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Original Article

Maggot extracts promote regulatory T cell differentiation by upregulating Foxp3 in allergic rhinitis

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Abstract: Background and objective: Maggots are the larval stage of *Lucilia sericata* and have strong antibacterial activity and immunomodulatory effects. The objective of our study was to investigate whether maggot extracts can modulate regulatory T cells (Tregs) and treat allergic rhinitis (AR). Methods: Mice were randomly assigned to five groups (n=6/group): normal, AR, Maggot, AR+ Maggot, and AR+ dexamethasone (DXM). The Total Nasal Symptom Score (TNSS), ovalbumin (OVA)-sIgE titers, histopathological characteristics and Th1-/Th2-/Th17-related cytokine levels were evaluated. The expression of T-bet, GATA3, RORγt and Foxp3 in the spleen and nasal mucosa of mice was detected, and the proportion of differentiated Tregs in the spleen of mice was determined. In addition, the effects of maggot extracts on the expression level of Foxp3 and the differentiation of Tregs in vitro were studied. Histological evaluation of the potential toxicity was also performed. Results: Compared with the AR group, the AR+ Maggot group showed reduction in histopathological inflammation, downregulated OVA-sIgE titers, and restoration of the imbalance in cytokine profiles ($P<0.05$). After treatment with maggot extracts, the proportions of Tregs and Foxp3 expression in the spleen were significantly increased, the expression of GATA3 and RORγt was decreased ($P<0.05$), and the expression of T-bet showed no significant change ($P>0.05$). In vitro, maggot extracts promoted the expression of Foxp3 and differentiation of Tregs in a dose- and time-dependent manner ($P<0.05$). In addition, no obvious organ damage was observed in mice treated with maggot extracts. Conclusion: Maggot extracts can inhibit the progression of AR by upregulating the level of Foxp3 and promoting the differentiation of Tregs, thus serving as an alternate treatment for AR.

Keywords: Maggot extracts, regulatory T cells, Foxp3, allergic rhinitis, immune imbalance

Introduction

Allergic rhinitis (AR) is a type I hypersensitivity mediated by immunoglobulin E (IgE) and characterized by persistent sneezing, clear nasal discharge and nasal congestion in an atopic individual exposed to an allergen [1-3]. The pathogenesis of AR is complex and involves multiple subsets of T cells [4]. It was previously believed that the onset of AR was caused by an immune imbalance between Th1 cells and Th2 cells and that AR was mainly related to an enhanced Th2 cell response [5]. However, further research revealed that the occurrence of AR cannot be completely explained by Th1/Th2 immune reaction imbalance, as an imbalance in Th17 cells and regulatory T cells (Tregs) is also involved in this process [6].

Th1/Th2 cells are a pair of major $CD4^+$ T cell subsets that usually show enhanced differentiation favoring Th2 cells and reduced differentiation of Th1 cells in allergic reactions [5, 7]. Th1 cells can secrete IFN-γ to eliminate intracellular pathogens [8], whereas Th2 cells release cytokines such as IL-4, IL-5, and IL-13 to promote the occurrence of allergic diseases and aggravate the symptoms of allergic reactions [9]. In models of allergic diseases such as asthma [10], allergic dermatitis [11] and food allergies

[12], the immune response shows increased Th2 cell differentiation and hypersecretion of cytokines; thus, specific suppression of the Th2 cell immune response has become a method for the rapid treatment of most allergic diseases [13]. Tregs/Th17 cells are another pair of $CD4^+$ T cells [7]. In allergic diseases [14], Tregs can regulate both innate immunity by blocking the degranulation of mast cells, the differentiation of type 2 innate lymphoid cells, and adaptive immunity by affecting effector T cells (Th1/Th2/Th17). Th17 cells express the specific transcription factor RORγt and can secrete IL-17, which has strong proinflammatory properties. Th17 cells and the levels of IL-17 in the nasal lavage fluid of AR model mice were significantly increased; these increases were correlated with allergic symptoms and the degree of eosinophilic infiltration. In AR model mice exposed to an allergen, the Th17 immune response was significantly overactive and could promote Th2 cytokine secretion and eosinophilic granulocyte aggregation [15]. Theoretically, an in-depth study of the pathogenesis of the imbalanced differentiation of $CD4^+$ T cells and the mechanism of this abnormal regulation could provide new options for treating and curing AR and other allergic diseases.

Maggots, also known as “Wu Gu Chong” in traditional Chinese medicine, were used as natural antibiotics in the 1930s [16]. Because of their durability, safety, effectiveness, lack of drug resistance and reasonable cost, maggot extracts have elicited widespread enthusiasm in the international medical community. At present, maggot extracts have been approved by the Food and Drug Administration for clinical use. They have a variety of beneficial biological activities, such as antibacterial and immunomodulatory activities [17]. Maggot extracts have been clinically used to treat tissue necrosis and wound infection [18] and can be combined with other drugs to treat gastric cancer and relieve coma [19]. The excreta and secretions in maggot extracts can also inhibit the proinflammatory responses of various neutrophils but do not compromise their antibacterial activity [20]. A recent study indicated that maggot extracts can ameliorate the inflammatory response in a mouse model of ulcerative colitis (UC) by regulating the Nrf2/HO-1 pathway to reduce inflammation and oxidative stress [21].

Th/Tr imbalance, B cell system imbalance, and innate immune system dysfunction are important factors leading to the occurrence of UC [22]. However, the pathogenesis of AR is currently unclear, although there are data suggesting that pathogenesis is related to the Th1/Th2 immune imbalance [23]. Based on the relationship between maggot extracts and the host immune system and the therapeutic effect of maggot extracts on UC, we wondered whether maggot extracts play regulatory roles in the occurrence and development of AR. To date, there is no research report on the influence of maggot extracts on AR. Our research was dedicated to elucidating the impact of maggot extracts on the AR inflammatory response in vivo and in vitro and to clarifying the potential mechanism by which maggot extracts inhibit inflammation in AR in order to identify alternate strategies and directions for the potential treatment of AR.

Materials and methods

Maggots and maggot powder

Blowflies were raised and propagated under aseptic conditions. After the flies were propagated, maggots were collected and processed in accordance with the traditional processing method. Maggots were washed several times with aseptic water and air dried. The dried maggots were gently heated until they became slightly yellow and then ground into powder. Finally, the maggot powder was disinfected and vacuum-packed.

An appropriate amount of maggot powder was taken and added to a 2-fold volume of phosphate-buffered saline (PBS) to prepare the maggot extract homogenate. The resulting supernatant was collected and incubated in a 70°C thermostatic water bath for 30 s. The sample was centrifuged at 15000×g for 10 min, and the collected supernatant was filtered through a 0.22 μm filter [21, 24].

Experimental animals

Sixty specific pathogen-free (SPF) C57BL/6 female mice aged 4 weeks and weighing 20 to 25 g were provided by Beijing Vitong Lihua Experimental Animal Technology Co., Ltd. (License No.: SCXK2016-0006). Mice were raised in an SPF barrier environment at the Animal Experimental Center of the Renmin Hospital of Wuhan University at a room temperature of 18-22°C and humidity of 50%-

60%. All of the mice had free access to a daily diet and activities. On the 29th day of modeling, the mice were anesthetized via intraperitoneal injection with 0.3 mL of 2% pentobarbital sodium and were sacrificed via cervical dislocation. All experimental procedures for mice were approved by the Animal Ethics Committee of the Renmin Hospital of Wuhan University in accordance with the guidelines of animal ethics (ethical approval number of WDRM (F) No. 20200602).

Animal models

Mice were fed adaptively in the SPF laboratory of the Animal Experimental Center, Renmin Hospital of Wuhan University for 1 week. They were randomly assigned to 5 groups: normal (normal saline only), AR (ovalbumin, OVA), Maggot (maggot extracts only), AR+ Maggot (OVA+ maggot extracts), and AR+ DXM (OVA+ dexamethasone), with 12 mice in each group. The mice in the AR, AR+ Maggot and AR+ DXM groups were intraperitoneally injected with 300 μL of normal saline containing 100 μg of OVA and 2 mg of aluminum hydroxide (basal sensitization) on days 0, 2, 4, 6, 8, 10 and 12. The mice in the normal and Maggot groups were intraperitoneally injected with 300 μL of normal saline at the same time. Beginning on the 14th day, the AR group was stimulated with 10% OVA in normal saline dropped into the nasal cavity (once a day, 10 μL per nostril), while the AR+ Maggot group were treated with maggot extracts via gavage (1 g/kg) 1 h before each OVA nasal drip [25]. Mice in the AR+ DXM group received intraperitoneal injection of DXM (2 mg/kg) 1 h before each OVA nasal drip [2], mice in the Maggot group received only gavage treatment with maggot extracts (1 g/kg), and mice in the normal group received normal saline as the nasal drip treatment. The treatment time was the same as that described above, and treatments ended on the 27th day. OVA and aluminum hydroxide were purchased from Sigma (USA) and Pierce Chemical Company (USA), respectively.

Observation of the biological behavior of the AR model

The biological behaviors of the mice (n=6/group) in each group, including nasal scratching, sneezing, and nasal discharge, were observed within 30 min after the last nasal stimulation. The Total Nasal Symptom Score (TNSS) was recorded as follows: 0: no sneezes, no nasal cleansing and no nasal scratching; 1: 1-3 sneezes, runny nose at nasal opening, and slight scratching nose several times; 2: 4-10 sneezes, nasal clearing over the nasal opening, and repeated nose scratching with both paws; and 3: >11 sneezes, runny cheeks, scratching and rubbing. A total score of more than 5 indicated successful modeling [26].

OVA-specific serum immunoglobulin E (IgE) assay

After the mice were deeply anesthetized, the eyeball was rapidly excised with tweezers, and peripheral blood was collected into an EP tube. The samples were incubated at room temperature for 3 h and then centrifuged at 4°C and 2500×g for 15 min (the centrifugation time was increased appropriately according to the sample condition) until the serum was completely separated. The serum was stored at -80°C until use in further experiments. The titers of OVA-specific IgE in the serum were detected using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Wuhan Hualianke Technology Co., Ltd. The experiment was repeated three times, with n=6 in each group.

Morphological observation of the nasal mucosa

After blood collection, mice were euthanized by cervical dislocation, and the complete nasal bone was excised. After fixation in paraformaldehyde (10%) for 24 h, the nasal bone was transferred to a 10% EDTA decalcification solution. After the bone was completely softened (approximately 3-4 weeks), it was paraffin embedded. Paraffin sections were used for hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining. Morphological changes in the nasal mucosa, including the conditions of the upper layer, basement membrane, lamina propria and submucosa, were observed in each group. The eosinophil infiltration status, goblet cell proliferation and secretion, the degrees of vascular dilatation and congestion, and gland hyperplasia were also assessed. The eosinophil count and goblet cell count in 5 randomly selected high-power (HP) fields (200×) were averaged. Polyformaldehyde and the 10% EDTA decalcification solution were

purchased from Wuhan Saiwell Biotechnology Co., Ltd. The experiment was repeated three times, with n=6 in each group.

Western blot detection

The nasal mucosal and spleen tissues were scraped, and the quality was recorded. Then, 80 μL of precooled RIPA lysis solution, 10 μL of protease inhibitor and 10 μL of phosphatase inhibitor were added to 10 mg of tissue to make homogenates. After ultrasonic lysis for 1 min, tissues were placed on ice for 30 min. Then, the homogenates were centrifuged at 11,000×g and 4°C for 30 min to collect the supernatant. The proteins were electrophoresed in 10% SDS-PAGE gels at 90 V for 2 h before they were transferred to PVDF membranes (GE Life, USA, #10600023) at a current of 200 mA. The PVDF membranes were placed in 5% skim milk for 1 h. After washing with TBS-Tween 20 three times, the membranes were incubated with primary antibodies targeting T-bet (1:1000; Proteintech; China; 13700-1-AP), GATA3 (1:1000; Absin; China; ABS131766-50 ug), RORγt (1:1000; Absin; ABS124841-50 UL), FOXP3 (1:1000; ServiceBio, China, GB112325) or GAPDH (1:1000; ServiceBio, GB11002) and incubated overnight at 4°C. On the following day, the membranes were placed in secondary antibody (1:20000) for 1 h at room temperature on a shaker. Finally, an ECL chemiluminescence solution (BioSharp, China) was used to visualize the protein bands, and the membranes were imaged with a chemiluminescence imager. ImageJ software was used for quantification. The relative expression levels of the target protein normalized to the levels of GAPDH were analyzed. The experiment was repeated three times, with n=6 in each group.

Treg detection

To determine the Treg counts in vivo, lymphocytes were obtained from the spleen tissue of mice. Then, half of the cell suspensions were separated for Treg staining using antibodies against CD4-FITC (BD553046) and CD25-APC (BD557192). After incubating for 30 min in the dark on ice, the cell suspension was treated with a cell disruption kit (EB00-5523-00) to disrupt the cell membranes, and the intramembrane antibody FOXP3-PE (BD, 563101) was used to stain Tregs. The cells were incubated again for 30 min in the dark on ice. Finally, after the samples were washed with PBS, the proportion of Tregs in the total $CD4^+$ T cell population was detected by a BD FACS Calibur flow cytometer (BD) [2]. The experiment was repeated three times, with n=6 in each group.

For detection of the Treg counts in vitro, cell culture induction was carried out first. The obtained cells were incubated with antibodies against CD4-FITC, CD25-APC and FOXP3-PE to identify the Treg population among spleen lymphocytes derived from mice treated with different concentrations of maggot extracts for different durations. A BD FACSCaliburTM flow cytometer (BD) was used for flow cytometric analysis, and the experiment was repeated three times.

Detection of cytokine levels

Lymphocyte suspensions were seeded in 6-well plates in an ultraclean environment, cultured in 2 mL of RPMI 1640 medium containing 10% fetal bovine serum and stimulated with 1 μL/mL lipopolysaccharide. The plates were then placed in a 5% CO_2 incubator at 37°C for 12 h. Cells were collected and incubated with reagents from the MS Th1/Th2/Th17 Cytocounting Bead Array Kit (BD). The levels of Th1/Th2/Th17 cell-related cytokines, including IL-2, IFN-γ, TNF-α, IL-4, IL-6, IL-17A, and IL-10, were detected using the manufacturer's method. The experiment was repeated three times. The data from three independent experiments are presented, with n=6 in each group.

Cell culture conditions

The spleens from 6 WT C57BL mice aged 8 to 12 weeks were ground into single-cell suspensions. The cell density was diluted to 1×10^6 cells/mL, and the suspensions were plated into complete medium (1640 medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 5 μm L-glutamine and 1× HEPES) at 2 mL/well. Maggot extracts (0, 1, 2 or 4 μg/μL) were transferred to 6-well plates, which were incubated in a CO_2 incubator at 37°C. During cell culture, the cell status was observed with an inverted microscope, and the culture medium was changed the next day. After 24 h, 48 h and 72 h of stimulation, the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was detected by flow cytometry.

Assessment of the toxicity of maggot extracts in mice

After mice were euthanized, their visceral organs (spleen, liver, and kidneys) were removed and fixed in formalin. Then, the organ tissues were embedded in paraffin prior to H&E staining. Ten percent neutral formalin was purchased from Wuhan Xavier Biological Technology Co., Ltd. The experiment was repeated three times, with n=6 in each group.

Statistical methods

Research data were analyzed by SPSS Software v. 20.0 (IBM Corp., Armonk, NY, USA). The normality and homogeneity of variance for all data were confirmed, and quantitative data with a normal distribution are presented as the mean ± standard deviation. One-factor analysis of variance was used to compare the differences among groups of data, and Tukey's post hoc test was used for pairwise comparisons. GraphPad Prism 8.0 (GraphPad Software, USA) was used for graphing the data. A value of $P<0.05$ indicated that the difference was significant.

Results

Maggot extracts could significantly reduce the allergic inflammatory response in AR mice

Mice were sensitized and stimulated before they were treated with either maggot extracts or DXM, and the therapeutic effect was observed (**Figure 1A**). Compared with the normal mice, the AR mice had a higher TNSS (**Figure 1B**; P<0.001), IgE titers (**Figure 1C**; P<0.001), eosinophil infiltration and goblet cell proliferation (**Figure 1D-G**; P<0.001), indicating that the AR model was successfully established.

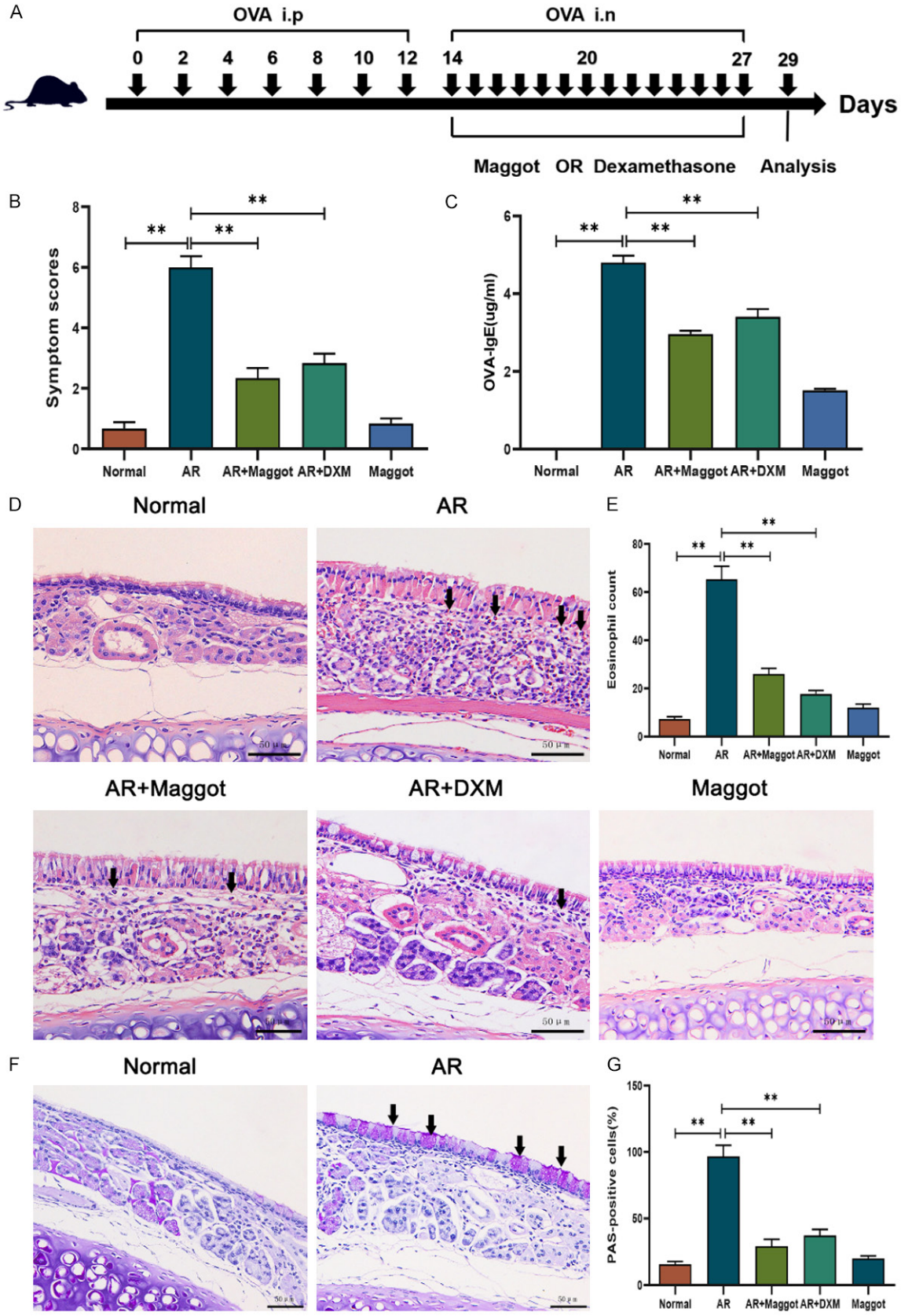
Compared to mice in the AR group, mice in the Maggot and DXM groups showed significantly lower TNSSs (**Figure 1B**; P<0.001) and IgE titers (**Figure 1C**; P<0.001). The infiltration of eosinophils and lymphocytes, lamina propria edema, vasodilation and glandular hyperplasia in the nasal cavity were also significantly reduced in these groups (**Figure 1D**, **1E**; P<0.001). In addition, mice treated with either maggot extracts or DXM showed reduced epithelial ciliary loss and no significant goblet cell proliferation (**Figure 1F**, **1G**; P<0.001). In terms of therapeutic efficacy, maggot extracts and DXM showed similar effects in the treatment of OVA-induced AR in mice.

The Th1/Th2/Th17 cytokine imbalance in the AR model could be effectively reversed by treatment with maggot extracts

The pathogenesis of AR is similar to that of bronchial asthma, involving other pathways in addition to an abnormal Th2 response [27]. The imbalance in $CD4^+$ T cells, especially in the Th1/Th2 and Th17/Treg ratios, can affect the inflammatory response of allergic diseases [1]. Next, to clarify the regulatory effect of maggot extracts on Th1/Th2/Th17 immune responses, we treated AR mice with maggot extracts and examined their effects on the Th1/Th2/Th17. The Th2 cytokines IL-4, IL-6, and IL-10, as well as the cytokine IL-17A, were apparently increased after OVA treatment (**Figure 2A-D**; P<0.001), and the Th1 cytokine IFN-γ was decreased (**Figure 2E**; P<0.01), which is indicative of typical Th2 polarization characteristics. Therapeutic administration of maggot extracts significantly reversed the Th1/Th2/Th17 cytokine imbalance during OVA stimulation, as indicated by the increased levels of IFN-γ and decreased levels of IL-4, IL-6, IL-10 and IL-17A (**Figure 2A-E**; P<0.001). The levels of TNF-α and IL-2 were not significantly different among all groups (**Figure 2F**, **2G**; P=0.475), suggesting that the Th1/Th2/Th17 cytokine imbalance in the AR mouse model can be reversed by treatment with maggot extracts. The inhibitory effects of maggot extracts on IL-4, IL-6 and IL-10 expression were particularly significant, with levels close to those observed in the control group. Correspondingly, although DXM also showed a significant therapeutic effect on AR, the inhibitory effects of maggot extracts on IL-4, IL-6, IL-10 and IL-17A levels were significantly better than those of DXM.

Maggot extracts could significantly upregulate Foxp3 expression and downregulate the expression of RORγt and GATA-3

Th1/Th2 and Th17/Treg imbalances have been observed in AR patients [1]. In addition, the above experiments showed that the Th1/Th2/Th17 cytokine imbalance could be reversed by maggot extracts. The levels of T-bet, GATA-3, RORγt and Foxp3 signals are determinants of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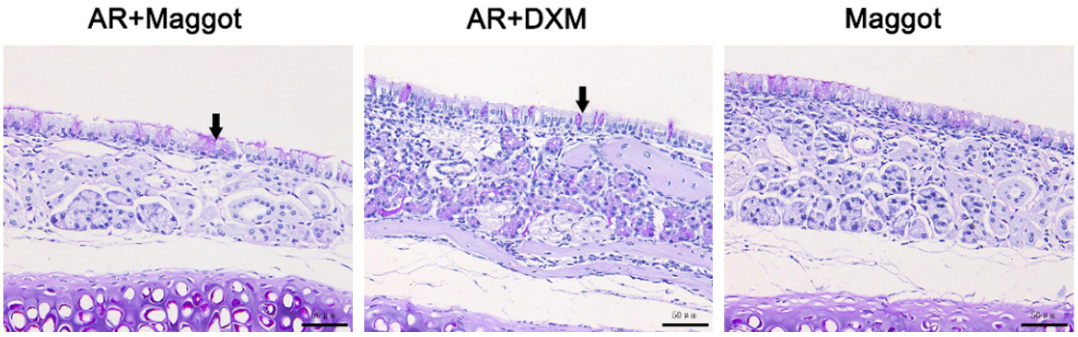

Figure 1. Maggot treatment can reduce the inflammatory response of AR in mice. A. C57BL/6 mice were sensitized with OVA on days 0, 2, 4, 6, 8, 10, and 12 and stimulated with OVA daily from day 14 to day 27. Mice in the DXM group were given daily injections of DXM or oral gavage of maggot extracts from day 14 to day 27. The mice were euthanized and analyzed on day 29. B. The TNSS was calculated for each group. C. Evaluation of serum IgE levels in OVA-treated mice using an ELISA. D. Nasal mucosal sections from mice were stained with H&E. Eosinophils were found in the lamina propria of the nasal mucosa (black arrow). The original magnification was 200×. Scale =50 μm. E. Quantification of eosinophilic infiltration. F. Nasal mucosal sections stained with periodic acid-Schiff (PAS) reagent. The magenta cells in the epidermis are goblet cells (black arrows). The original magnification was 200×. Scale =50 μm. G. Quantification of goblet cell hyperplasia. **P<0.001, n=6, Tukey's test was used for pairwise comparisons. AR: allergic rhinitis; DXM: dexamethasone; OVA: ovalbumin; Maggot: maggot extracts.

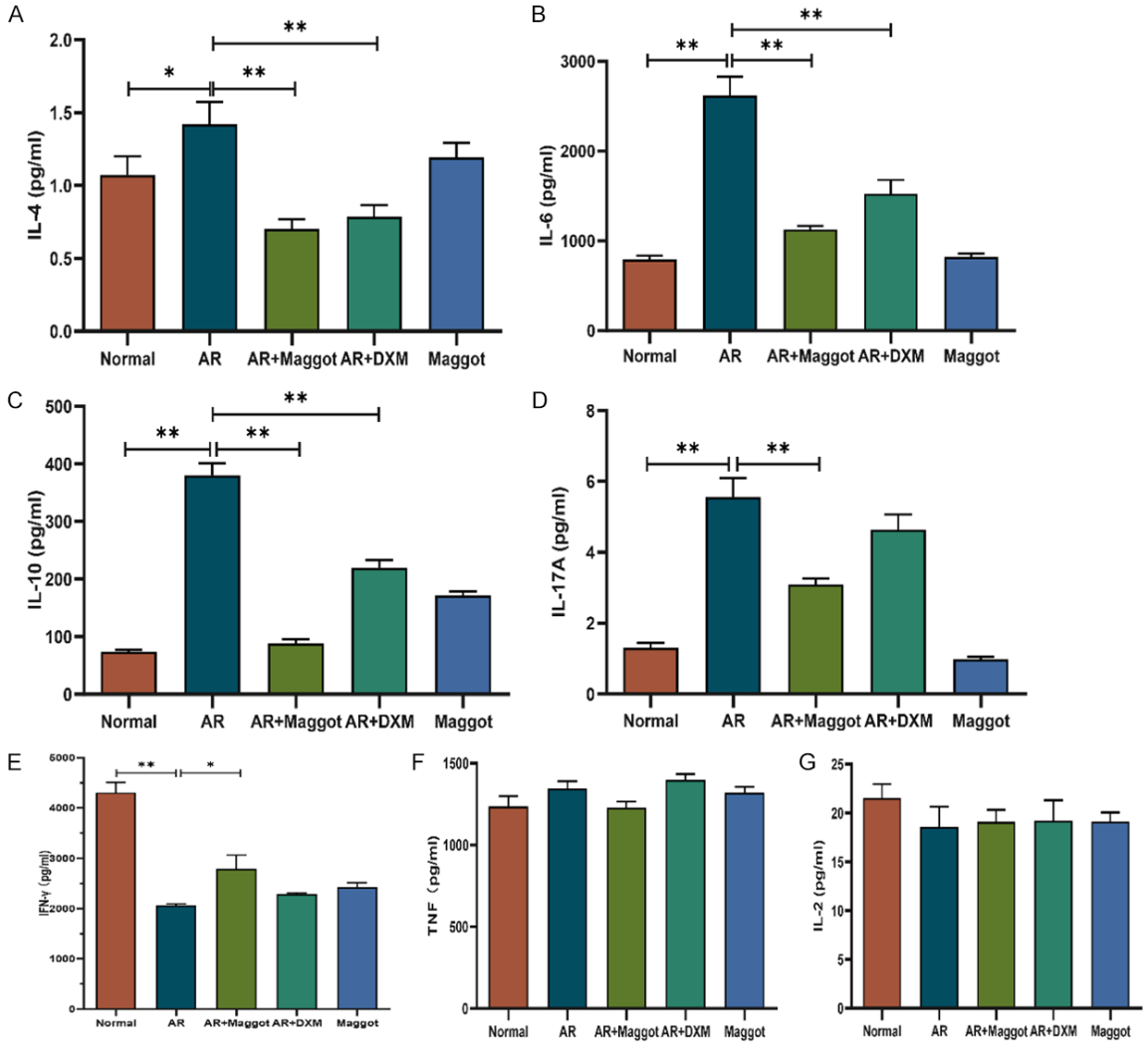

Figure 2. The imbalance in Th1/Th2/Th17 cytokines in the AR model could be effectively reversed by treatment with maggot extracts. A-G. Bead-based cytokine analysis (IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF and IL-2) by flow cytometry. Statistical analysis of the levels of cytokine expression in splenocytes from each group (IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF and IL-2). *P<0.01, **P<0.001, n=6, Tukey's test was used for pairwise comparisons. AR: allergic rhinitis; DXM: dexamethasone; Maggot: maggot extracts.

Th cell differentiation [28]. Therefore, the levels of these proteins in the nasal mucosa and spleen of mice were detected by Western blotting to investigate whether maggot extracts inhibit airway inflammation and regulate the production of cytokines in AR model mice by regulating Th cell subsets. In comparison with those in normal mice, the levels of GATA3 and RORγt expression in the spleen and nasal mucosa of mice in the AR group were apparently increased, while the levels of T-bet and Foxp3 signals were decreased (**Figure 3A-D**; P<0.001). However, these trends were reversed after treatment with maggot extracts: the expression of GATA3 and RORγt in the spleen and nasal mucosa of AR mice was decreased, while the expression of Foxp3 was remarkably increased (**Figure 3A-D**; P<0.001) and the expression of T-bet showed no substantial changes (**Figure 3A-D**; P=0.079).

Maggot extracts significantly promoted the differentiation of Tregs in the spleen of AR model mice

Next, we investigated the biological effects of maggot extracts on Tregs in AR model mice. In comparison with normal mice, AR mice presented an appreciable reduction in the number of $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ Tregs; however, AR mice treated with maggot extracts had significantly higher Treg counts close to those in the normal group (**Figure 4A**, **4B**; P<0.01). In comparison with untreated mice, DXM-treated AR mice did not exhibit a significant recovery in the proportion of Tregs, suggesting that the effect of DXM on AR was independent of Treg function. In addition, the Treg proportion in normal mice was not significantly affected by treatment with maggot extracts (**Figure 4A**, **4B**; P<0.01). These results indicate that maggot extracts could effectively promote the differentiation of splenic Tregs in the AR model. The augmented Foxp3 expression and promotion of Treg differentiation might be the main mechanism by which maggot extracts alleviate the pathology of AR. By contrast, the effect of DXM on AR does not involve Tregs.

Maggot extracts promoted the proliferation and differentiation of Tregs by upregulating the level of Foxp3 in $CD4^{+}$ T cells

Next, to further explore the differentiation effect of maggot extracts on Tregs, we treated splenic lymphocytes in culture with different concentrations of maggot extracts. Additionally, we selected culture conditions that produced a high differentiation percentage of Tregs to detect the effects of different durations of maggot extract stimulation on splenic lymphocyte differentiation in vitro. As shown in **Figure 5**, the percentage of differentiated Tregs increased in a dose-dependent manner (**Figure 5A-C**; P<0.01). Furthermore, the expression level of Foxp3 also increased with higher doses of maggot extracts, as determined by Western blotting (**Figure 5D**; P<0.01).

The percentage of differentiated Tregs increased with increasing treatment time (**Figure 5E**, **5F**; P<0.001), which was consistent with the expression of Foxp3 detected by Western blotting (**Figure 5G**; P<0.01). In summary, we determined that maggot extracts appreciably promoted the differentiation of Tregs from initial $CD4^{+}$ precursors in a time- and dose-dependent manner.

The main organs of mice with OVA-induced AR were not affected by maggot extracts

Next, to determine whether the extracts exhibited toxicity in mice, selected tissues were processed for microscopic examination. We found no significant signs of clinical toxicity in mice after treatment with maggot extracts. After 14 days of nasal drip stimulation with OVA, mononuclear cell infiltration was present in the spleen in the AR group, with the AR+ Maggot and AR+ DXM groups showing a smaller amount of infiltration, but no related abnormalities were observed in the Maggot group (**Figure 6**). Histopathological examination of mice from each group revealed no apparent pathological changes or inflammation in the liver or kidneys. Overall, after 14 days of treatment with maggot extracts, no significant pathologic abnormalities were observed.

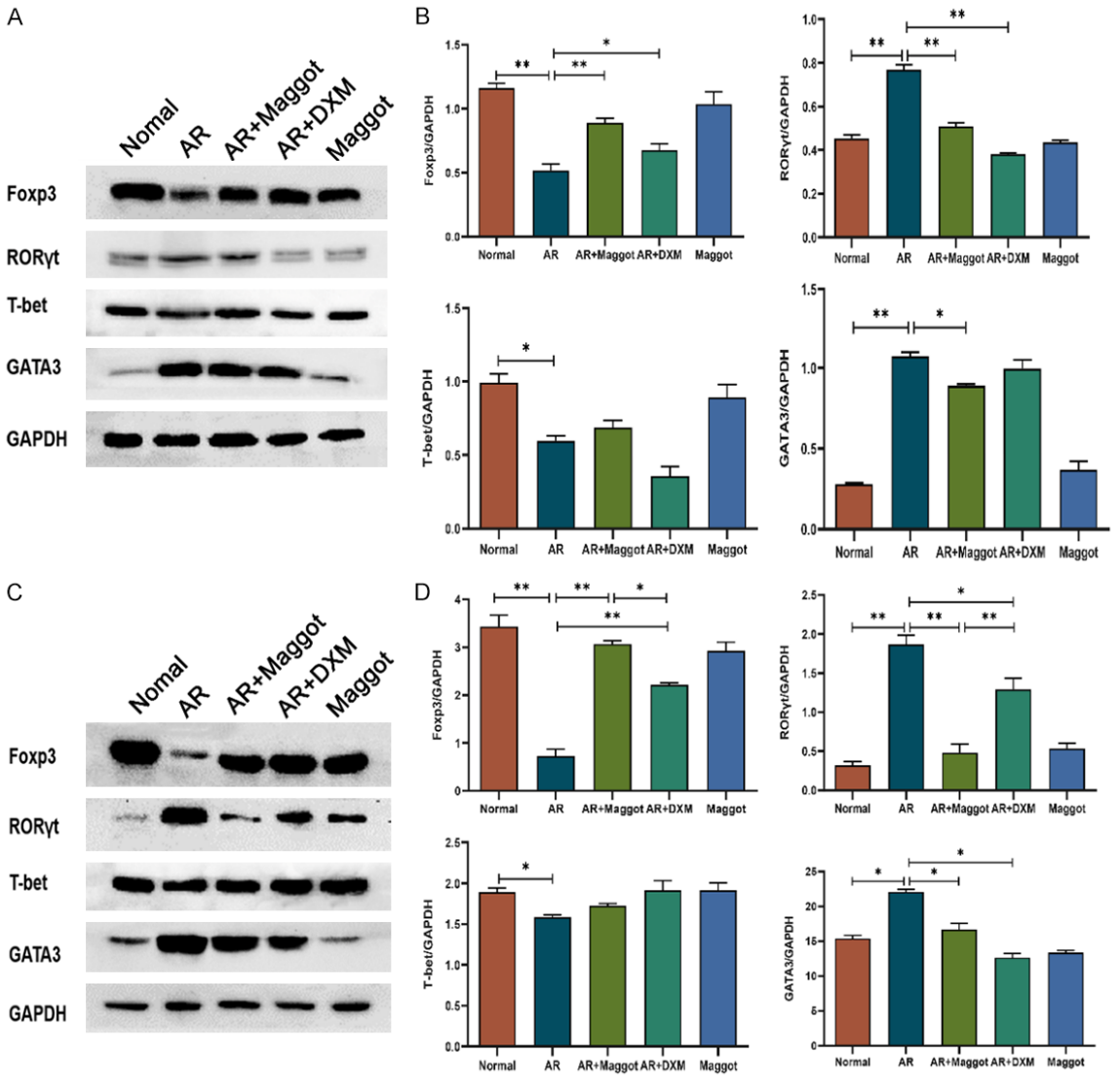

Figure 3. Maggot extracts significantly upregulated Foxp3 expression and downregulated RORγt and GATA-3 expression. A, B. Relative protein expression levels of Foxp3/GAPDH, RORγt/GAPDH, T-bet/GAPDH and GATA3/GAPDH in the nasal mucosa were assessed by Western blotting. C, D. Relative protein expression levels of Foxp3/GAPDH, RORγt/GAPDH, T-bet/GAPDH and GATA3/GAPDH in the spleen were assessed by Western blotting. *P<0.01, **P<0.001, n=3, Tukey's test was used for pairwise comparisons. AR: allergic rhinitis; DXM: dexamethasone; Maggot: maggot extracts.

Discussion

There are an increasing number of patients with AR worldwide [29]. As a result, AR has become a global health problem that needs to be addressed. Because the pathogenesis of AR is complex and has many influencing factors, including the genetic and environmental factors, there is no ideal treatment method. At present, comprehensive treatments, including avoiding exposure to allergens and environmental irritants, drug therapy, immunotherapy, surgical treatment and patient health education, are mainly used for AR. While these treatments are effective in controlling symptoms in most patients, approximately 20% still have uncontrolled disease, and many patients require long-term medical therapy. AR not only seriously affects the quality of life of patients but also causes heavy social and economic burdens [30]. Thus, it is important to explore safer, more efficient and targeted treatments for AR.

Studies have shown that a cecropin-like peptide from *Lucilia eximia* (Lucilin peptide) has

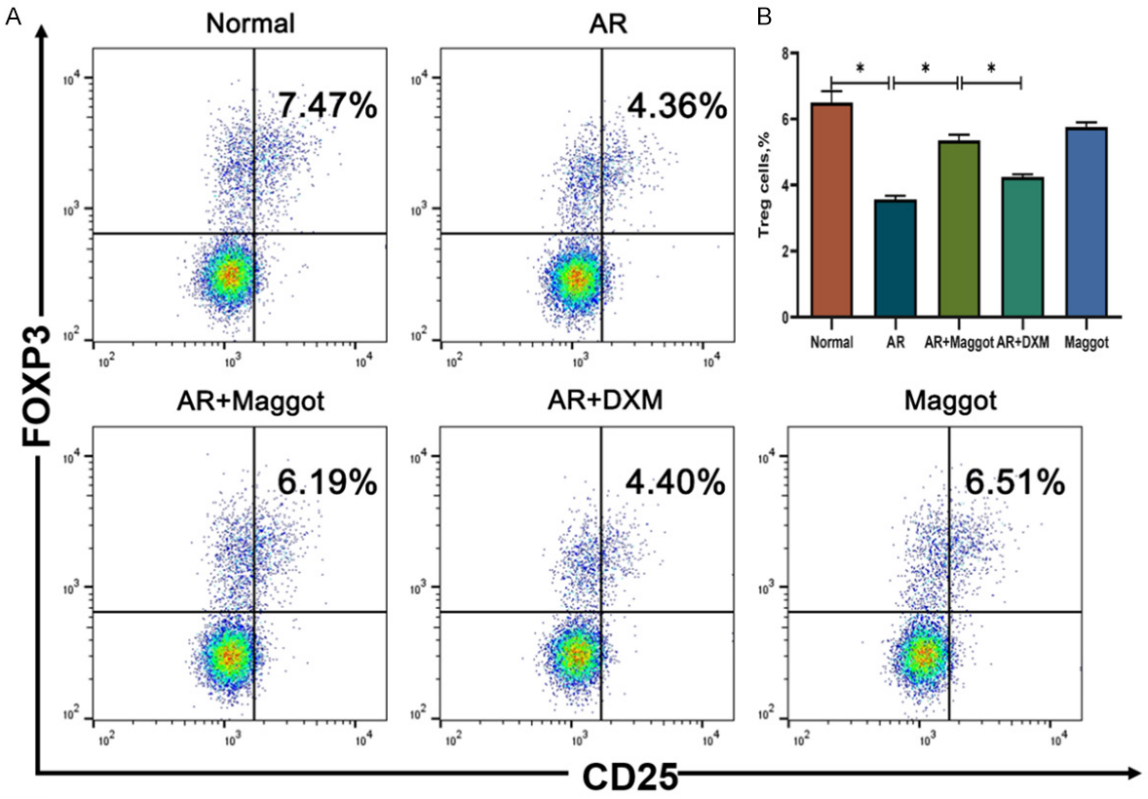

Figure 4. Treatment with maggot extracts can promote the differentiation of Tregs. A. Treg gating strategy. B. Statistical analysis. In comparison with the normal mice, the AR group mice had an increased proportion of Tregs. In comparison with untreated AR mice, the AR+ Maggot mice had an appreciably decreased proportion of Tregs. *P<0.01, n=3, Tukey's test was used for pairwise comparisons. AR: allergic rhinitis; DXM: dexamethasone; Maggot: maggot extracts.

strong antibacterial and immunomodulatory activities [17]. Recent studies have found that an imbalance in Th17/Tregs was detected in the intestinal mucosa of patients with UC [31, 32] and that treatment with a maggot-derived protein ameliorates acute colon injury in UC [21, 24]. Similar to the onset of UC, an imbalance in Th17/Tregs also affects the onset of AR. Therefore, we speculated that maggot extracts may also exert an anti-inflammatory effect on AR.

In this study, we first established an OVA-induced AR animal model and found that treatment with maggot extracts reduced TNSSs and decreased serum IgE levels in mice. These results confirmed that maggot extracts have an effective therapeutic effect on OVA-induced AR. When we observed the nasal mucosa and spleen of AR model mice, we found that maggot extracts could inhibit the infiltration of eosinophils and proliferation of goblet cells. These results provided clear evidence that maggot extracts reduce inflammation in AR and that the anti-inflammatory effect of maggot extracts was comparable to that of the positive control DXM.

After entering the body, allergens stimulate epithelial cells to produce inflammatory mediators, which trigger the release of various cytokines from innate immune cells. These cytokines lead to the increased differentiation of $CD4^+$ T cells toward Th2 cells and Th17 cells, which then produce more proinflammatory cytokines to cause the occurrence of allergic diseases and aggravate the symptoms of allergic reactions [9, 15]. Therefore, in this study, we detected differences of Th1-/Th2-/Th17-related cytokines in mouse spleen cells. In AR mice, the levels of Th2 cytokines and proinflammatory Th17 cytokines were significantly increased while the levels of Th1 cytokines were decreased. Therapeutic administration

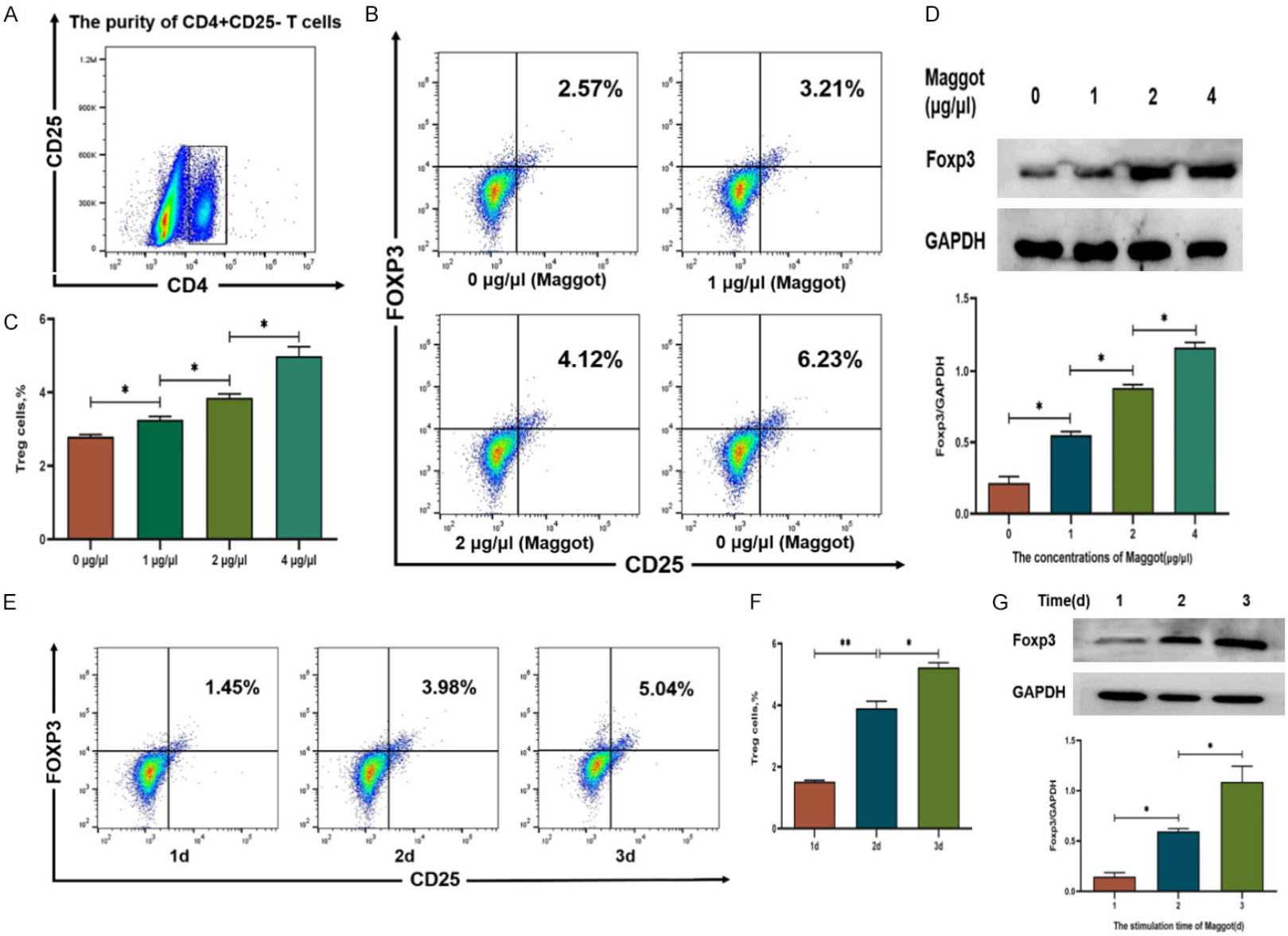

Figure 5. In vitro experiments confirmed that maggot extracts promoted the proliferation and differentiation of Tregs by increasing the expression of Foxp3 in $CD4^+$ T cells. A. Flow cytometric analysis of $CD4^+CD25^-$ T lymphocytes. B. Flow cytometric analysis of the concentration-dependent effect of maggot extracts on Treg differen-

tiation. C. Statistical graph showing the concentration-dependent effects of maggot extracts. In comparison with the 0 μg/μL group, the 1, 2 and 4 μg/μL groups had significantly higher proportions of Tregs, with gradual increases in response to increasing concentrations of maggot extracts. D. Western blotting was performed to detect the expression of Foxp3 in cells treated with different concentrations of maggot extracts. E. Flow cytometric analysis of the time-dependent effect on Tregs. F. Statistical graph showing the time-dependent effects of treatment with maggot extracts. The proportion of Tregs increased with increasing stimulation time. G. Western blotting was performed to detect the expression of Foxp3 in cells treated for different times. $^{*}P<0.01$, $^{**}P<0.001$, C, F: n=6; D, G: n=3, Tukey's test was used for pairwise comparisons.

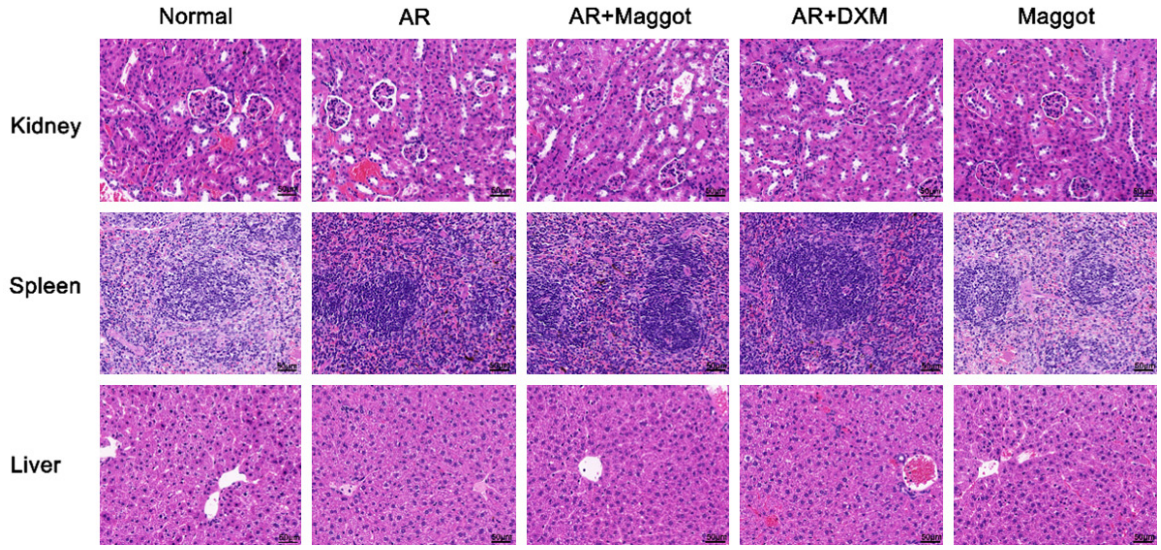

Figure 6. There were no obvious abnormalities in the main organs of AR mice treated with maggot extracts. The kidneys, spleen, and liver were fixed, sectioned and stained with H&E. Scale =50 μm, n=6. AR: allergic rhinitis; DXM: dexamethasone; OVA: ovalbumin; Maggot: maggot extracts.

of maggot extracts during OVA stimulation reversed this imbalance in cytokine production. Moreover, the levels of IL-4, IL-6 and IL-10 in the AR mice treated with maggot extracts were very similar to those in the normal control mice, which confirmed that maggot extracts had a strong inhibitory effect on the production of inflammatory cytokines by Th2 cells. Additionally, this study found no significant differences in IL-2 or TNF expression among the experimental groups, which indicated that treatment with maggot extracts or DXM had no effect on Th1 cytokine production in the AR inflammatory response.

Previous studies have confirmed that $CD4^{+}$ T cells are involved to the pathogenesis of AR. The expression levels of T-bet, GATA-3, RORγt and Foxp3 are a determinant of Th cell differentiation [33]. We discovered that treatment with maggot extracts could substantially upregulate the expression level of Foxp3 and downregulate the expression levels of RORγt and GATA-3 in AR mice but had no significant effect on T-bet. These findings were consistent with the above results for related cytokine expression, suggesting that maggot extracts inhibit the transcription and differentiation of proinflammatory Th2 cells and Th17 cells by promoting the development of Tregs at the inflamed site in allergic mice but have little effect on the Th1 pathway.

Tregs are essential for preventing the differentiation and migration of Th1, Th2, and Th17 cells in autoimmune inflammatory diseases [14, 25, 34, 35]. In anaphylactic mouse models, reduced Treg counts are associated with anaphylactic inflammatory responses driven by Th2 cells in the local mucosa, lungs and gastrointestinal tract [25]. Tregs can inhibit eosinophil aggregation, excessive mucus production and Th2 cytokine release [36]. In this study, it was found that maggot extracts could effectively promote the expression of Foxp3 in mice such that more Tregs could differentiate and conse-

quently exert an inhibitory effect on T cells. When $CD4^+$ T cells were cultured in vitro, the expression of the transcription factor Foxp3 and the differentiation of Tregs increased with increasing concentrations and treatment times of maggot extracts, indicating dose- and time-dependent effects. Previously, the differentiation of $CD4^+$ T cells regulated by maggot extracts has not been reported. Our current in vivo and in vitro studies provide a new target and strategy for promoting Treg differentiation.

Recent studies by Wang et al. [24] showed that there was no toxic effect of maggot extracts on the main organs of the experimental mice after oral administration. In our experiment, normal mice treated with only maggot extracts showed no significant abnormalities or significant differences compared to the untreated control mice. No significant weight loss or major organ changes were observed in our experimental mice, and we did not observe obvious signs of toxicity in the kidneys, spleen, or liver, suggesting that treatment with maggot extracts does not elicit significant toxic effects.

Because this is the first study of maggot extracts in an AR model, our study had some limitations. The maggot extract was a crude extract, which is not suitable for intravenous or intradermal injection. In this study, we extracted and then delivered maggot extracts to mice via oral gavage [24] and did not compare the effects of different routes of administration on treatment outcomes. In future research, it will be indispensable to purify and identify the key bioactive proteins in maggot extracts.

In conclusion, this study suggested that maggot extracts have a therapeutic effect on AR. Maggot extracts contribute to the differentiation of Tregs in vivo and in vitro by upregulating Foxp3 to regulate the balance of other effector T cells in the inflammatory response, thus significantly reducing the inflammatory response in AR. The inhibition of inflammation induced by maggot extracts is not specific to a single molecule or pathway but is the result of a complex and multifaceted immune response, and the specific mechanism(s) need to be further explored. This study could be considered as the basis for developing a novel therapeutic strategy for the treatment of AR using maggot extracts.

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Disclosure of conflict of interest

None.

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