IL-17 Exacerbates Experimental Autoimmune Prostatitis via CXCL1/CXCL2-Mediated Neutrophil Infiltration

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Research Article

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Abstract

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a poorly understood disease. Accumulating evidence suggests that autoimmune dysfunction is involved in the development of CP/CPPS. IL-17 is associated with the occurrence and development of several chronic autoimmune inflammatory diseases. However, the molecular mechanisms underlying the role of IL-17 in CP/CPPS remain unclear. Herein, we first confirmed that IL-17 was increased in the prostate tissues of experimental autoimmune prostatitis (EAP) mice. Corresponding to the increase of IL-17 in the prostate of EAP, neutrophil infiltration and the levels of CXCL1 and CXCL2 (CXC chemokine ligands 1 and 2) were also increased. Treatment of EAP mice with IL-17-neutralizing monoclonal antibody (mAb) resulted in a decreased number of infiltrated neutrophils, as well as the CXCL1 and CXCL2 level. Depletion of neutrophil by anti-Ly6G antibodies ameliorated inflammatory changes and hyperalgesia caused by EAP. Fucoidan, which could potently inhibit neutrophil migration, could also ameliorate the manifestations of EAP. Our finding suggested that IL-17 promoted the production of CXCL1 and CXCL2, which subsequently triggered neutrophil chemotaxis to prostate tissues. And fucoidan might be a potential drug for the therapy of EAP by the effectively inhibiting on neutrophil infiltration.

Introduction

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a frequently occurring disease of urologic morbidity in men younger than 50 years old, accounting for 90-95% of all prostatitis diagnoses[1–3]. It is characterized by chronic pelvic pain and symptoms of prostatic inflammation. The incidence of CP/CPPS varies from 2-16% in previous reports[4, 5]. In China, the incidence of CP/CPPS was reported to be 8.4% with a mean age of 34.56±13.48 years by our group[4]. The etiology of CP/CPPS has yet to be elucidated. Currently, several hypotheses have been proposed to explain CP/CPPS pathogenesis including cryptic infections, abnormal pelvic floor neuromuscular activity, and autoimmune mechanism[6–8].

Interleukin-17 (IL-17), a pro-inflammatory cytokine mainly secreted by T helper 17 (Th17) cell subsets is relevant to the occurrence and development of several autoimmune inflammatory diseases. IL-17 targets various inflammatory cell types and induces proinflammatory cytokines (such as IL-1β, G-CSF, GM-CSF) and chemokines (such as CXCL1, CXCL2) in some inflammatory diseases[9, 10]. Although some studies have reported controversial results regarding the role of IL-17 in experimental autoimmune prostatitis (EAP), a noninfectious autoimmune-driven mice model of CP/CPPS[11, 12]. The aforementioned research conclusions were drawn based on C57BL/6 mice, which were proved less susceptible to EAP induction than non-obese diabetic (NOD) mice[13]. We have demonstrated that IL-17 presented a positive effecet in EAP-NOD mice[14]. The finding suggested that the levels of IL-17 in the prostate tissues from EAP mice were higher than that of control mice[14]. We also found that treatment with a neutralizing antibody to IL-17 decreased the level of IL-17 in prostate tissues and ameliorated the inflammatory changes and the pelvic pain caused by EAP[14]. However, the exact mechanisms of IL-17 involved in the inflammatory manifestations and pelvic pain in the EAP mice model and CP/CPPS diseases are still uncertain.

In the current study, we demonstrated the mechanisms by which IL-17 modulated the infiltration of neutrophils into prostate tissues in an EAP model. Depletion of neutrophil by anti-Ly6G antibodies ameliorated various symptoms of EAP. Our study indicated that IL-17-neutrophil might play an essential role in mediating prostatic inflammation and pelvic pain in CP/CPPS. These findings might improve the understanding of CP/CPPS pathogenesis and therapeutic intervention.

Materials And Methods

1. Mice and EAP induction

The animal experiments were approved by the Committee for Animal Care and Use of the Animal Center of Anhui Medical University (No.LLSC20190651). Six to eight-week-old male NOD mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All mice were maintained under specific pathogen-free conditions at the animal facility of our institution. NOD mice were induced to develop EAP model as previously reported[13, 14]; briefly, NOD mice were administered with 0.1 ml mixed emulsion prostate antigens (PAg) and complete Freund's adjuvant (CFA; Sigma-Aldrich) by subcutaneous injection on days 0 and 14. Mice were sacrificed by an overdose of chloral hydrate (Aladdin Biotechnology) and euthanized by cervical dislocation at different times after 1st immunization. Blockade of IL-17 was achieved by intraperitoneal injection of 10 μg anti-mouse IL-17 neutralizing antibodies (R&D System, MAB421) or isotype-matched rat IgG (R&D System, 6-001-A) one day before EAP induction and then once per week[11]. After EAP induction, parts of EAP mice were injected intraperitoneally with recombinant mouse IL-17 (rIL-17, 0.1 mg/mouse) every day for 1 week after 1st and 2nd immunization[15]. Fucoidan (Sigma-Aldrich, F5631) or its vehicle was administered by intraperitoneal injection at a dose of 20 mg/kg body weight one day before EAP induction and then once per week thereafter[16].

2. Tactile allodynia assessment

The mice were tested before PAg injection (baseline, day 0) and at 14, 28, 42 days after EAP induction. Referred hyperalgesia and tactile allodynia were tested using von Frey filaments applied to the abdomen region near the prostate according to previous references[14]. Three types of behaviors were considered to be positive responses to von Frey filament stimulation: 1) sharp retraction of the abdomen; 2) immediate licking or scratching of the area of filament stimulation; or 3) jumping. The response frequency was calculated as the percentage of positive responses and data were reported as the mean ± SEM.

3. Inflammation scoring

Prostate tissues were fixed in 10% formalin and embedded in paraffin. 5 μm sections were stained with hematoxylin and eoxin (HE) and scored blindly using the histopathological classification system for chronic prostatic inflammation[17]. Briefly, the extent of chronic inflammation was graded from 0-3: a) 0, no inflammation; b) 1, mild but definite perivascular cuffing with mononuclear cells; c) 2, moderate

perivascular cuffing with mononuclear cells; d) 3, marked perivascular cuffing, hemorrhage, and numerous mononuclear cells in the parenchyma.

4. Immunohistochemistry and immunofluorescence assays

Immunohistochemistry (IHC) and immunofluorescence (IF) assays were performed as described previously[14, 18]. Briefly, prostate tissues were fixed in 10% formalin and embedded in paraffin. Prostate sections were incubated overnight at 4℃ with anti-IL-17 (Abcam, Ab79056) primary antibodies at a dilution of 1:500. Then, an IHC kit (ZSGB-Bio, SP9000) and 3,3'-diaminobenzidine (ZSGB-Bio, ZLI-0918) were used for subsequent immunohistochemical analysis according to the manufacturer's instructions.

Prostate sections were blocked with 5% bovine serum albumin and incubated overnight at 4℃ with rabbit anti-mouse Ly6G primary antibodies (1:200, Elabscience, E-AB-70094). The sections were then incubated with Cy3-conjugated goat anti-rabbit IgG (1:400, Beyotime, A0516) and DAPI (1:1000, Beyotime, C1005) after washed with tris-buffered saline (TBS). Fluorescence images were acquired using a Zeiss LSM510 confocal microscope.

5. RNA isolation and quantitative real-time RT-PCR

Total RNA from prostate tissues were extracted with Trizol reagent according to the manufacturer's instructions. Total RNA was synthesized into cDNA using the Fast Quant RT kit (Tiangen, KR106-02). Quantitative real-time PCR was performed with Super Real Premix Plus (SYBR green) (Tiangen, FP205-02). The gene-specific primer sets used to amplify each gene were synthesized by Sangon Biotech Co., Ltd (Shanghai) (**Supplemental Table 1**). Gene expression was assessed by the comparative C_T method. The results were normalized to *Gapdh*.

6. Analysis of chemokine and cytokine expression in prostate tissue

The concentrations of cytokines in prostate tissue homogenate samples were determined by enzyme-linked immunosorbent assay (ELISA). The levels of IL-17 (Elabscience, E-EL-M0047c), CXCL1 (Elabscience, E-EL-M0018c), and CXCL2 (Elabscience, E-EL-M0019c) in the prostate tissues were measured by ELISA kits according to the manufacturer's instructions.

7. Analysis of prostate-infiltrating neutrophil

Freshly harvested prostate tissues were mechanically disrupted and enzymatically digested in RPMI 1640 medium containing 1mg/ml collagenase D (Sigma-Aldrich, C9891) and 0.05% DNase Ⅰ (Sigma-Aldrich, D5025) for 45 min at 37℃. After digestion, suspensions were filtered through 75-μm cell strainers and single-cell suspensions were washed with PBS. Then, cells were stained with APC-conjugated anti-Ly6G (BD Bioscience, 560599) for FACS analysis. FACS Calibur flow cytometer (BD Bioscience) was used to analyze the stained cells, and the data were analyzed by FlowJo Software X (Tree Star).

8. Analysis of MPO activity

Myeloperoxidase (MPO) is produced by activated neutrophils and is an established marker of neutrophil migration. MPO activity in prostate tissues was analyzed by an MPO activity measurement kit (Elabscience, E-BC-K074) according to the manufacturer's instructions. MPO activity was determined by a spectrophotometer at 450 nm and expressed as U/g prostate tissues.

9. Neutrophil depletion assays

For neutrophil depletion studies, we used anti-Ly6G antibody (clone 1A8; Bio-X-Cell)[19, 20]. Briefly, EAP mice were treated with intraperitoneal injection of 0.25 mg/dose of anti-Ly6G or control rat IgG (clone GL117, Bio-X-Cell) one day before EAP induction and then once a week. Peripheral blood from mice was collected by cardiac puncture for leukocyte quantification using a HEMAVET 950 multispecies hematology cell counter.

10. Statistical analysis

Statistical analysis was performed by two-tailed Student's *t*-test and two-way ANOVA with Bonferroni post hoc test. Data are representative of three independent experiments with 4 mice per group. The data are expressed as mean ± SEM. *P<0.05 was considered to indicate a significant difference in the analyses. Statistical analyses were performed using SPSS software version 21.0 and GraphPad Prism 6 software.

Results

1. IL-17 was increased in the prostate tissues of EAP mice

We first analyzed the changes between control and EAP mice at day 42 after EAP 1st immunization. As shown in Fig. 1A, pathological changes were observed in EAP mice, including a large amount of inflammatory cells infiltration and tissues disorder. The inflammation scores of control and EAP groups were 0.40±0.16 vs 2.50±0.17, respectively (Fig. 1B, P<0.01). Behavioral tests showed significant increases in the response frequencies to von Frey filament stimulation of EAP mice on day 42 after 1st immunization with a force of 0.4, 1, 2, 4 g compared to that of control mice (Fig. 1C, P<0.05). As shown in Fig. 2, the IL-17 mRNA and protein expression level, the inflammation scores, response frequency to von Frey filaments stimulation of EAP mice were consistently increased during days 0-42. According to the results of Fig. 2, we found a positive correlation between the increased expression of IL-17 in the prostate tissues and the development of prostatitis in EAP mice. We then test the IL-17 expression in the prostate tissues of control and EAP mice at day 42 after 1st immunization. The mRNA and protein levels of IL-17 in the prostate tissues were significantly increased in EAP group compared to that of control group (Fig. 1D and Fig. 1E, P<0.05). The immunohistochemistry result also verified that IL-17 positive staining cells were increased in the prostate tissues of EAP mice compared to those of control mice (Fig. 1F).

2. Neutrophil infiltration and the levels of CXCL1 and CXCL2 were increased in the prostate of EAP mice

Prior studies have suggested that IL-17 promoted the recruitment of neutrophils to the inflammatory sites [21, 22]. We were interested in whether the high level of IL-17 in the prostate tissues of EAP could increased neutrophil infiltration. Therefore, we analyzed neutrophil infiltration into the prostate tissues by some methods. As shown in Fig. 3A, the percentage of $Ly6G^+$ cells in prostate tissues from the EAP mice was significantly higher than that of the control mice (P<0.05). The immunofluorescence (IF) staining results for the neutrophil marker Ly6G indicated that EAP prostate tissue sections showed significantly elevated levels of Ly6G-positive neutrophils around the prostatic glandular cavity (Fig. 3B). MPO is produced by activated neutrophils and is an established marker of neutrophil migration. To quantitatively assess neutrophil migration, we measured MPO activity in prostate tissues from the control and EAP mice. The results suggested that MPO activity in prostate tissues of the EAP mice was higher than that of control mice(Fig. 3C, P<0.01). In summary, the results of Fig. 3A-3C suggested that the neutrophil infiltration was increased in EAP mice. Neutrophils are not direct targets of IL-17, because they do not express the IL-17 receptor subunit [23]. While neutrophils express chemokine receptors, such as CXC chemokine receptor 2 (CXCR2), they are known to readily react to the chemokines, CXCL1 and CXCL2 [24]. Neutrophil recruitment from peripheral blood is a consequence of the local production of chemokines, mainly CXCL1 and CXCL2[25], which can be elicited by IL-17[9, 10]. Therefore, we measured the mRNA and protein level of CXCL1, CXCL2, which are downstream of the IL-17 signaling pathway, in prostate tissues from control and EAP mice. The mRNA and protein levels of CXCL1, CXCL2 in the prostate tissues of EAP mice were higher than those of control mice (Fig. 3D-3E, P<0.05). Thus, we hypothesized that IL-17 promoted neutrophil infiltration of the prostate tissues via the high levels of CXCL1 and CXCL2 in EAP mice rather than control mice.

3. IL-17 neutralization reduced the production of CXCL1/CXCL2, and the recruitment of neutrophils

To verify our hypothesis that IL-17 attracted neutrophil infiltration through CXCL1 and CXCL2, we treated EAP mice with an IL-17-neutralizing mAb. As a result, IL-17 neutralization decreased the IL-17 levels in the prostate tissues and ameliorated the inflammatory changes and pelvic pain associated with EAP (**Supplemental Fig. 2, P**<0.05).

Then we measured neutrophil infiltration into the prostate in the EAP+IgG and EAP+anti-IL-17 groups by flow cytometry and IF staining. As shown in Fig. 4A, the percentage of $Ly6G^+$ cells in prostate tissues from the EAP+anti-IL-17 group was significantly lower than that of the EAP+IgG group (P<0.05). The IF staining results for Ly6G in prostate tissues suggested that Ly6G-positive neutrophils were decreased in the prostate sections of EAP+anti-IL-17 group by IL-17 neutralization (Fig. 4B). We also found that MPO activity in the prostate tissues from the EAP+anti-IL-17 group was decreased compared to that of EAP+IgG group (Fig. 4C, P<0.01). These results confirmed that blocking IL-17 reduced neutrophil infiltration in the prostate tissues of EAP mice.

Furthermore, we analyzed the effect of IL-17 neutralization on the expression of the chemokines, CXCL1 and CXCL2. The qRT-PCR results suggested that blocking IL-17 decreased the mRNA level of CXCL1 and CXCL2 (Fig. 4D, P<0.01). Consistent with the qRT-PCR results, the protein levels of CXCL1 and CXCL2

were also decreased by anti-IL-17 treatment (Fig. 4E, P<0.01). Taken together, these results suggested that IL-17 promoted the recruitment of neutrophils through the inflammatory chemokines, CXCL1 and CXCL2. Additionally, we hypothesized that the amelioration of inflammatory changes and pelvic pain in EAP+anti-IL17 mice was because of the decrease of neutrophil infiltration in prostate tissues.

4. Depletion of neutrophil ameliorated inflammatory changes and hyperalgesia caused by EAP

Recent studies have documented that neutrophil infiltration plays a vital role in inflammatory disease[26]. We hypothesized that the depletion of neutrophils might alleviate the severity of inflammation caused by EAP. To verify this hypothesis, we treated EAP mice with anti-Ly6G or IgG isotype control. As shown in Fig. 5A, the anti-Ly6G-treatment decreased neutrophils without affecting lymphocytes and monocytes in circulating blood. We then examined the effect of depletion of neutrophil treatment on the manifestation of EAP. HE staining showed that the pathological alterations were significantly alleviated in the EAP+anti-Ly6G mice compared to the EAP+IgG mice (Fig. 5B-5C). And response frequencies to mechanical filament stimulation of the pelvic area also exhibited a significant decrease compared to that of EAP+IgG mice (Fig. 5D, P<0.05). Taken together, these data suggested that neutrophils were fundamental to the development of EAP and decreasing the neutrophil in the prostate tissues might be a promising therapeutic strategy for EAP or CPPS.

5. Fucoidan ameliorated inflammatory changes and pain symptoms caused by EAP by decreasing the infiltration of neutrophil

Fucoidan has been proved to ameliorate inflammation and hyperalgesia in various inflammatory disease[16, 27, 28]. The most important one of fucoidan's therapeutic effects is that fucoidan is potent inhibition neutrophils recruitment to the inflammatory sites, as fucoidan is a competitive inhibitor of selectins, which are necessary for neutrophil migration[16]. We were curious whether fucoidan was effective for the treatment of CPPS. So we treated EAP mice with rIL-17 (to enhance neutrophil recruitment to prostate tissues) or fucoidan as previous studies described[15, 16]. The percentage of $Ly6G^+$ cells in the prostate tissues in the four groups were 5.92%±0.31% for EAP+vehicle, 7.45%±0.44% for EAP+rIL-17, 4.50%±0.14% for EAP+fucoidan, 4.33%±0.67% for EAP+rIL-17+fucoidan group, respectively (Fig. 5A-5B). These results suggested that fucoidan treatment decreased the percentage of $Ly6G^+$ neutrophil infiltration in the prostate tissues (Fig. 5A-5B, P<0.05). Similarly, the MPO activity in prostate tissues was also decreased by fucoidan treatment (Fig. 5C, P<0.01). These results confirmed the efficiency of fucoidan-mediated neutrophil inhibition.

Finally, we investigated the effect of fucoidan treatment on the severity of prostate inflammation and chronic pain behavior. The HE staining results showed that the histological appearance of prostate tissues from fucoidan-treated-EAP mice was alleviated, as evidenced by a reduction of stromal mononuclear cell infiltration (Fig. 5D-5E, P<0.01). Moreover, chronic pain development analysis suggested that the response frequency was ameliorated in the EAP+fucoidan group compared to the EAP+vehicle group (Fig. 5F, $^{*}P<0.05$, $^{**}P<0.01$). Compared to the EAP+rIL-17 group, the inflammation changes and pain

symptoms were significantly decreased in EAP+rIL-17+fucoidan group. (Fig. 5D-5F, $P<0.05$). These results raised the possibility that fucoidan might be a potential drug for EAP treatment by inhibiting neutrophil infiltration into the prostate tissues.

Discussion

CP/CPPS is a complex syndrome with unclear etiology, while the involvement of immune dysfunction, especially autoimmunity, has received considerable support from a variety of human and mice studies[6, 29, 30]. We recently demonstrated that the characteristic features of CPPS, namely pelvic pain and infiltration of inflammatory cells into the prostate, were observed in the EAP mice model[14]. The pelvic pain in this model is represented by referred visceral hyperalgesia of the somatic area. Our previous study has demonstrated that IL-17 presented a positive effect in EAP mouse model[14]. The objective of this study was to examine the molecular mechanism of IL-17, the main effector cytokine of Th17 cells, in the development of pelvic pain and prostate inflammation in EAP model mice. Here, we first noticed the connection between the dynamic expression of IL-17, the inflammation scores of prostates tissues, and the response frequencies to pelvic pain stimulation during the development of EAP. The results indicated that IL-17 was associated with the development of EAP. Additionally, we found that the levels of CXCL1 and CXCL2, and neutrophil infiltration were increased in the prostate tissues in EAP mice, compared to that of control mice.

To confirm the direct involvement of IL-17 in the development of EAP, we administered an IL-17-specific neutralizing antibody to EAP mice. We found that IL-17 neutralization significantly mitigated the inflammatory changes and the pelvic pain, which was accompanied by the reduction of neutrophil chemoattractants (CXCL1 and CXCL2) and neutrophil infiltration. Combined with previous publications suggesting neutrophils are necessary for inflammatory diseases[16, 26], we hypothesized that neutrophil infiltration was fundamental to EAP development and the decrease of neutrophil infiltration might ameliorate inflammatory changes and hyperalgesia in the context of EAP.

To verify this hypothesis, we treated EAP mice with anti-Ly6G antibodies to deplete neutrophils. The results suggested that depletion of neutrophils ameliorated inflammatory changes and hyperalgesia caused by EAP. These results suggested that neutrophil is necessary to the development of EAP. Neutrophil recruitment was considered to be important in an early step of inflammatory response or sterile tissue damage. Scalerandi et al. found that higher neutrophil accumulation was associated with severer inflammatory signs and tissue damage in a bacteria-induced mice model of prostate inflammation[25], which was largely consistent with our findings.

Notably, we found that fucoidan treatment ameliorated the severity of prostate inflammation and chronic pain behavior in EAP by inhibiting neutrophils recruitment to prostate tissues. Park et al. confirmed that fucoidan treatment inhibited neutrophil recruitment in the gingival tissue of mice and inhibit gingival inflammation in a periodontitis mice model[28]. Fucoidan could also inhibit radiation-induced pneumonitis by decreasing neutrophil and macrophage accumulation[27].

In conclusion, the current study has revealed that IL-17 exacerbated the manifestation of EAP via neutrophil infiltration in the prostate tissues. Neutrophil recruitment was necessary to the development of EAP. And the depletion of neutrophils by anti-Ly6G antibodies and the decreasing of neutrophil infiltration by fucoidan could effectively ameliorate prostate inflammation and chronic pain in EAP mice. In addition, this study also indicated that fucoidan might be a potential therapeutic drug for the treatment of CP/CPPS in the near future.

Declarations

Ethics approval and consent to participate

The animal experiments were approved by the Committee for Animal Care and Use of the Animal Center of Anhui Medical University (No.LLSC20190651).

Consent for publication

Not applicable.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Competing of interest

The authors declare that they have no conflicts of interest in the data of this article.

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Authors' contributions

Chang-Sheng Zhan, Chao-Zhao Liang, Li Zhang designed and conceived the project. Chang-Sheng Zhan, Cheng Zhang and Jia Chen acquired the data, analyzed the data and wrote the manuscript. Hui Wang, Jing Chen collected the samples. Mei-Juan Zheng and Xian-Guo Chen provided technical support.

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Figures

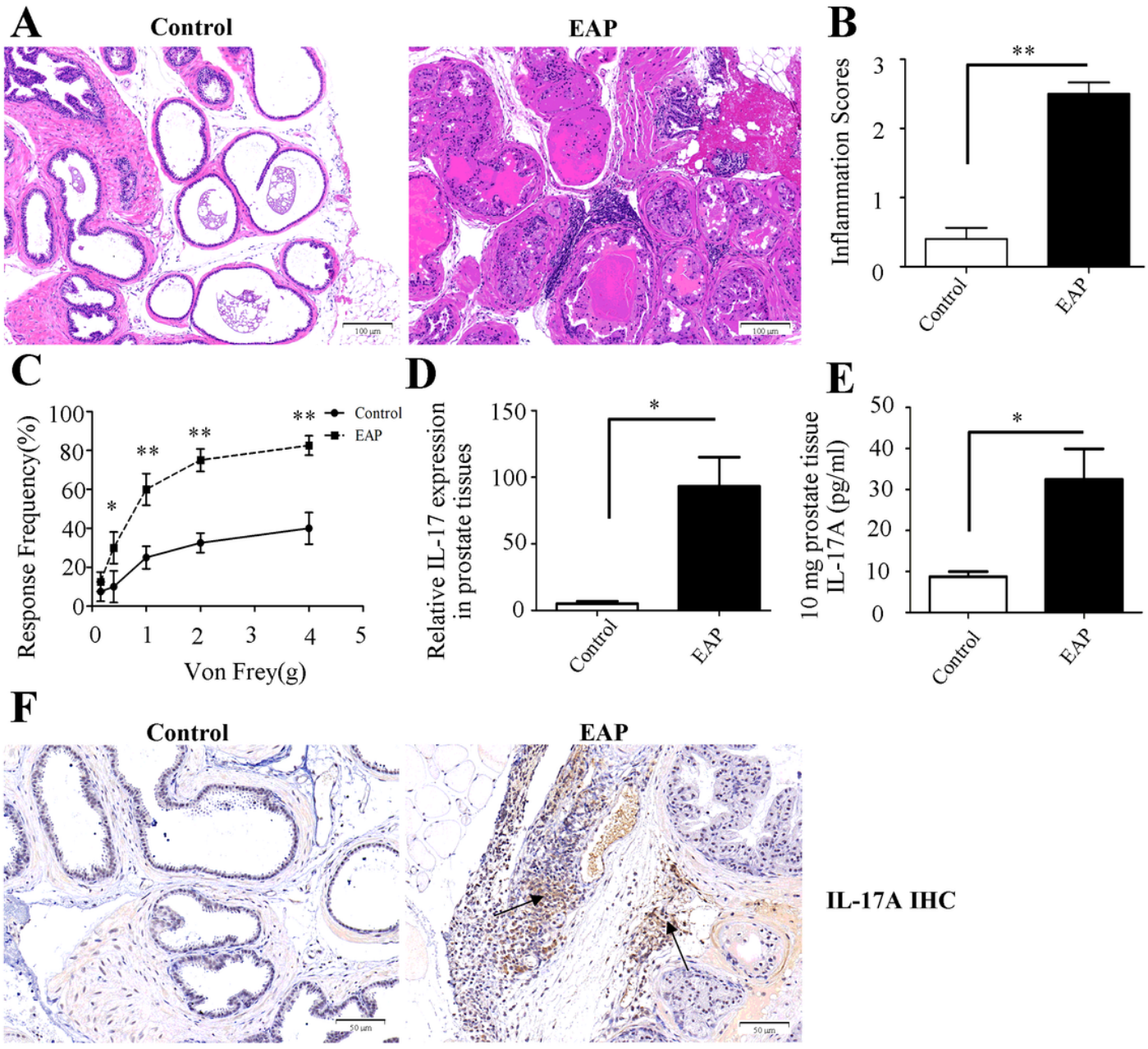

Figure 1

EAP induction caused histological changes in prostate tissues, pelvic pain, and increased IL-17 in prostate tissues. (A-B) Representative images of HE staining (A) and inflammation scores (B) of prostate tissue sections from the control or EAP group on day 42 after EAP induction. (magnification×100, scale bar=100 μm). (C) The response frequencies to individual filament stimulation of the pelvic area on day 42. (D) IL-17 mRNA expression in prostate tissues on day 42 was measured by real-time PCR. (E) The level of IL-17 in prostate tissues on day 42 was analyzed by ELISA. (F) Immunohistochemical analysis of IL-17 in prostate sections from the control and EAP groups on day 42 after EAP induction. *P<0.05, **P<0.01. Data represent the mean ± SEM for 4 mice per treatment group of three independent experiments.

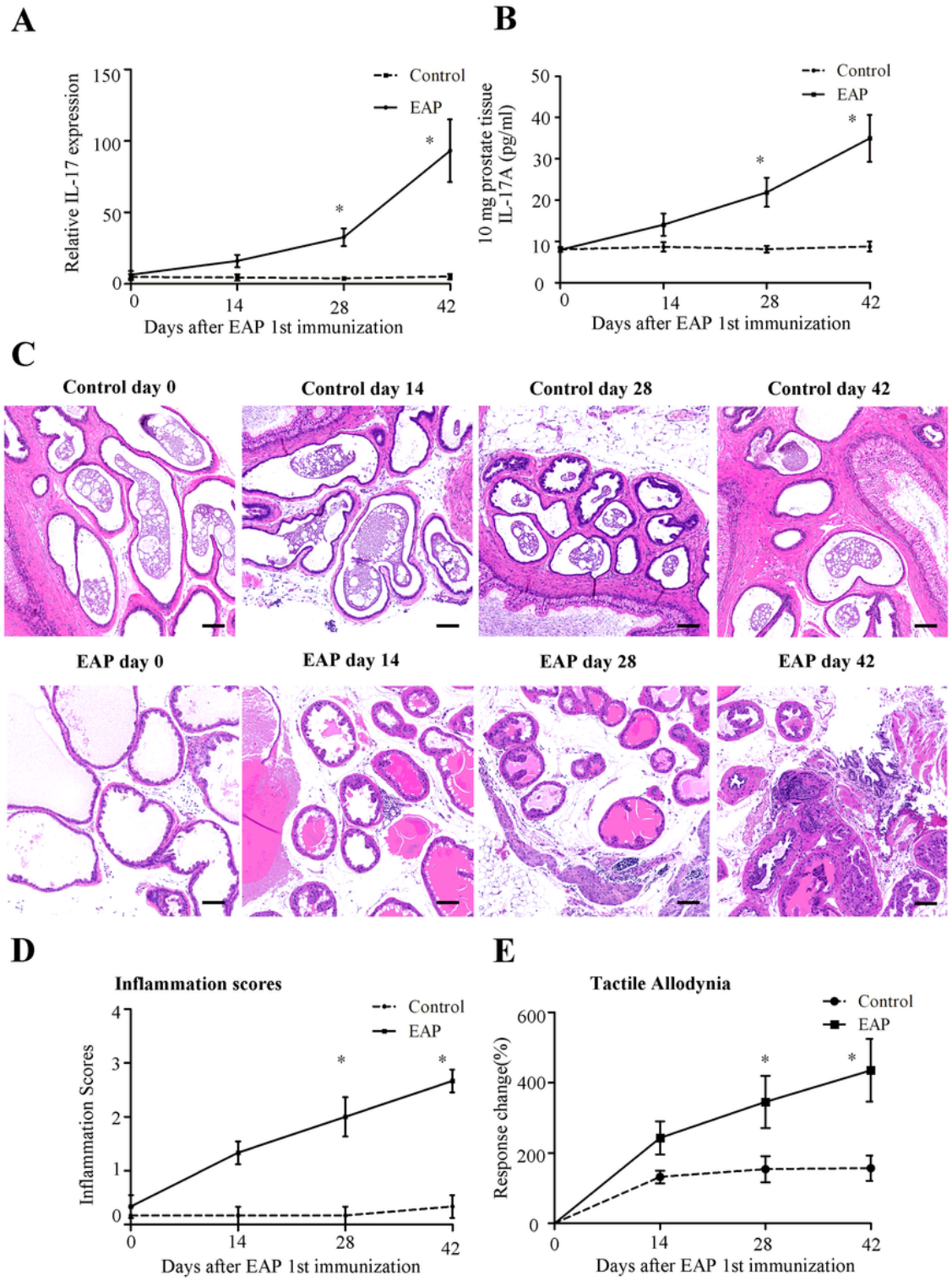

Figure 2

IL-17 mRNA and protein expression paralleled EAP-induced prostate inflammation and pain symptoms. (A) IL-17 mRNA expression in prostate tissues was measured by real-time PCR at the indicated times after EAP 1st immunization. (B) IL-17 levels in prostate homogenates were determined by ELISA at the indicated times after EAP 1st immunization. (C-D) The mice were sacrificed at the indicated times after EAP 1st immunization and formalin-fixed prostate sections were stained with HE staining. Representative

images of HE staining (C) and inflammation scores (D) of prostate tissue sections from the control or EAP mice at different times after EAP induction. Original magnification: ×100. Scale bars= 100 μm. (E) Chronic pelvic pain was assessed by tactile allodynia using von Frey filaments stimulation at different times after EAP 1st immunization. *P<0.05, **P<0.01. Data represent the mean ± SEM for 4 mice per treatment group of three independent experiments.

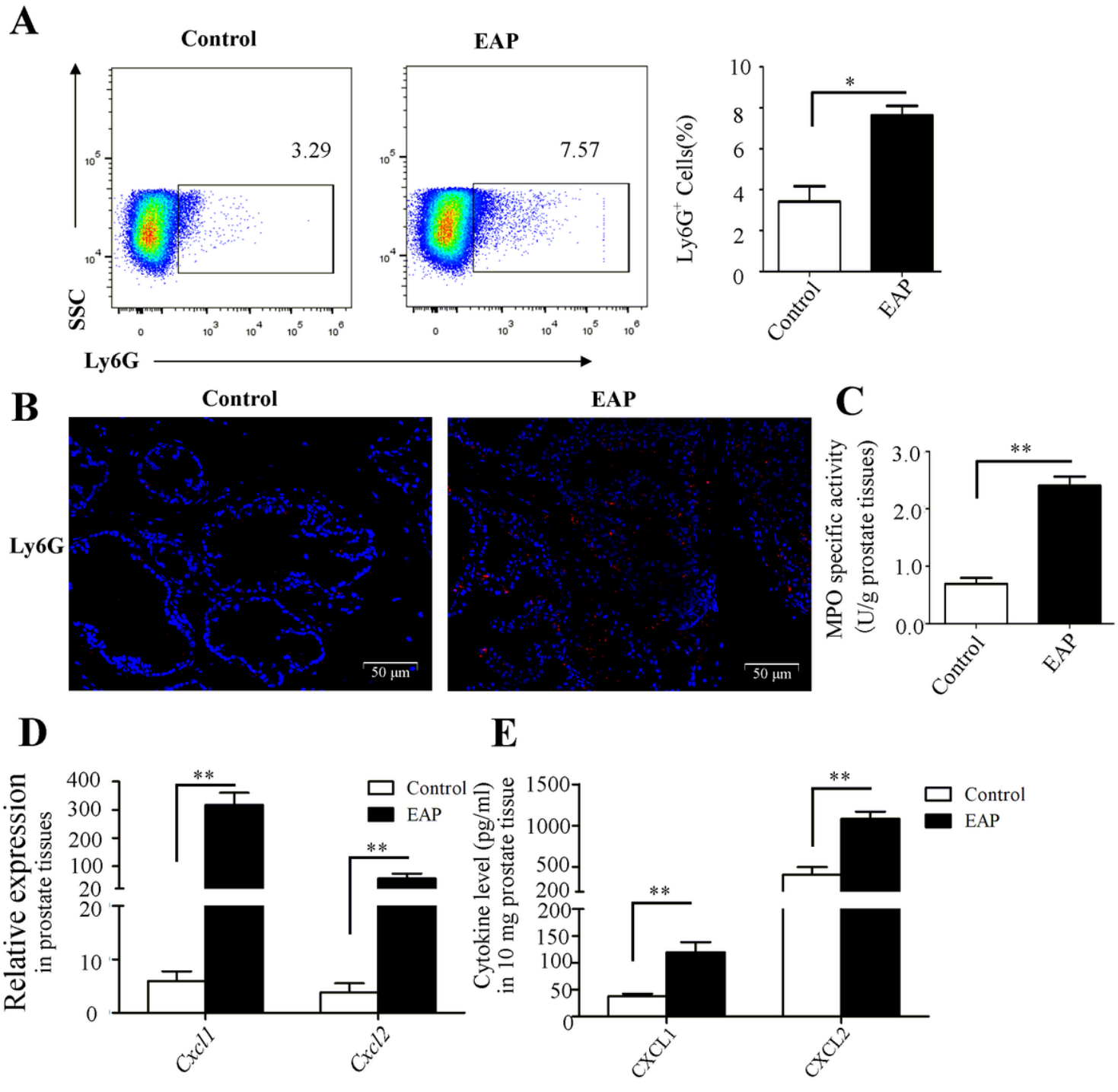

Figure 3

Neutrophil infiltration and the expression levels of CXCL1, and CXCL2 were increased in the prostate tissues of EAP mice. (A) Ly6G+ neutrophils in prostate tissues were analyzed by flow cytometry on day 42

after EAP 1st induction. (B) Immunofluorescent images of neutrophil infiltration in prostate tissues on day 42 after EAP 1st induction. Representative images of anti-Ly6G staining in control and EAP mice are shown. (C) The MPO activity in the prostate tissues from control and EAP mice was determined by MPO colorimetric assay kit on day 42 after EAP 1st induction. (D) The mRNA levels of Cxcl1 and Cxcl2 in the prostate tissues from control and EAP mice were determined by qRT-PCR on day 42 after EAP 1st induction. The results were normalized to Gapdh. (E) The levels of CXCL1 and CXCL2 in prostate tissues were determined by ELISA on day 42 after EAP induction.*P<0.05, **P<0.01. Data represent the mean ± SEM for 4 mice per treatment group of three independent experiments.

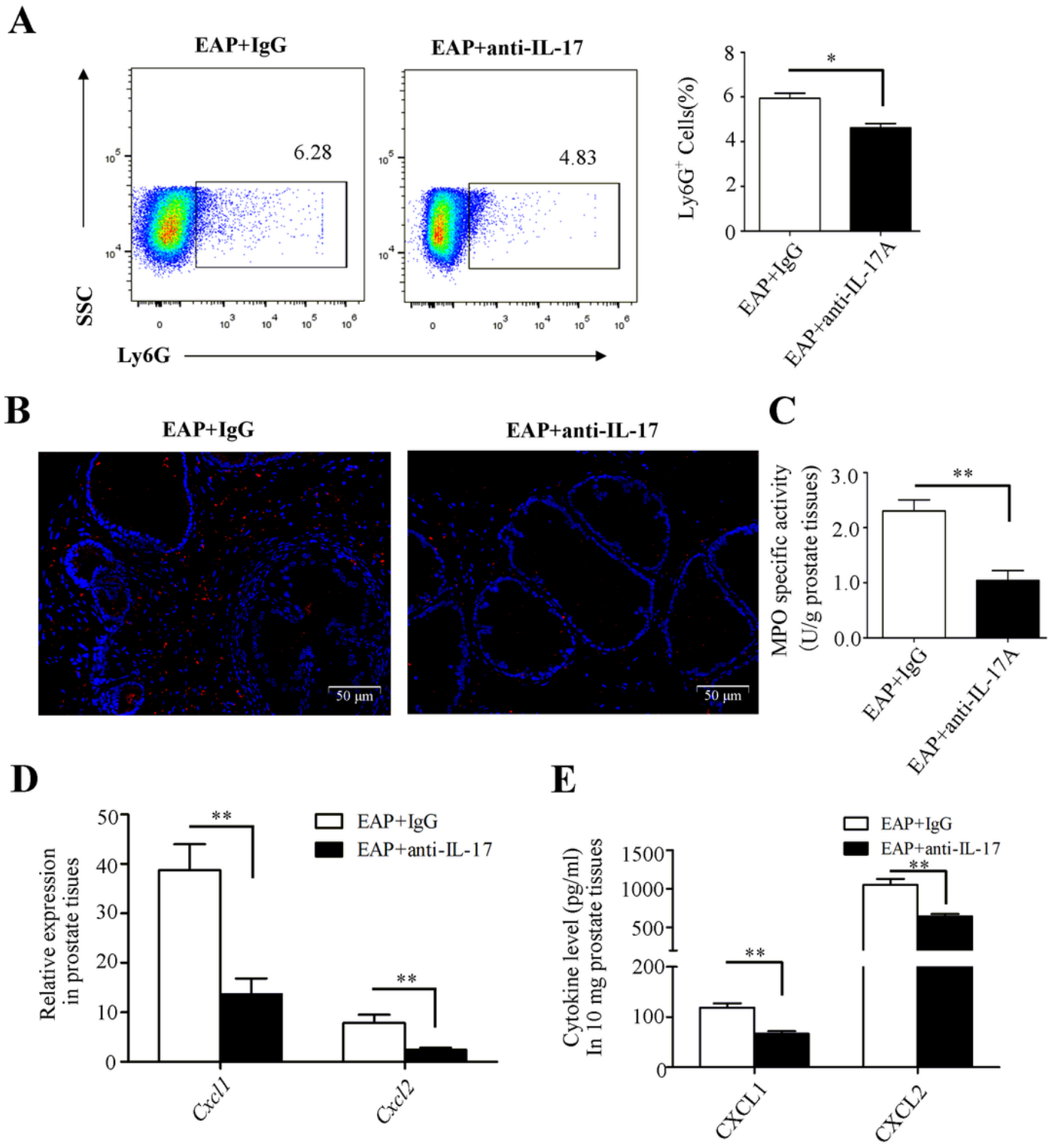

Figure 4

Neutralization of IL-17 reduced the infiltration of neutrophils, and the levels of CXCL1 and CXCL2. (A) Ly6G+ neutrophils in prostate tissues were analyzed by flow cytometry on day 42 after EAP 1st immunization. (B) Representative immunofluorescent images of neutrophil infiltration (anti-Ly6G) in the prostate tissues from the EAP+IgG group and the EAP+anti-IL-17 group on day 42 after EAP 1st immunization. (C) The MPO activity in the prostate tissues from mice in the EAP+IgG and EAP+anti-IL-17

groups was determined by MPO colorimetric assay kit on day 42 after EAP 1st immunization. (D) The mRNA levels of Cxcl1 and Cxcl2 in the prostate tissues from mice in the EAP+IgG and EAP+anti-IL-17 groups were determined by qRT-PCR on day 42 after EAP 1st immunization. The results were normalized to Gapdh. (E) The levels of CXCL1 and CXCL2 in prostate tissues were analyzed by ELISA on day 42 after EAP 1st induction. *P<0.05, **P<0.01. Data represent the mean ± SEM for 4 mice per treatment group of three independent experiments.

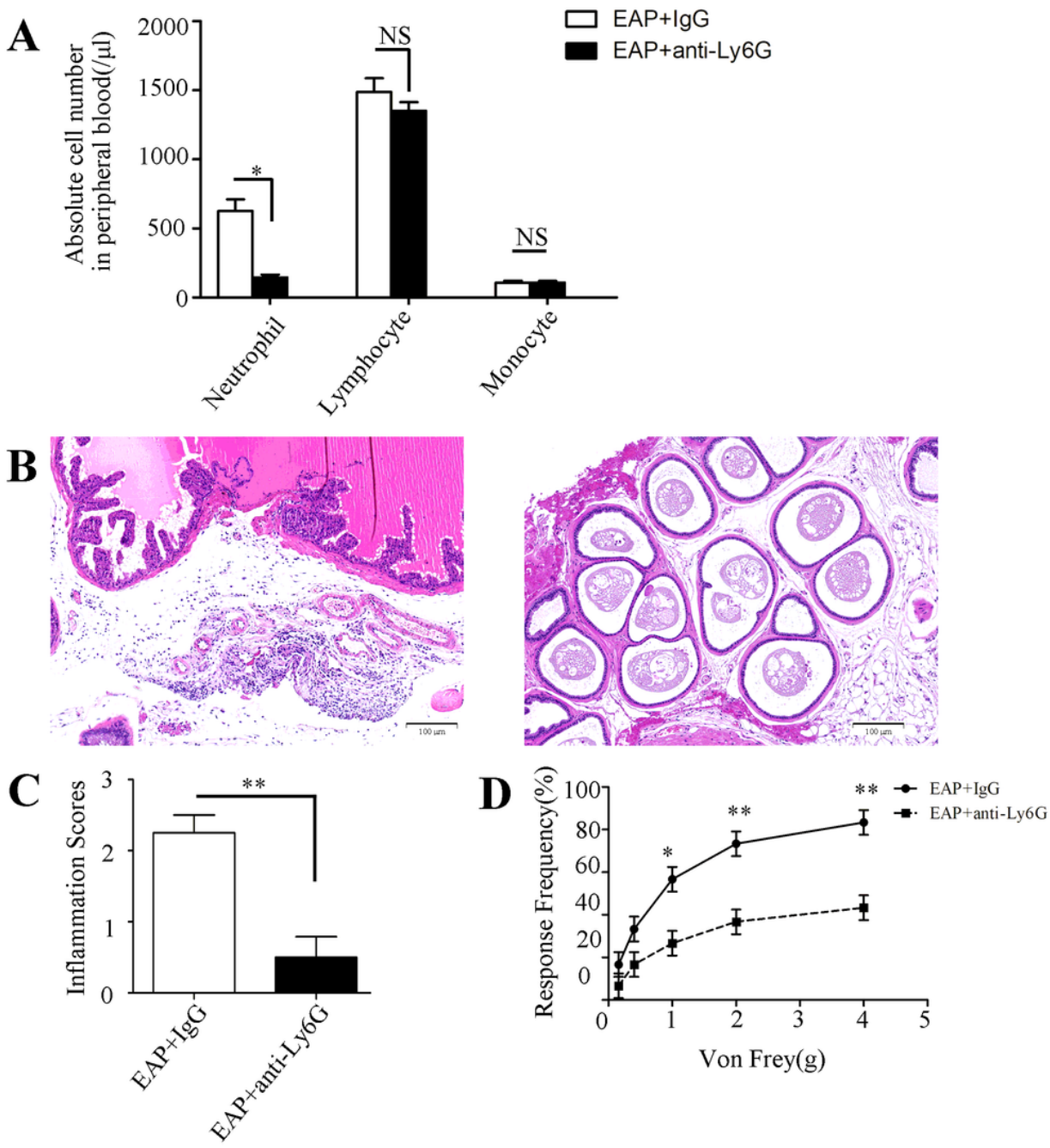

Figure 5

Depletion of Neutrophils by anti-Ly6G antibody protects NOD mice from EAP. (A) Number of neutrophils, lymphocytes and monocytes in the peripheral blood from EAP+IgG and EAP+anti-Ly6G mice measured via hematology cell counter at day 42 after EAP 1st immunization. (B-C) Representative HE staining

images (B) and inflammation scoces (C) of prostate tissue of EAP+IgG and EAP+anti-Ly6G mice on day 42 after EAP 1st immunization. (D) The response frequencies to individual filament stimulation of the pelvic area on day 42 after EAP 1st immunization. *P<0.05, **P<0.01. Data represent the mean ± SEM for 4 mice per treatment group of three independent experiments.

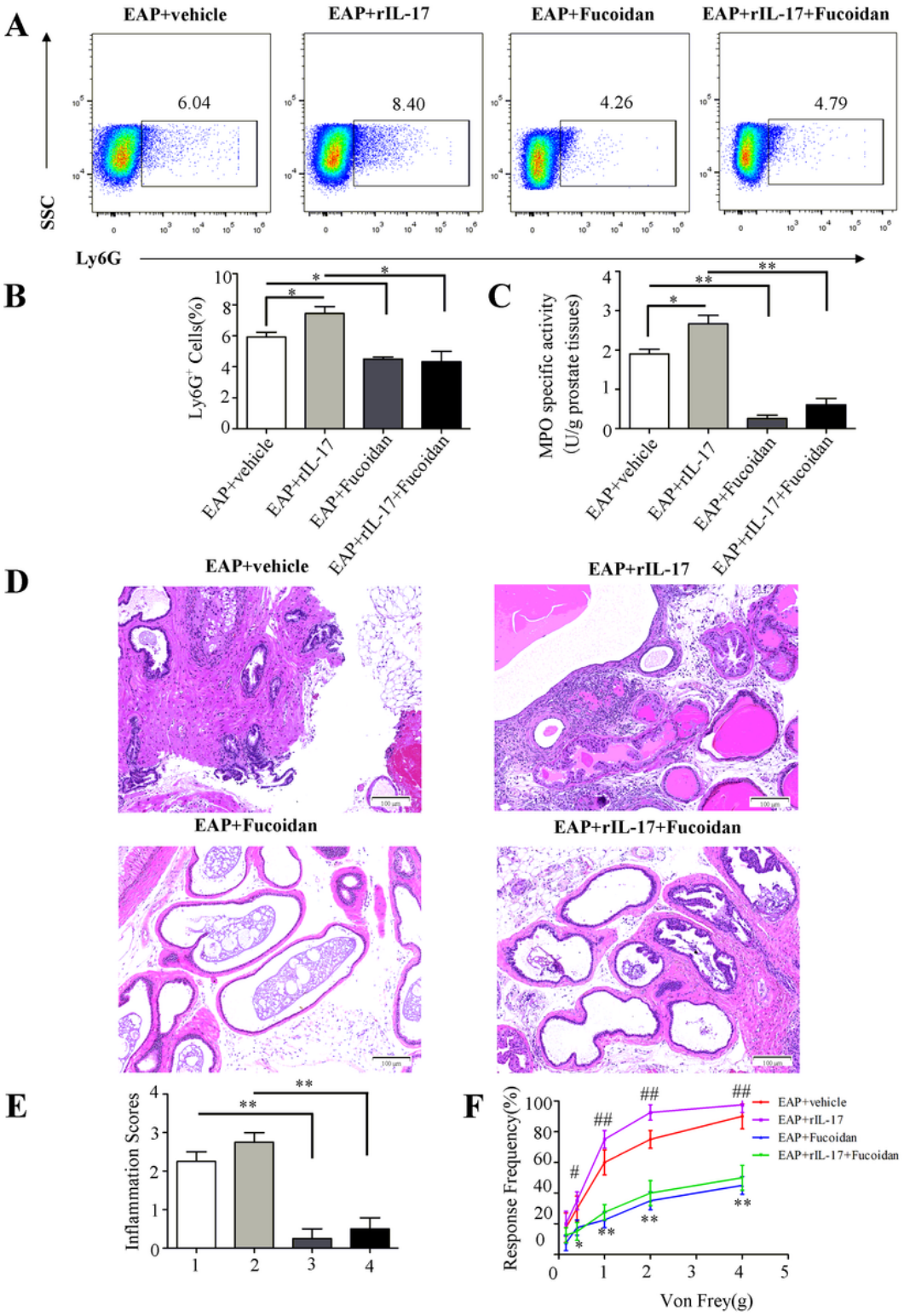

Figure 6

Fucoidan treatment decreased the severity of prostate inflammation and chronic pain behavior. (A-B) Ly6G+ neutrophils in the prostate tissues from each group were analyzed by flow cytometry on day 42 after EAP 1st immunization. (C) The MPO activity of the prostate tissues from each group analyzed by the MPO colorimetric assay kit on day 42 after EAP 1st immunization. (D-E) Representative HE staining images (D) and inflammation scores (E) of prostate tissue from each group on day 42 after EAP 1st immunization: 1, EAP+vehicle; 2, EAP+rIL-17; 3, EAP+fucoidan; 4, EAP+rIL-17+fucoidan. (F) The response frequencies to individual filament stimulation of the pelvic area on day 42 after EAP 1st immunization. (EAP+fucoidan vs EAP+vehicle, *P<0.05, **P<0.01 and EAP+rIL-17+fucoidan vs EAP+rIL-17, #P<0.05, ##P<0.01). *P<0.05, **P<0.01. Data represent the mean ± SEM for 4 mice per treatment group of three independent experiments.

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