previous studies have demonstrated that oral administration of *E. granulosus* cystic fluid improved allergic airway inflammation through pulmonary Treg cell activation and Th2 cell inhibition²⁵. In this study, we investigated the mechanism by which EV extracted from *E. granulosus* cystic fluid also suppressed allergic airway inflammation. As a result, EV can serve as a useful therapeutic agent for treating immune disorders, including asthma.

Allergic asthma exhibits AHR and plays an important role in eosinophil recruitment and IgE production due to the activation of Th2 cell-derived cytokines such as IL-4, IL-5, and IL-13²⁷. There has been considerable research on immunomodulators that treat allergic diseases and asthma. EV, such as exosomes, which are released outside the cell, are also known to act as immunomodulators. Various EV have been shown to play a role in alleviating inflammatory responses in lung diseases. Mesenchymal stem cell-derived extracellular vesicles (MSC-EV) have been shown to eliminate the inflammatory response by reducing the release of IL-10 and TGF- β and cytokines related to Th2 and Th17²⁸. Adipose tissue-derived-MSCs reduce eosinophilic infiltration into lung tissue and parenchymal collagen content in a mouse model of asthma²⁹. EV isolated from *Pseudomonas aeruginosa* led to increased Treg cells and an attenuated Th2 response, along with reduced inflammation in the perivascular and peribronchial spaces in the allergic asthma model³⁰.

Parasitic infections play an important role in relieving allergic inflammation such as asthma. *E. granulosus* infection increases Th2 and Treg cell cytokine levels and downregulates the expression of IL-5 and IL-17A in OVA-induced asthmatic mice. This significantly reduced the severity of OVA-induced airway inflammation, including reduction of eosinophil cell infiltration and mucus production³¹. These immunological changes were confirmed to be caused by parasite-derived substances that occur during communication between the parasite and the host. Many studies have shown that parasite-derived substances play a role in alleviating asthma, an autoimmune disease. In several studies, soluble products derived from parasitic nematodes improved airway inflammatory responses by suppressing Th2 immune responses and activating Treg cells in OVA-induced asthma mouse models³². Research on EV, a representative material of parasite-derived substances, is also being conducted. EV isolated from *E. granulosus* induced an increase in the levels of CD86 and a decrease in levels of MHC class II in dendritic cells, thereby inducing dendritic cell differentiation and maturation³³.

We hypothesized that EV regulate airway inflammatory responses through the same mechanism as *E. granulosus* cystic fluid allergic airway inflammatory downregulation. After induction of an inflammatory reaction to ASP in MLE-12 cells, treatment with of 1 μ g EV increased the secretion of IL-10 (Fig. 3). This also suggests that administration of *E. granulosus* EV can significantly reduce the severity of OVA-induced airway inflammation, including reduction of eosinophil cell infiltration and mucus production and improvement of AHR (Fig. 2). Wang, Li et al. demonstrated that increased levels of IL-10 and downregulated IL-5 and IL-17A expression reduced the severity of allergic airway inflammation in mice infected with *E. granulosus*³¹. Our study also showed that the administration of EV to the spleen (Fig. 4A) and LLN (Fig. 4B) significantly lowered the concentration of IL-17A in OVA-induced asthmatic mice. Haniloo et al. demonstrated that antigens derived from *Echinococcus* stimulate Treg cells and regulatory B cells to produce immune regulatory cytokines such as TGF- β and IL-10, which block inflammation and protective immunity³⁴. Our study showed that the levels of TGF- β and IL-10 in BALF (Fig. 4C) were significantly higher after EV administration than in OVA-induced asthma mice. In addition, secretion of these cytokines was increased in the spleen (Fig. 4A) and LLN (Fig. 4B). Additionally, although the effect was not significant, this confirmed that EV-only administration increased the levels of these cytokines in not only BALF (Fig. 4C) but also in the spleen and LLN compared with those in the control group. In addition, the population of CD4⁺IL-4⁺T cells in the spleen (Fig. 5A) and LLN (Fig. 6A) significantly increased in the OVA-only treated group

compared with that in the control group but decreased in the EV pretreated group while the population of Treg cells was increased. This suggests that EV induce the production of immunomodulatory cytokines, thereby reducing airway inflammatory responses. Studies have shown that parasite-derived EV secrete immunomodulatory cytokines such as IL-10. EV from *Leishmania amazonensis* increase the expression of IL-6 and IL-10 by macrophages³⁵. In addition, EV generated during parasitic infection communicate through cell-cell interactions and act as immunomodulatory agents by altering the immune system of the host³⁶.

Our study confirmed that a therapeutic effect can be induced by altering the function of immune cells in the allergic airway inflammatory response using EV derived from *E. granulosus* cystic fluid. We found that EV, isolated from cystic fluid, induced the expression of immunomodulatory cytokines such as IL-10 and TGF- β , thereby altering the activation of immune cells. This suggests that EV may be useful in controlling the host immune system, similar to the immune system regulation by cystic fluid demonstrated in previous studies. This also means that the immune modulatory response in cystic fluid is likely to be influenced by EV. In this respect, EV will contribute to the development of new therapeutics for immune disorders. Exosome research including EV has emerged as an interesting research field, and exosomes have been found to have therapeutic effects in biological processes including human diseases such as antigen presentation, immune response, cancer metastasis, and inflammation treatment through cell-to-cell communication^{37 38}. Considering that EV carries not only RNA but also various proteins, lipids and cellular metabolites, further investigation is needed to determine which components of the component perform their functions.

References

1. Vuitton, D. A.; McManus, D. P.; Rogan, M. T.; Romig, T.; Gottstein, B.; Naidich, A.; Tuxun, T.; Wen, H.; Menezes da Silva, A.; World Association of, E., International consensus on terminology to be used in the field of echinococcoses. *Parasite* **2020**, 27, 41.
2. De, N. V.; Minh, P. N.; Duyet, L. V.; Bich, N. N.; Son, T. N.; Jung, B. K.; Chai, J. Y., Two Human Cases of Echinococcus orteppi Infection in the Lung and Heart in Vietnam. *Korean J Parasitol* **2020**, 58 (4), 451-456.
3. Soba, B.; Gaspersic, S.; Kese, D.; Kotar, T., Molecular Characterization of

Echinococcus granulosus sensu lato from Humans in Slovenia. *Pathogens* **2020**, *9* (7).

4. Fotiadis, C.; Sergiou, C.; Kyrou, I.; Troupis, T. G.; Tselentis, J.; Doussaitou, P.; Gorgoulis, V. G.; Sechas, M. N., Experimental echinococcus infection in the mouse model: pericystic cellular immunity reaction and effects on the lymphoid organs of immunocompetent and thymectomized mice. *In Vivo* **1999**, *13* (6), 541-6.
5. Zhang, W.; Ross, A. G.; McManus, D. P., Mechanisms of immunity in hydatid disease: implications for vaccine development. *J Immunol* **2008**, *181* (10), 6679-85.
6. Zhang, W.; Wen, H.; Li, J.; Lin, R.; McManus, D. P., Immunology and immunodiagnosis of cystic echinococcosis: an update. *Clin Dev Immunol* **2012**, *2012*, 101895.
7. Amri, M.; Mezioug, D.; Touil-Boukoffa, C., Involvement of IL-10 and IL-4 in evasion strategies of Echinococcus granulosus to host immune response. *Eur Cytokine Netw* **2009**, *20* (2), 63-8.
8. Tuxun, T.; Wang, J. H.; Lin, R. Y.; Shan, J. Y.; Tai, Q. W.; Li, T.; Zhang, J. H.; Zhao, J. M.; Wen, H., Th17/Treg imbalance in patients with liver cystic echinococcosis. *Parasite Immunol* **2012**, *34* (11), 520-7.
9. Wu, Z.; Wang, L.; Tang, Y.; Sun, X., Parasite-Derived Proteins for the Treatment of Allergies and Autoimmune Diseases. *Front Microbiol* **2017**, *8*, 2164.
10. Ouaisi, A., Regulatory cells and immunosuppressive cytokines: parasite-derived factors induce immune polarization. *J Biomed Biotechnol* **2007**, *2007* (4), 94971.
11. Gao, X.; Ren, X.; Wang, Q.; Yang, Z.; Li, Y.; Su, Z.; Li, J., Critical roles of regulatory B and T cells in helminth parasite-induced protection against allergic airway inflammation. *Clin Exp Immunol* **2019**, *198* (3), 390-402.
12. Coakley, G.; McCaskill, J. L.; Borger, J. G.; Simbari, F.; Robertson, E.; Millar, M.; Marcus, Y.; McSorley, H. J.; Maizels, R. M.; Buck, A. H., Extracellular Vesicles from a Helminth Parasite Suppress Macrophage Activation and Constitute an Effective Vaccine for Protective Immunity. *Cell Rep* **2017**, *19* (8), 1545-1557.
13. Khelifi, L.; Soufli, I.; Labsi, M.; Touil-Boukoffa, C., Immune-protective effect of echinococcosis on colitis experimental model is dependent of down regulation of TNF-alpha and NO production. *Acta Trop* **2017**, *166*, 7-15.
14. Zhang, W.; Li, J.; McManus, D. P., Concepts in immunology and diagnosis of hydatid disease. *Clin Microbiol Rev* **2003**, *16* (1), 18-36.
15. Bayraktar, M. R.; Mehmet, N.; Durmaz, R., Th1 and Th2 inducing cytokines in Cystic

echinococcosis. *Turkiye Parazitol Derg* **2005**, 29 (3), 167-70.

16. Raposo, G.; Stoorvogel, W., Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* **2013**, 200 (4), 373-83.
17. Maas, S. L. N.; Breakefield, X. O.; Weaver, A. M., Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends Cell Biol* **2017**, 27 (3), 172-188.
18. Zaborowski, M. P.; Balaj, L.; Breakefield, X. O.; Lai, C. P., Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience* **2015**, 65 (8), 783-797.
19. Hansen, E. P.; Fromm, B.; Andersen, S. D.; Marcilla, A.; Andersen, K. L.; Borup, A.; Williams, A. R.; Jex, A. R.; Gasser, R. B.; Young, N. D.; Hall, R. S.; Stensballe, A.; Ovchinnikov, V.; Yan, Y.; Fredholm, M.; Thamsborg, S. M.; Nejsun, P., Exploration of extracellular vesicles from *Ascaris suum* provides evidence of parasite-host cross talk. *J Extracell Vesicles* **2019**, 8 (1), 1578116.
20. Coakley, G.; Maizels, R. M.; Buck, A. H., Exosomes and Other Extracellular Vesicles: The New Communicators in Parasite Infections. *Trends Parasitol* **2015**, 31 (10), 477-489.
21. Twu, O.; de Miguel, N.; Lustig, G.; Stevens, G. C.; Vashisht, A. A.; Wohlschlegel, J. A.; Johnson, P. J., *Trichomonas vaginalis* exosomes deliver cargo to host cells and mediate host-parasite interactions. *PLoS Pathog* **2013**, 9 (7), e1003482.
22. Ofir-Birin, Y.; Regev-Rudzki, N., Extracellular vesicles in parasite survival. *Science* **2019**, 363 (6429), 817-818.
23. Wu, Z.; Wang, L.; Li, J.; Wang, L.; Wu, Z.; Sun, X., Extracellular Vesicle-Mediated Communication Within Host-Parasite Interactions. *Front Immunol* **2018**, 9, 3066.
24. Khosravi, M.; Mirsamadi, E. S.; Mirjalali, H.; Zali, M. R., Isolation and Functions of Extracellular Vesicles Derived from Parasites: The Promise of a New Era in Immunotherapy, Vaccination, and Diagnosis. *Int J Nanomedicine* **2020**, 15, 2957-2969.
25. Kim, H. J.; Kang, S. A.; Yong, T. S.; Shin, M. H.; Lee, K. J.; Park, G. M.; Suvonkulov, U.; Yu, H. S., Therapeutic effects of *Echinococcus granulosus* cystic fluid on allergic airway inflammation. *Exp Parasitol* **2019**, 198, 63-70.
26. Cho, K. S.; Kang, S. A.; Kim, S. D.; Mun, S. J.; Yu, H. S.; Roh, H. J., Dendritic cells and M2 macrophage play an important role in suppression of Th2-mediated inflammation by adipose stem cells-derived extracellular vesicles. *Stem Cell Res* **2019**, 39, 101500.
27. Nakae, S.; Ho, L. H.; Yu, M.; Monteforte, R.; Iikura, M.; Suto, H.; Galli, S. J., Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine

production in an asthma model in mice. *J Allergy Clin Immunol* **2007**, *120* (1), 48-55.

28. Mohan, A.; Agarwal, S.; Clauss, M.; Britt, N. S.; Dhillon, N. K., Extracellular vesicles: novel communicators in lung diseases. *Respir Res* **2020**, *21* (1), 175.
29. Du, Y. M.; Zhuansun, Y. X.; Chen, R.; Lin, L.; Lin, Y.; Li, J. G., Mesenchymal stem cell exosomes promote immunosuppression of regulatory T cells in asthma. *Exp Cell Res* **2018**, *363* (1), 114-120.
30. Ding, F. X.; Liu, B.; Zou, W. J.; Li, Q. B.; Tian, D. Y.; Fu, Z., Pseudomonas aeruginosa-derived exosomes ameliorates allergic reactions via inducing the Treg response in asthma. *Pediatr Res* **2018**, *84* (1), 125-133.
31. Wang, H.; Li, J.; Pu, H.; Hasan, B.; Ma, J.; Jones, M. K.; Zheng, K.; Zhang, X.; Ma, H.; McManus, D. P.; Lin, R.; Wen, H.; Zhang, W., Echinococcus granulosus infection reduces airway inflammation of mice likely through enhancing IL-10 and down-regulation of IL-5 and IL-17A. *Parasit Vectors* **2014**, *7*, 522.
32. Sun, S.; Li, H.; Yuan, Y.; Wang, L.; He, W.; Xie, H.; Gao, S.; Cheng, R.; Qian, H.; Jiang, H.; Wang, X.; Zhan, B.; Fang, Q.; Yang, X., Preventive and therapeutic effects of Trichinella spiralis adult extracts on allergic inflammation in an experimental asthma mouse model. *Parasit Vectors* **2019**, *12* (1), 326.
33. Nicolao, M. C.; Rodriguez Rodrigues, C.; Cumino, A. C., Extracellular vesicles from Echinococcus granulosus larval stage: Isolation, characterization and uptake by dendritic cells. *PLoS Negl Trop Dis* **2019**, *13* (1), e0007032.
34. Haniloo, A.; Ghasemi, F.; Shikhi, A.; Ghavami, M. B., Immunoregulatory Cytokine (TGF-beta And IL-10) Responses in Mice Inoculated With Protoscoleces and Major Hydatid Fluid Antigens of Cystic Echinococcosis. *Iranian Journal of Parasitology* **2008**, *3* (3), 18-23.
35. Barbosa, F. M. C.; Dupin, T. V.; Toledo, M. D. S.; Reis, N.; Ribeiro, K.; Cronemberger-Andrade, A.; Rugani, J. N.; De Lorenzo, B. H. P.; Novaes, E. B. R. R.; Soares, R. P.; Torrecilhas, A. C.; Xander, P., Extracellular Vesicles Released by Leishmania (Leishmania) amazonensis Promote Disease Progression and Induce the Production of Different Cytokines in Macrophages and B-1 Cells. *Front Microbiol* **2018**, *9*, 3056.
36. Montaner, S.; Galiano, A.; Trelis, M.; Martin-Jaular, L.; Del Portillo, H. A.; Bernal, D.; Marcilla, A., The Role of Extracellular Vesicles in Modulating the Host Immune Response during Parasitic Infections. *Front Immunol* **2014**, *5*, 433.
37. Schorey, J. S.; Harding, C. V., Extracellular vesicles and infectious diseases: new

complexity to an old story. *J Clin Invest* **2016**, *126* (4), 1181-9.

38. Zhou, X.; Xie, F.; Wang, L.; Zhang, L.; Zhang, S.; Fang, M.; Zhou, F., The function and clinical application of extracellular vesicles in innate immune regulation. *Cell Mol Immunol* **2020**, *17* (4), 323-334.

Table 1. Primers Used Real-Time PCR

Primer	Sequence
GAPDH-for*	5'- TAC CCC CAA TGT GTC CGT C -3'
GAPDH-rev [#]	5'- AAG AGT GGG AGT TGC TGA AG -3'
IL10-for	5'-GCT ATG CTG CCT GGT CTT ACT G-3'
IL10-rev	5'-TCC AGC TGG TCC TTT GTT TG-3'
IL4-for	5'-CGT CTG TAG GGC TTC CAA GGT G-3'
IL4-rev	5'-GTG CAG CTT ATC GAT GAA TCC AGC-3'
Eotaxin-for	5'-GCG CTT CTA TTC CTG CTG CTC ACG G-3'
Eotaxin-rev	5'-GTG GCA TCC TGG ACC CAC TTC TTC-3'
TSLP-for	5'-GGA GAT TTG AAA GGG GCT AAG-3'
TSLP-rev	5'-TGG GCA GTG GTC ATT GAG-3'
IL25-for	5'-TGG CAA TGA TCG TGG GAA CC -3'
IL25-rev	5'-GAG AGA TGG CCC TGC TGT TGA-3'
IL33-for	5'-GTA GTA GCA CCT GGT CTT GCT CTT-3'
IL33-rev	5'-CCT CCC TGA GTA CAT ACA ATG ACC CTT-3'
IL35-for	5'-CCG GTC CAG CAT GTG TCA A-3'
IL35-rev	5'-CAG GTT TCG GGA CTG GCT AAG A-3'

*for; forward,

[#]rev; reverse

Figure legends

Fig. 1. Morphological study of extracellular vesicles derived from *E. granulosus* cystic fluid. Transmission electron microscopy shows that EV are spherical and have a lipid bilayer (original magnification $\times 40,000$) (A). The average diameter of the EV was $33.93 \pm 27.87\text{nm}$, and dynamic light scattering was used (B).

Fig. 2. Chemokine gene expression in MLE-12 cells after EV treatment. After pretreatment with *Aspergillus* protease antigen at a concentration of 200 ng, MLE-12 cells were treated with EV at 0.5, 1, and 2 μg to evaluate the expression of Th2- and Treg cell-related chemokine and cytokine genes by real-time PCR. All data analysis of real-time PCR was calculated through the Gene-x program (Bio-Rad laboratories, Inc., USA). (* $p < 0.05$ **; $p < 0.01$, ***; $p < 0.001$).

Fig. 3. Effect of EV treatment on OVA-induced allergic airway inflammation. Schedule of allergic airway inflammation mouse model (A). EV (1 mg) or PBS was orally administered seven times, once every other day. Mice were sensitized by intraperitoneal injection of OVA-alum or PBS on days 14, 15, 21, and 22, and challenged intra nasally with OVA on days 29, 30, 36, and 37 (I.P; intraperitoneal, I.N; intra nasal). The value of PenH was evaluated at baseline and after treatment of aerosolized methacholine at increasing doses (0, 12.5, 25, and 50 mg/mL) (B). The number of inflammatory cells in BALF was performed by Diff-Quik staining and counted. (Control group; PBS I.P and PBS I.N, OVA group; OVA and alum I.P and OVA I.N., EV + OVA group; seven times pretreatment of EV and OVA and alum I.P and OVA I.N., only EV group; seven times pretreatment of EV and PBS I.P and PBS I.N, **; $p < 0.01$, ***; $p < 0.001$).

Fig. 4. Expression levels of cytokines in the spleen. Cytokine concentration was measured by CD3 stimulation in lymphocytes isolated from the spleen (A) and LLNs (B). And cytokine concentration was measured in the BALF (C) using the supernatant. To confirm the effect of EV administration on cytokine levels in the spleen, the levels of IL-4, IL-5, IL-13, IL-17A, IL-10, and TGF- β were measured using ELISA (* $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$).

Fig. 5. Cell population of CD4⁺IL-4⁺T cells and CD4⁺CD25⁺Foxp3⁺ T cells in the spleen.

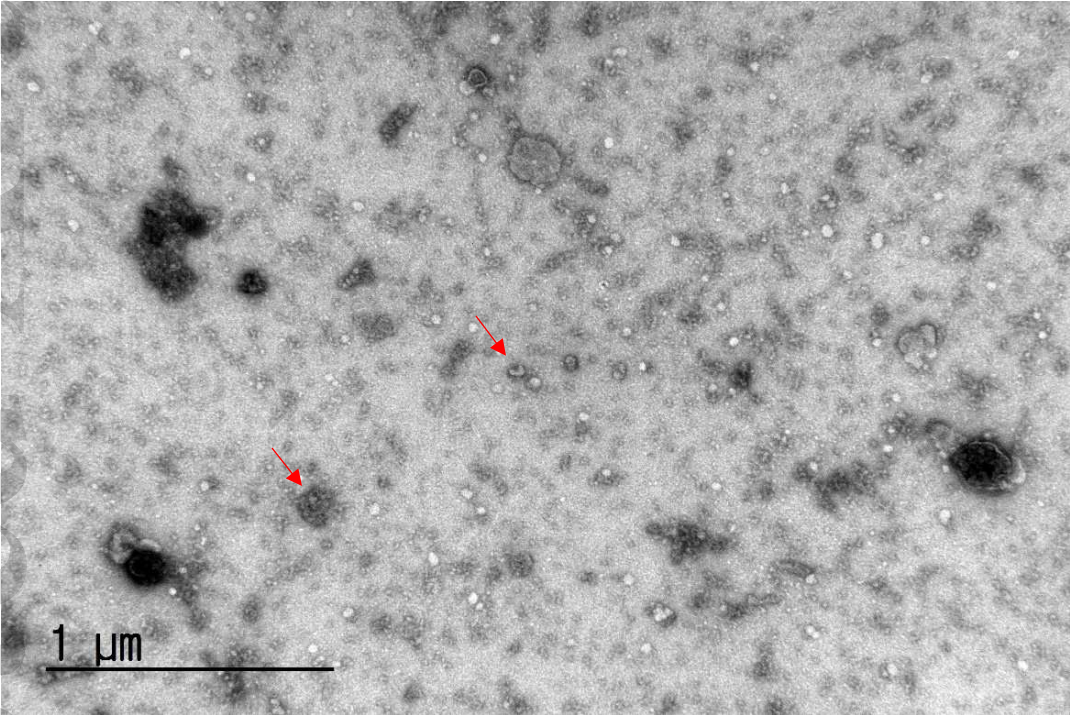
The cell population of CD4⁺IL-4⁺T cells (A) and CD4⁺CD25⁺Foxp3⁺ T cells (B) was obtained through FACS analysis. Cells isolated from mouse spleens were fluorescently stained. The stained cells were analyzed for the ratio of IL-4 cells and CD25⁺Foxp3⁺ cells through gated CD4 cells. Experimental data was analyzed using V10 (FLOWJO). (* $p < 0.05$, *** $p < 0.001$).

Fig. 6. Cell population of CD4⁺IL-4⁺T cells and CD4⁺CD25⁺Foxp3⁺ T cells in the LLN.

The cell population of CD4⁺IL-4⁺T cells (A) and CD4⁺CD25⁺Foxp3⁺ T cells (B) was obtained through FACS analysis. Cells isolated from mouse LLNs were fluorescently stained. The stained cells were analyzed for the ratio of IL-4 cells and CD25⁺Foxp3⁺ cells through gated CD4 cells. Experimental data was analyzed using V10 (FLOWJO). (* $p < 0.05$, *** $p < 0.001$).

S1 Fig. Histological changes in mouse lung

A



B

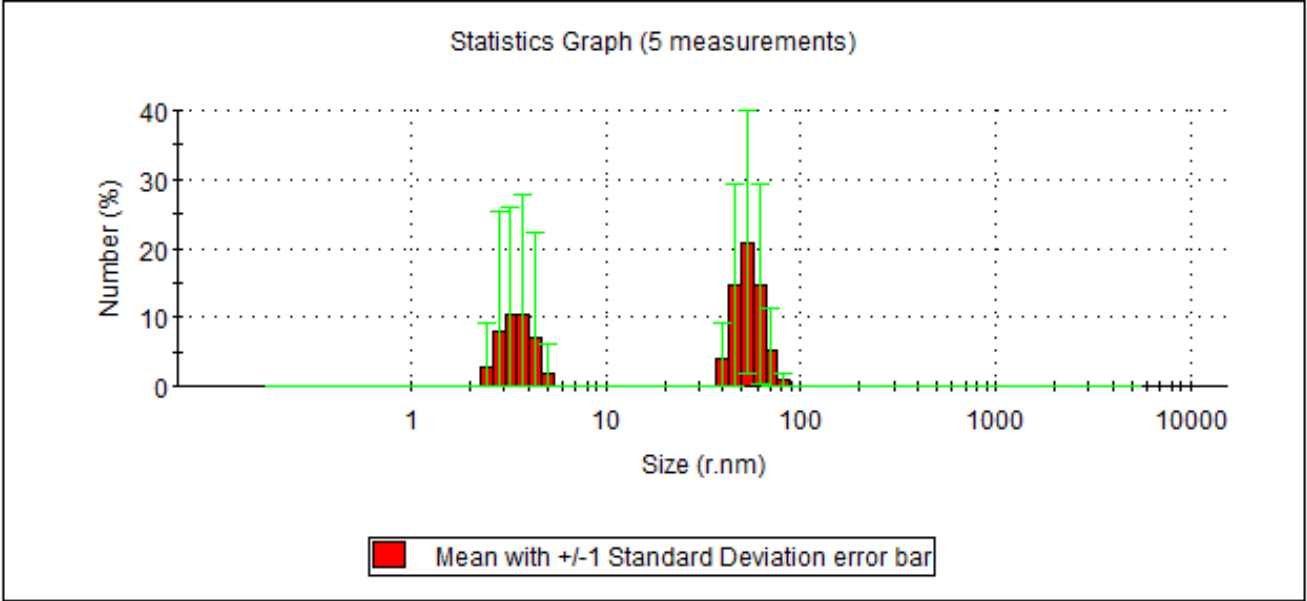


Figure 1

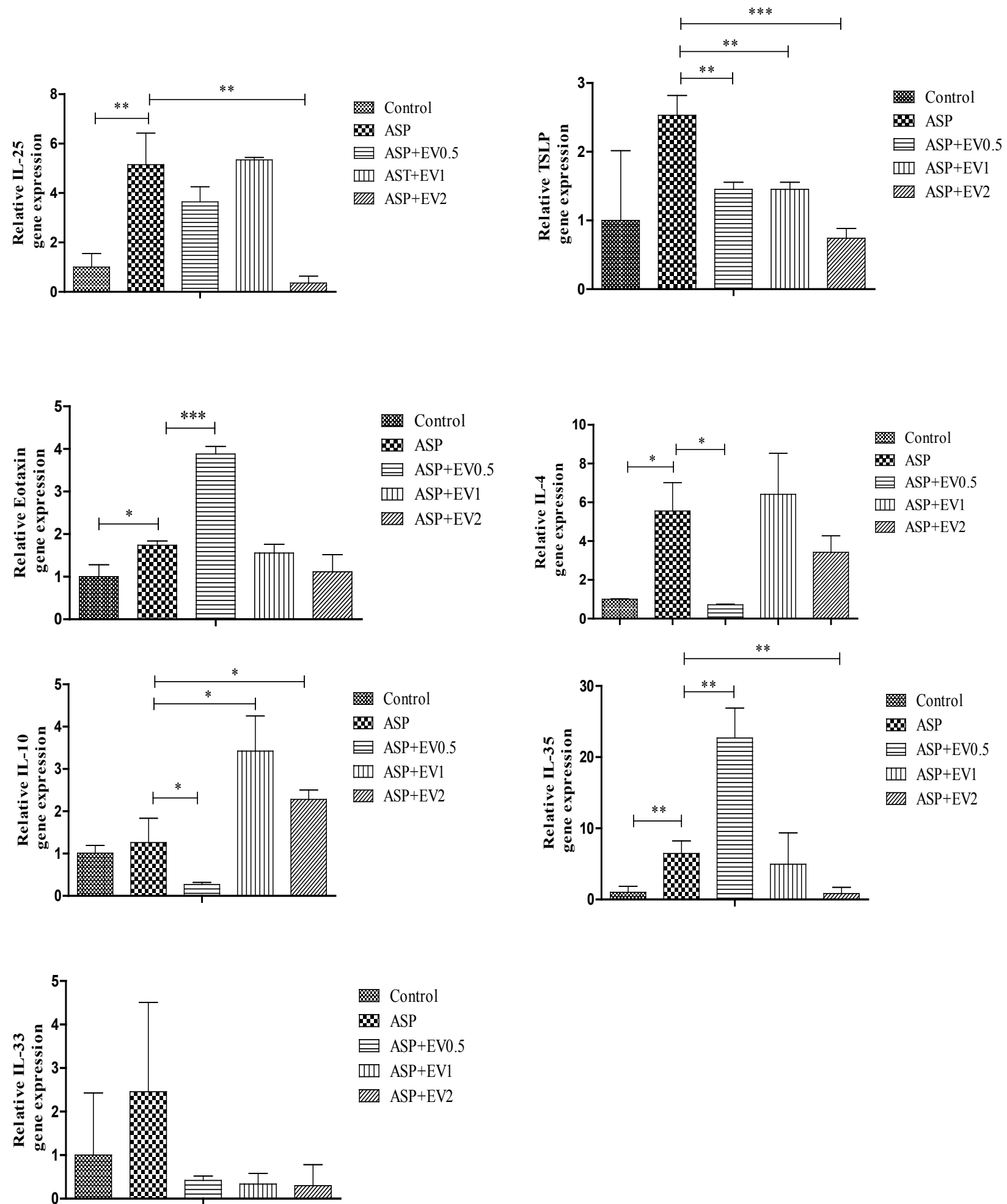
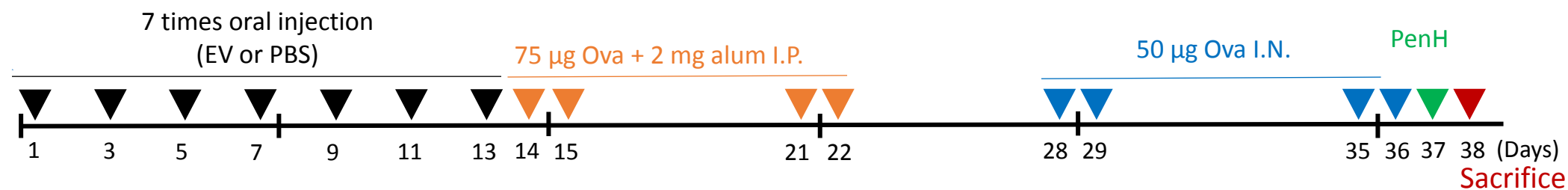
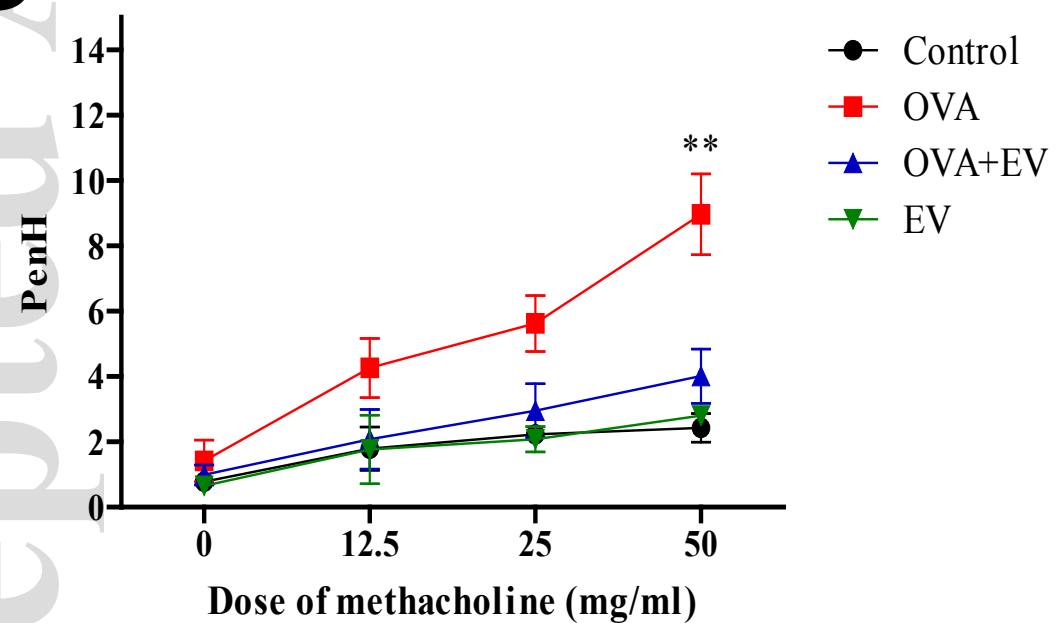


Figure 2

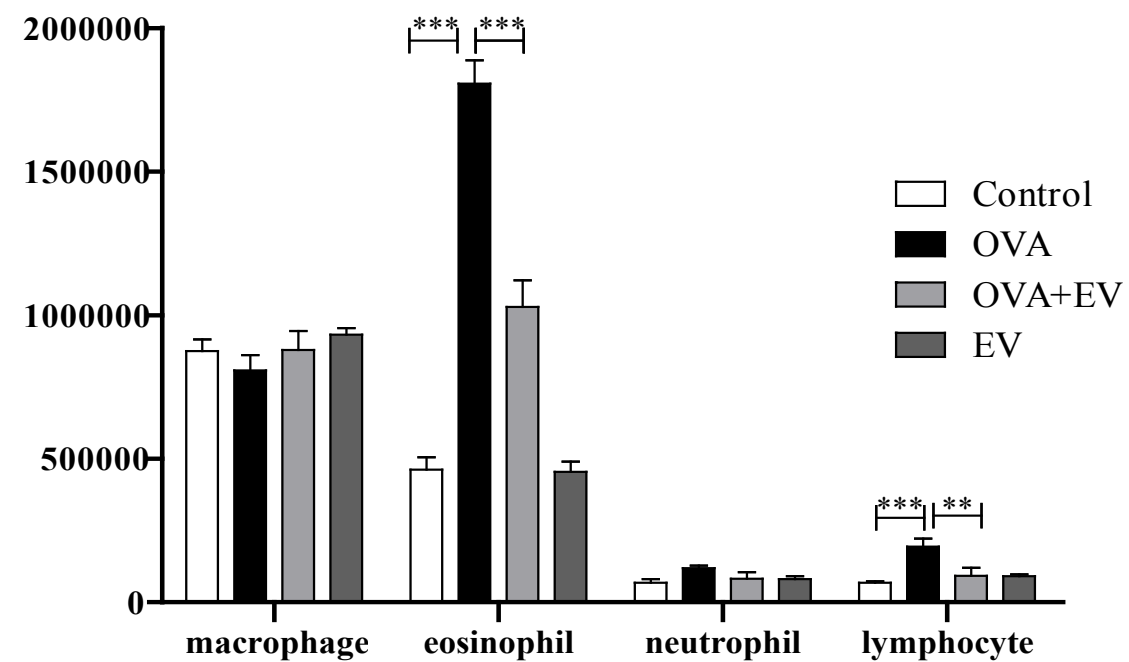
A

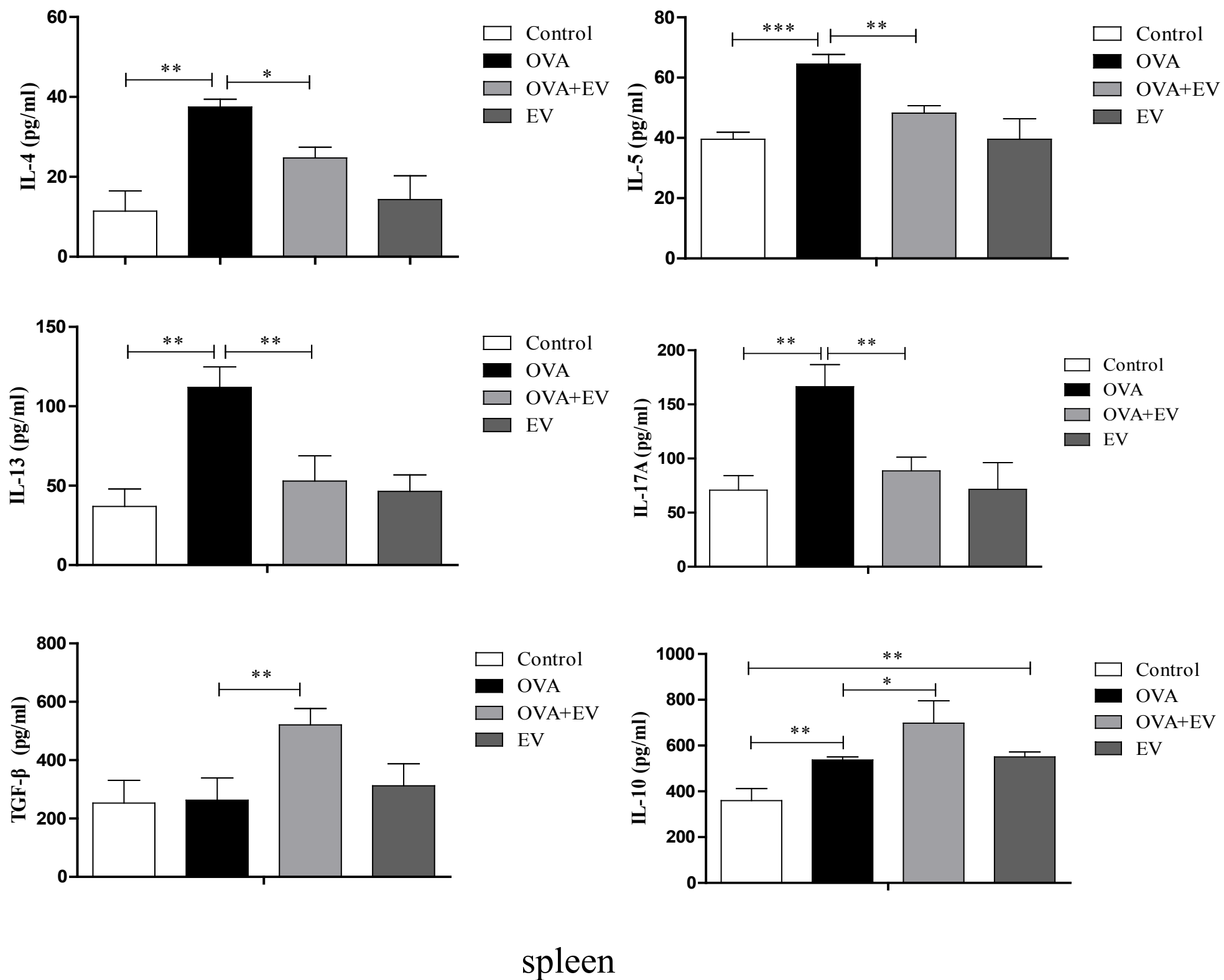


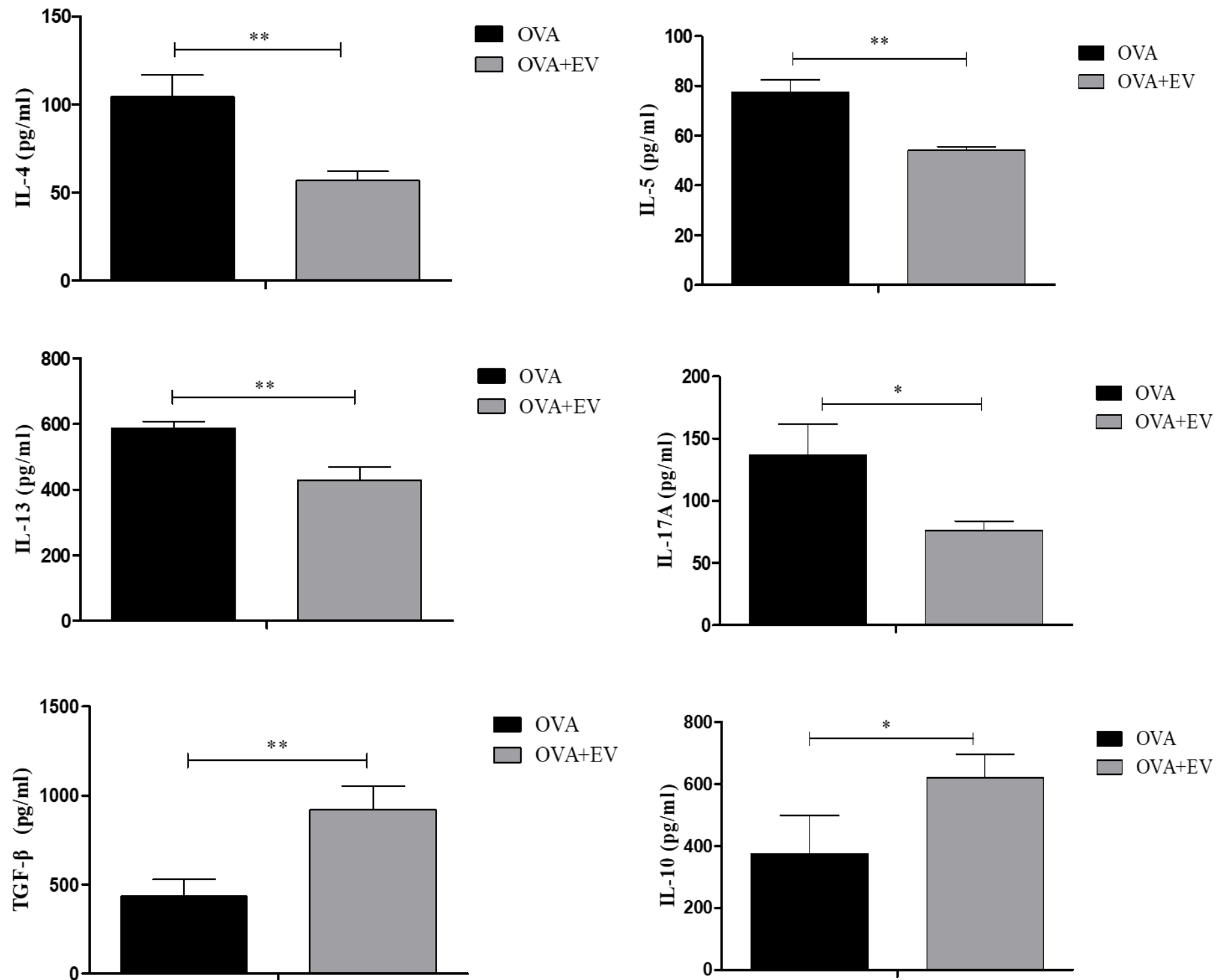
B



C



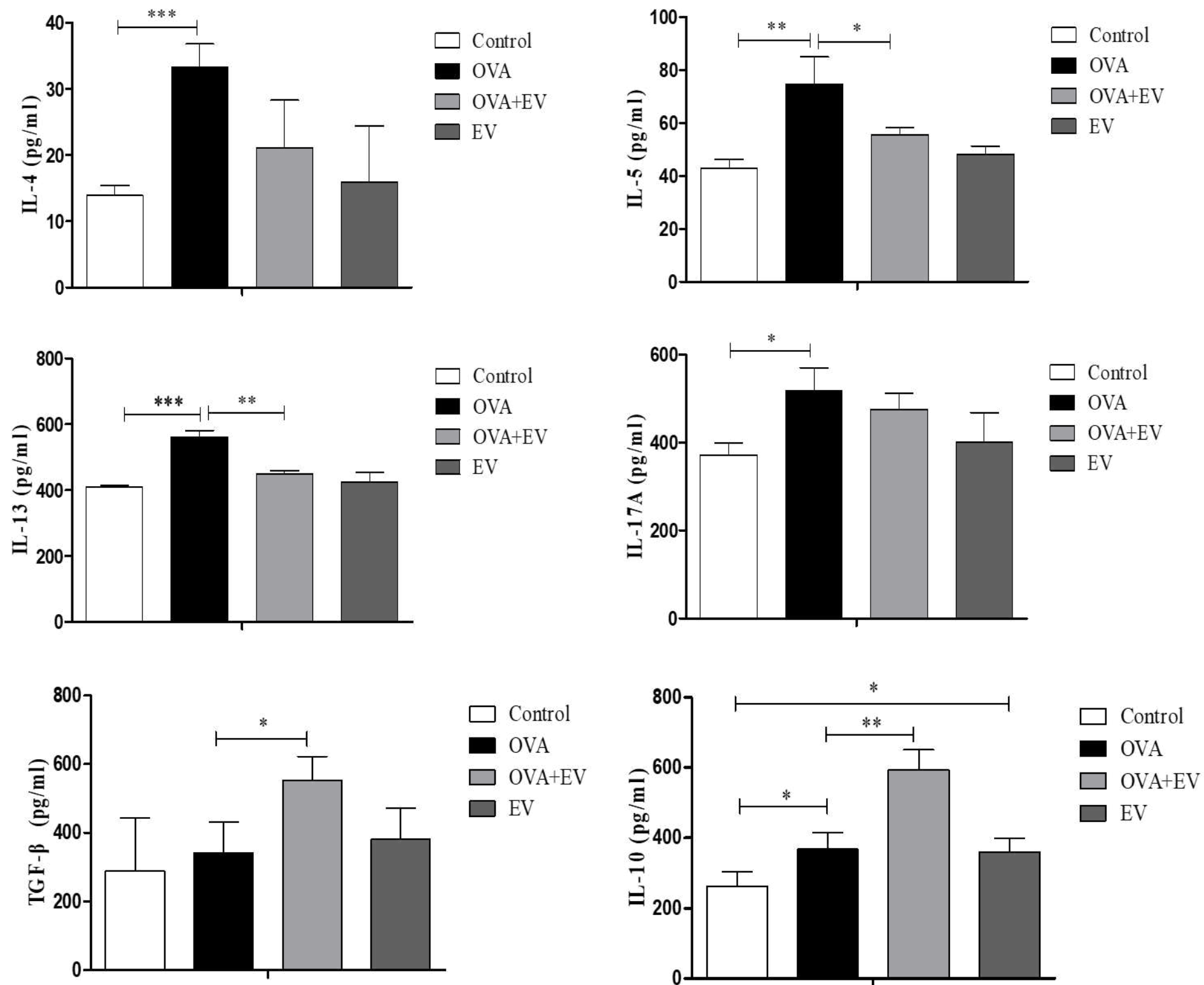
A

B

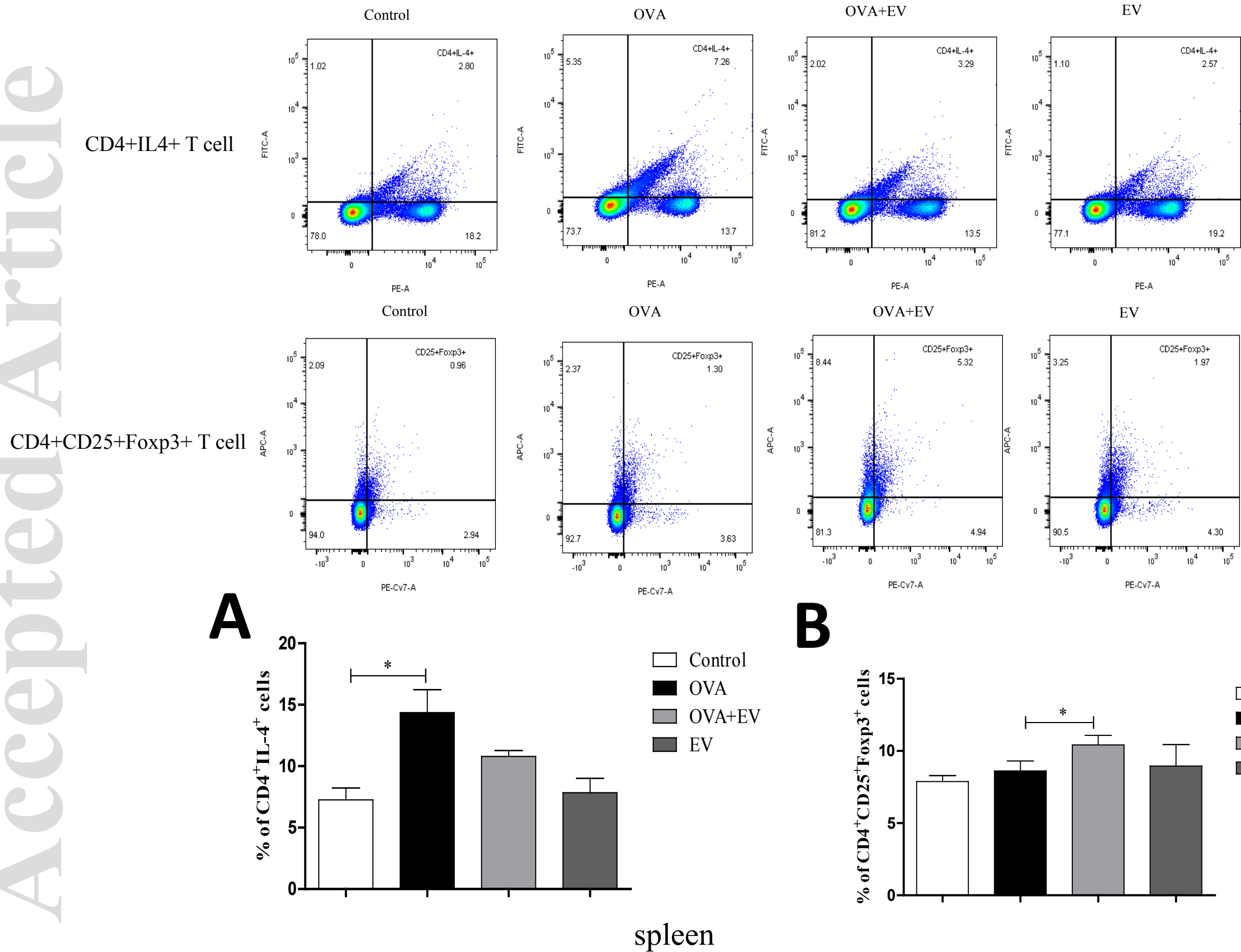
Lung draining lymph node (LLN)

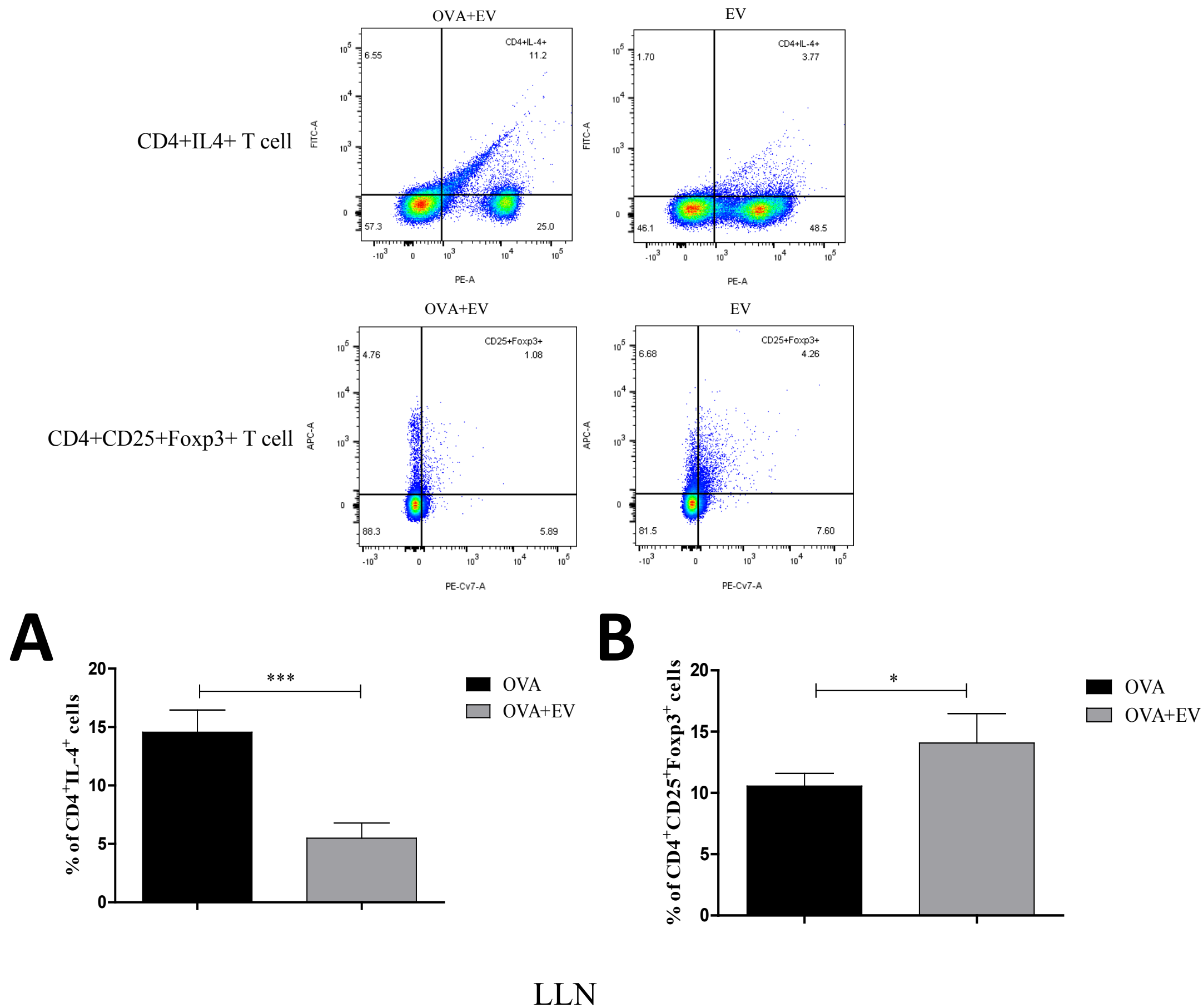
This article is protected by copyright. All rights reserved

C

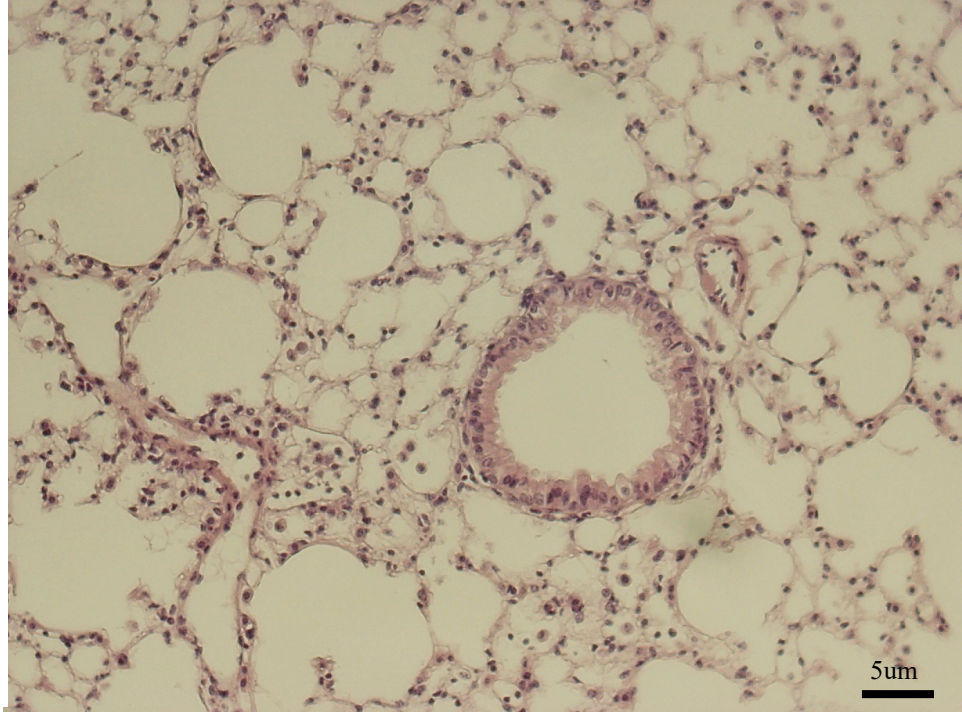


BALF

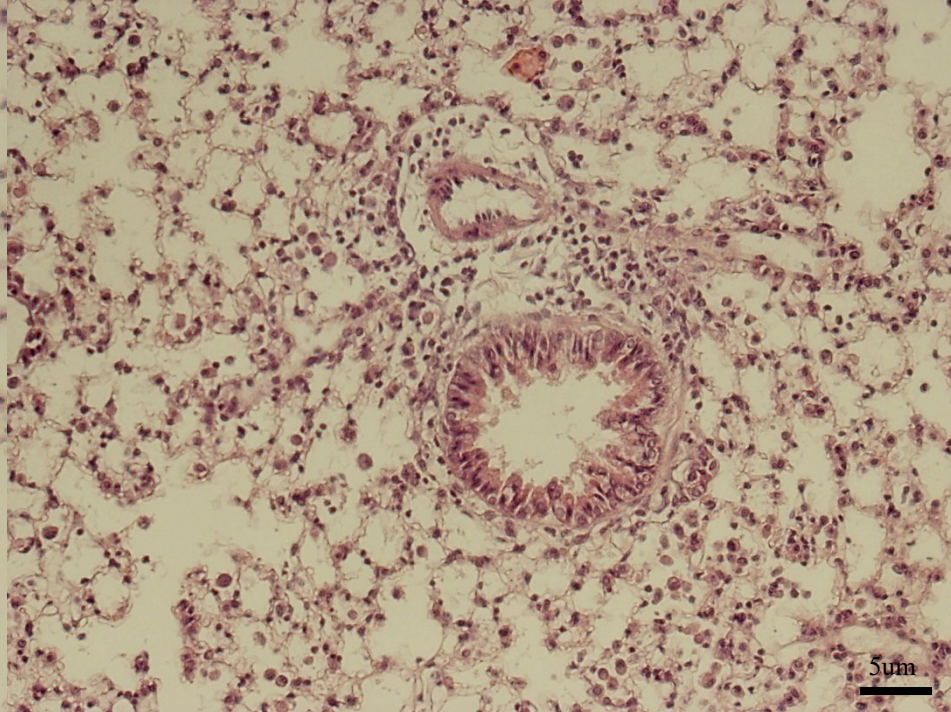




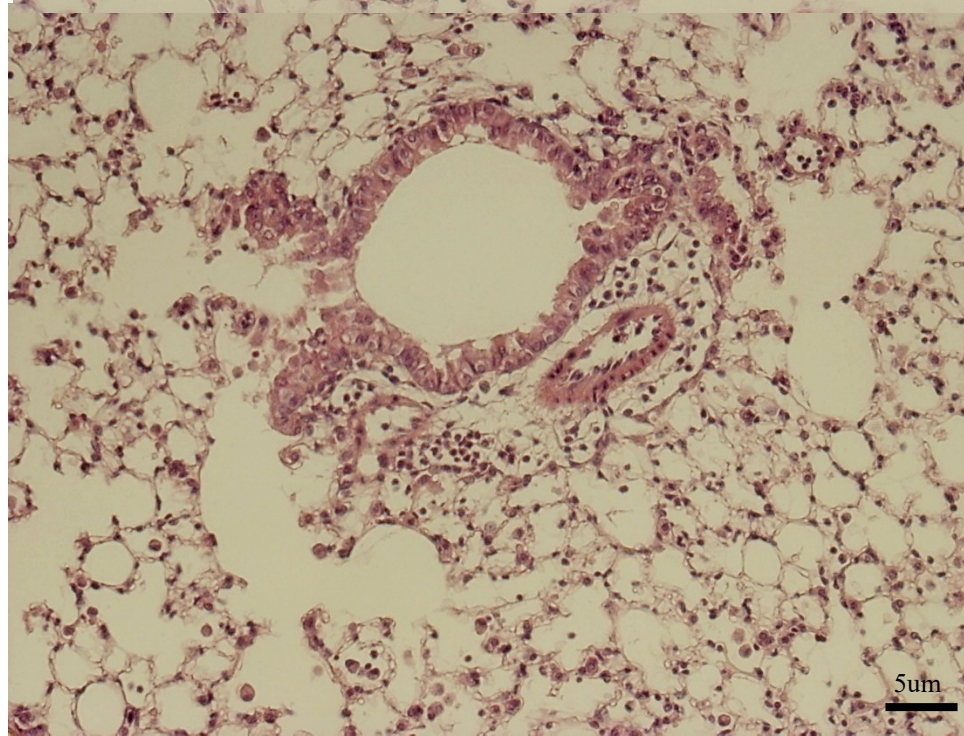
Control



OVA



OVA + EV



EV

