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ORIGINAL ARTICLE

The Hypermethylation of Foxp3 Promoter Impairs the Function of Treg Cells in EAP

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Abstract— Treg cells are crucial for maintaining immune homeostasis in CP/CPPS, but the molecular mechanisms underlying the modulation of the function of Treg in CP/CPPS remain unclear. The main purpose of this study is to investigate the relationship between immunosuppressive function of Treg and the methylation level of Foxp3 promoter in experimental autoimmune prostatitis (EAP) mouse model. EAP model was induced by subcutaneous injecting prostate-steroid-binding protein (PSBP) and complete Freund's adjuvant with NOD mice. Histological analysis revealed that EAP model was successfully induced. The expression of IFN-γ was increased, and TGF-β was decreased in the serum of EAP, respectively. The percentage of Tregs in splenic lymphocyte was increased in EAP. The suppressive ability of Tregs on Teffs was impaired in EAP. The methylation level of Foxp3 promoter was increased, and the expression of Foxp3 was decreased in EAP. By injection AZA which was DNA-methylation inhibitor into EAP mice, prostate inflammation was alleviated, expressions of TGF-β and Foxp3 were increased, and the suppressive function of Tregs was improved *in vitro* and *in vivo*. Thus, we concluded that aberrant increased methylation of Foxp3 promoter in Treg cells leads to the impaired suppressive function of Treg cells, exacerbating autoimmune inflammatory injury in EAP.

KEY WORDS: CP/CPPS; EAP; Treg cells; Foxp3; methylation.

INTRODUCTION

Prostatitis is a very common urologic diagnosis disease [1, 2], which reported that about 35–50% of men are afflicted with prostatitis during their lifetime [1, 3]. Especially in men younger than 50 years old, the morbidity of prostatitis will be even higher [1]. According to the proposal of the National Institutes of Health (NIH) in 1999 [4], prostatitis is classified into four categories. Category III prostatitis or chronic pelvic pain syndrome (CP/CPPS) accounts for most cases of chronic prostatitis, representing 90% of all chronic prostatitis cases [5], with the prevalence

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rate of about 8.4% in Chinese [6]. It is characterized by pelvic or perineal pain, irritative voiding symptoms, and sexual dysfunction, without evidence of urinary tract infection [2, 7]. The most frequent symptom of CP/CPPS is chronic pelvic pain which occurs in the region of pelvis, perineum, scrotum, testes, penis, rectum, and anus, and radiates to the lower abdomen. The chronic pelvic pain is often supposed to relate to lower urinary tract symptoms, erectile dysfunction, and psychosocial symptoms [8, 9], and leads to depression, psychosocial maladjustment, and diminished quality of life in CP/CPPS patient [3, 10]. Since the etiology of CP/CPPS is still not well established so far, available therapeutic options for CP/CPPS including alpha blocker, anti-inflammatory, physiotherapy, and neuroleptics are far from satisfactory for either patients or doctor [11]. In order to explore an effective therapeutic approach for CP/CPPS, deeper research to the etiology of CP/CPPS is particularly needed.

During the past decades, cumulative evidence obtained from studies of patients and animal models indicated that CP/CPPS was a consequence of dysregulated inflammation in the form of autoimmunity against prostate which may trigger chronic non-infectious inflammation [12–18]. Several studies had reported that autoimmune T cell from CP/CPPS patients could respond to PSA, prostatic acid phosphatase, seminal plasma protein, and some other prostate peptides [12–15]. Experimental autoimmune prostatitis (EAP) models achieved by immunization of mice with prostate antigen plus Folin adjuvants reflected the typical characteristics of human CP/CPPS disease. And EAP model is crucial for understanding pathophysiological immune mechanisms in the development of CP/CPPS [16–18].

Regulatory $CD4^+CD25^+Foxp3^+$Treg cells (Tregs) are a subgroup of T helper (Th) cells which are responsible for inhibiting the inflammation process [19–23]. Tregs are crucial in the balance of immunity and self-tolerance in healthy individuals [21–23]. Dysregulation of Treg function was considered to have a closed connection to autoimmunity and inflammatory diseases, such as systemic lupus erythematosus (SLE) [24] and multiple sclerosis (MS) [25]. Recently, some studies suggested that the decreased function of Tregs might contribute to the development and progression in CP/CPPS [18, 26]. Tregs play an immunological negative regulatory role which depended on the high-level expression of Foxp3 [27, 28]. Foxp3 plays a key role in the formation and immune tolerance function of Tregs [22, 29]. Inflammation is beneficial for pathogen clearance and protection against infection, but excessive inflammation can damage the tissues; hence, the activation of $Foxp3^+$Treg cells may be important for allowing effective immune responses to occur during inflammation [30]. Nonetheless, the molecular mechanism of how Foxp3 protein and Tregs function are regulated during inflammation is still unknown. An evolutionarily conserved CpG island which remained non-methylated in native Tregs was found in Foxp3 promoter [31]. The change of methylation level of Foxp3 promoter not only modifies the expression of Foxp3 but also affects the immune suppressive function of Tregs. Some studies found that abnormal epigenetic modification of Foxp3 such as promoter CpG-island methylation or histone acetylation happened in many autoimmune diseases [31, 32]. However, research concerning the relationship between epigenetic regulatory role of Foxp3 and CP/CPPS is still rare for now.

Here, we investigated the methylation status of Foxp3 promoter, the expression of Foxp3, and the immunosuppressive activity of Treg on Teff in EAP. Our result suggested that methylation of Foxp3 promoter was increased, expression of Foxp3 was decreased, and the immunosuppressive activity of Tregs on Teffs was decreased in EAP mice compared with the control group. This study provided an indication that the abnormal increased methylation of Foxp3 promoter in Tregs can reduce the immunosuppressive function of Treg in EAP.

MATERIALS AND METHODS

Mice and Mouse Model of EAP

Nod-shiltjnju (NOD) mice were purchased from the Animal Experiment Center of Nanjing University (Nanjing, China). All mice were housed and maintained under SPF conditions in Animal Facility of Anhui Medical University (Hefei, China) and used at the age of 6–8 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee of Anhui Medical University. The preparation of the mixture of prostate antigen (PAg) and purification of prostate-steroid-binding protein (PSBP) were carried out as previously described [33]. The PSBP was fully emulsified with complete Freund's adjuvant (Sigma-Aldrich, St. Louis, USA). NOD mice were randomly divided into three groups: control group received subcutaneous injection of 0.2 ml saline solution in the hind footpad, base of the tail, and shoulder at days 0 and day 15; EAP group received subcutaneous injection of 0.2 ml PSBP emulsion at days 0 and day 15; EAP+AZA group received subcutaneous injection of 0.2 ml PSBP at days 0 and day 15 with intraperitoneal injection 2.5 mg/kg AZA (5-Aza-2′-deoxycytidine, Sigma-Aldrich, St. Louis, USA) from day 1 to 10. Finally, all the mice were anesthetized

with 4% chloral hydrate (Aladdin Biotechnology Co. Ltd., Shanghai, China), killed using cervical dislocation, and sacrificed for subsequent analysis on day 42.

Histopathological Examination

Prostates separated from mice were fixed in 4% paraformaldehyde solution for 1 day and embedded in paraffin. The prostate tissues sectioned into 5-um-thick slices were stained with hematoxylin-eosin (HE) and then used for pathological and morphological evaluation.

Cytokine Measurements

The peripheral blood samples were got from mice in each group, and the serum was collected by centrifugation at 800*g* for 20 min. ELISA kits were used to detect the concentration of cytokines in the serum of mice from all groups, including IFN-γ, TGF-β, and IL-10, according to the manufacturer's protocols (all kits were purchased from Elabscience, Wuhan, China). The detection range of kits was 15.63–1000 pg/ml.

$CD4^+CD25^+$Tregs Isolation and Flow Cytometry Analysis

Fresh isolated $CD4^+CD25^+$Treg cells from spleen were stained for surface marker as previously described[18]. The spleens were ground in PBS. Then, the grinding fluid was centrifuged at 1000 rpm for 5 min at 4 °C. We discarded the supernatant and resuspended cells in red blood cell lysing buffer. After incubation for 15 min at 4 °C, cells were centrifuged and washed twice with PBS. Treg cells were stained with antibodies against anti-mouse CD25-APC (No. 557192; BD Biosciences, New York, USA), CD4-FITC (No. 553047; BD Biosciences, New York, USA), CD4-PE-Cy7 (No. 56333; BD Biosciences, New York, USA), and Foxp3-PE (No. 563101; BD Biosciences, New York, USA). FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, USA) was used to analyze those stained cells, and the data was analyzed by FlowJo Software 7.6.1(Tree Star, Ashland, USA).

Methylation Levels of the Foxp3 Upstream Promoter

Bisulfite sequencing was used to detect the methylation analysis. Genomic DNA extracted from $CD4^+CD25^+$Treg cells was used in the DNA methylation assay. EZ DNA methylation kit (QIAGEN) was used to perform the bisulfite conversion of genomic DNA. We used the method of PCR to amplify the Foxp3 promoter region by using the primer sequences (forward 5′-TAGT TGTTGTAGTTGTTGTTGTTGG-3′, reverse 5′-CTAA CCAAATATCAACCTCCTCAC-3′, fragment size 286 bp), the purified PCR products were cloned into pMD19-Tvector (TaKaRa, Tokyo, Japan), and single clones were selected for sequencing. All sequencing results of bisulfite-converted Foxp3 region were analyzed on BDPC DNA methylation analysis platform, 5500xl Genetic Analyzer (ThermoFisher Scientific), and Chromas software (2.4.4, South Brisbane, Queensland, Australia).

Detection of the $CD4^+CD25^+$Tregs Suppression Function by Personal Co-culture Studies

In order to compare the suppressive effect of Tregs obtained from mice of the control group, the EAP model group and EAP treatment group, Treg cells obtained from three groups were co-cultured with $CD4^+CD25^-$T cells (Teffs) which were obtained from the same groups at different ratio (Tregs:Teffs = 0:1, 1:2, 1:1), with the number of Teffs being held constant at 1.0×10^5 cells/well. Treg cells and $CD4^+CD25^-$ cells were co-cultured with monoclonal antibodies anti-CD3 (2 μg/ml, No.100202, Biolegend, USA), anti-CD28 (1 μg/ml, No.102102, Biolegend, USA), and IL2 (10 ng/ml, 402-ML, RD, USA) for 72 h at 37 °C with 5% CO_2. After culture, flow cytometry (BD Biosciences, USA) was used to test the proliferation index of Teffs which was labeled with CFSE (No. 565082; BD Biosciences, New York, USA).

Western Blotting

The protein levels of Foxp3 were determined by Western blot analysis. The protein samples were extracted from the $CD4^+CD25^+$Tregs which were isolated from the spleen of mice. Proteins (8 μl) were separated by 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. After blocking with 5% non-fat dry milk which was diluted with TBST for 1 h at room temperature, the membranes were incubated with primary antibodies against Foxp3 (1:1000; No. 22228-1-AP; Proteintech Group, Inc., Chicago, USA) or GAPDH (1:2000; No.1049-1-AP; Proteintech Group, Inc., Chicago, USA) overnight at 4 °C, and then incubated with secondary antibodies (1:5000; No. SA00004-2; goat anti-rabbit; Proteintech Group, Inc., Chicago, USA) for 1.5 h at room temperature. Protein bands were visualized by enchanced chemiluminescence using ECL reagents (Pierce; Thermo Fisher Scientific, Inc., USA) and exposed to film (Canon, Tokyo, Japan). GAPDH was used as the internal reference. Blots were semi-quantified by densitometric analysis using the Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, USA).

Adoptive Transfer of Treg Cells

EAP group and EAP+AZA group mice were treated with PSBP and AZA as mentioned above. Treg cells were stained with antibody against mouse CD4-FITC (No. 553047; BD Biosciences, New York, USA), CD8-PE (No. 553032; BD Biosciences, New York, USA), CD25-APC (No. 557192; BD Biosciences, New York, USA), and CD44-PE-Cy7 (No. 103029; Biolegend, California, USA). $CD4^+CD8^-CD25^+CD44^+$Treg cells isolated by FACS from EAP group and EAP+AZA group were named aTreg and bTreg respectively. After culture of 2 days, isolated Treg cells (3×10^5) were injected intravenously in the tail of EAP mice in a 100-μl suspension on day 35. The absolute number of cells was calculated by Olympus cell counter model R1. The control group and EAP group were used as negative control and positive control, respectively. Seven days later, mice were killed using cervical dislocation and sacrificed for subsequent analysis.

Immunohistochemistry

The formalin-fixed and paraffin-embedded prostate tissues collected from each group mice were examined using anti-mouse CD4 (1:100, No. E-AB-30827 Elabscience, Wuhan, China), anti-mouse CD8a (1:500, No. E-AB-70025, Elabscience, Wuhan, China), and anti-mouse Foxp3 (1:100, No.12653, CST, Massachusetts, USA). The SP-9000 IHC immunochemistry kit (ZSGB-Bio, China) and 3,3′-diaminobenzidine (ZLI-9018, ZS-Bio, China) were used to detect the immune complexes in prostate tissues finally.

Statistical Analysis

All experiments were repeated at least three times independently. The data are expressed as the mean ± SD. Statistical analysis was performed using the Student-Neuman-Keuls test and one-way ANOVA with the Bonferroni *post hoc* test. $^*P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Induction of the EAP Model

According to researches described previously [16, 34], we used prostatein-steroid binding protein (PSBP) to induce experimental autoimmune prostatitis (EAP) in NOD mice. In order to assess the effect of PSBP on the prostate tissues of mice, we analyzed the histologic alterations in prostate histologic sections by HE staining. In the control group, normal appearances of gland epithelium and stroma, without any inflammatory cell infiltration, were observed (Fig. 1a, b). While in the EAP group, congestion and edema of prostatic stroma, and a large number of inflammatory cell infiltration around the prostatic glandular cavity were observed (Fig. 1c, d). Significant pathological alternations were observed in the EAP group compared with the control group. Thus, we concluded that the EAP model was successfully induced with PSBP.

The Level of IFN-γ, TGF-β, and IL-10 in Serum of Mice

As we all know, IFN-γ is an inflammatory cytokine secreted by Th1 cell, while TGF-β and IL-10 are immune negative regulation cytokines secreted by Treg cells. In order to assess the effect of inflammation on EAP mice, we performed enzyme-linked immunosorbent assays (ELISA) to detect the levels of IFN-γ, TGF-β, and IL-10 in serum of mice. The result showed that the level of IFN-γ in the serum of EAP mice was significantly increased compared with the control group (Fig. 1e). While the level of TGF-β in the serum of EAP mice was significantly decreased compared with the control groups (Fig. 1f). The level of IL-10 in these two groups had no significant difference (Fig. 1g). In brief, the level of IFN-γ was increased and the level of TGF-β was decreased in the EAP group.

Treg Cells Percentage Was Increased in EAP Mice

Due to the importance of Tregs for EAP, we detected the percentage of Tregs in splenic lymphocytes from EAP and control groups by flow cytometry. The percentage of Treg cells in the splenic lymphocytes from EAP and control groups were measured at day 42 after PSBP or NS injection. Compared with the control group, the percentage of Treg cells in the EAP group was significantly increased (2.72 ± 0.08 *vs* 1.91 ± 0.13) (Fig. 2). This results showed that Tregs may be increased by responding to inflammation.

The Suppressive Function of Treg Was Impaired in EAP Mice

As the percentage of Treg cells in the EAP group was increased compared with the control group, we examined the ability of Tregs to suppress Teffs ($CD4^+CD25^-$T cells) by conducting *in vitro* co-culture studies. Tregs and Teff cells were isolated from the mouse spleen at day 42 after PSBP or NS injection. Treg cells from EAP mice had a lower

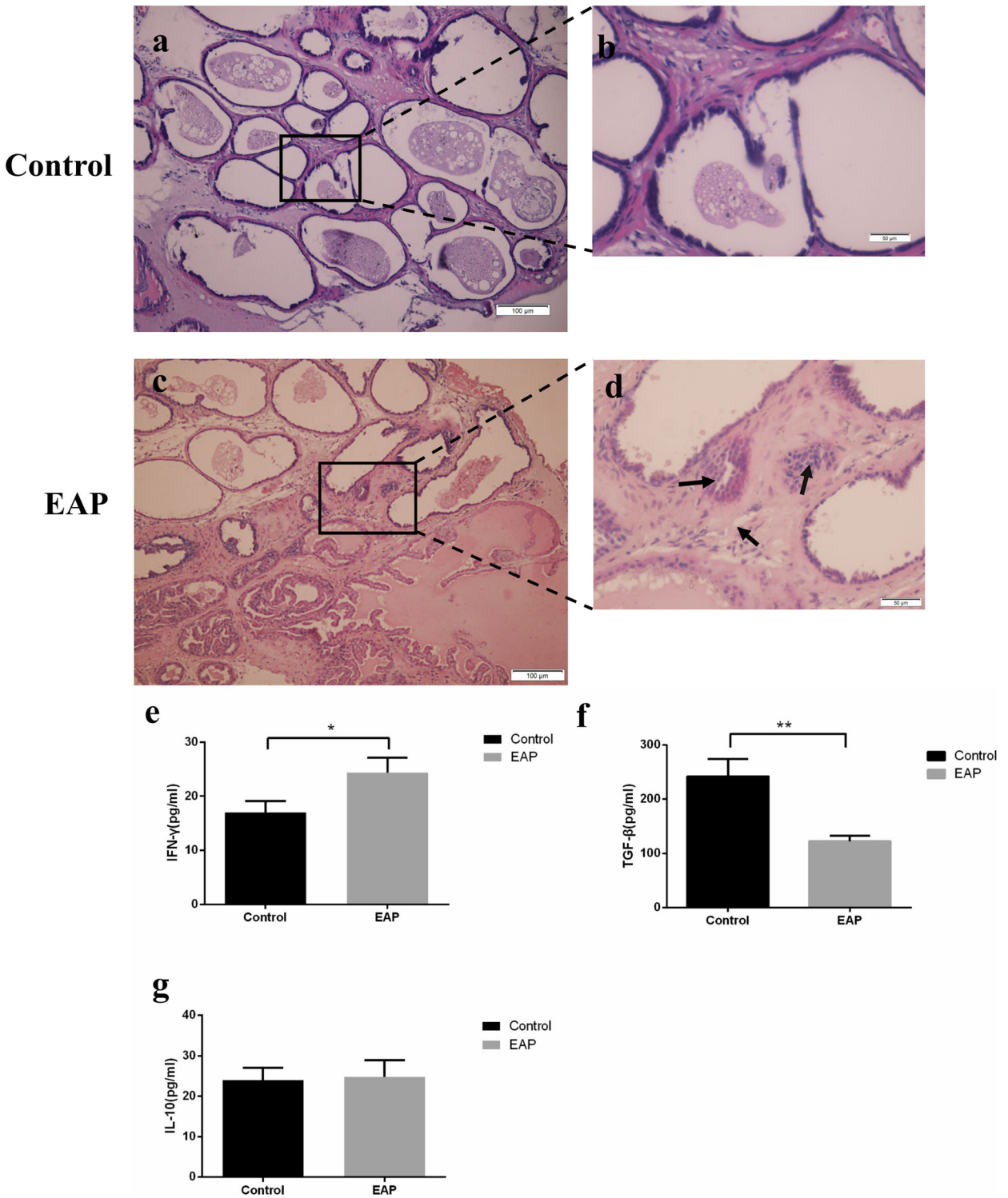

Fig. 1. Histological alterations in prostate tissues and inflammation cytokines in serum of mice from control and EAP group. a–d Section of prostate tissues from control group (a, b) and EAP mice (c, d). Arrows represent areas of leukocytic infiltrate in the prostate. (a, c: magnification × 100, bar = 100 μm; b, d: magnification × 400, bar = 50 μm). e–g Quantitative analysis of IFN-γ (e), TGF-β (f), IL-10 (g) in serum of mice determined by ELISA and reported as mean ± SD; all experiments were repeated at least two times and had 3 animals per group.*$P < 0.05$, **$P < 0.01$.

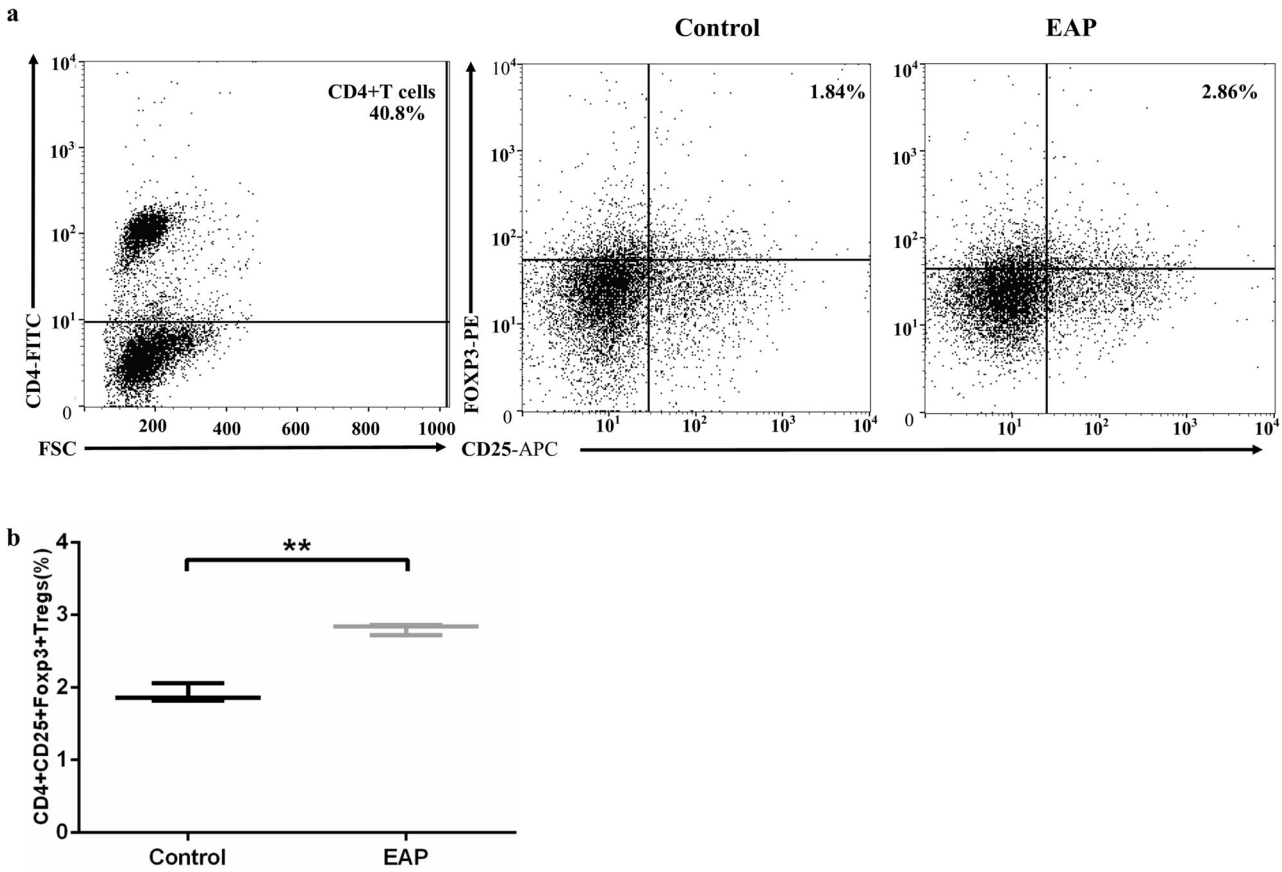

Fig. 2. The percentage of Treg cells in the splenic lymphocytes was increased in EAP mice. a The frequency of $CD4^+CD25^+Foxp3^+$ cells in splenic lymphocytes in control, EAP groups, respectively. b The percentage of $CD4^+CD25^+Foxp3^+$Treg cells in splenic lymphocytes in the two groups, corresponding data in (a). Data are representative of 3 independent experiments, $^{**}P < 0.01$.

suppressive ability when compared with Treg cells from control groups ($29.33 \pm 8.51\%$ *vs* $40.56 \pm 8.92\%$, Tregs:Teffs = 1:1,$^*P < 0.05$, Fig. 3). The impaired suppressive function of Tregs was also observed at Tregs:Teffs = 1:2 ($18.56 \pm 1.05\%$ *vs* $27.16 \pm 3.45\%$, Tregs:Teffs = 1:2, $^*P<0.05$, Fig. 3). But the capacity for proliferation of Teffs showed no significant difference (Tregs:Teffs = 0:1, $^*P > 0.05$, Fig. 3). All in all, the result showed that the suppressive function of Tregs was impaired in the EAP group.

Methylation Status of CpG Islands within Foxp3 Promoter Was Increased in Treg from EAP Mice

In our above experimental results, the increasing Treg percentage in the EAP group was inconsistent with the damaging of Tregs suppressive function in the EAP group. The immunological suppressive function of Treg depends on the high-level expression of Foxp3. Foxp3 plays a key role in the formation and immune tolerance function of Tregs [27, 28]. An evolutionarily conserved CpG island which remains non-methylated in native Tregs was found in Foxp3 promoter. The methylation level of CpG island not only modifies the expression of Foxp3 but also affects the immune suppressive function of Tregs [35]. To investigate the molecular mechanisms that regulate Foxp3 expression during Treg differentiation, we examined the methylation levels of CpG islands within the Foxp3 promoter of Tregs by bisulfite sequencing. The results indicated that the EAP mice had significantly higher methylation of Foxp3 promoter compared with the control group (Fig. 4).

AZA Injection Alleviated Prostate Inflammation of EAP

Our results above indicated that the suppressive function of Treg was impaired and methylation of Foxp3

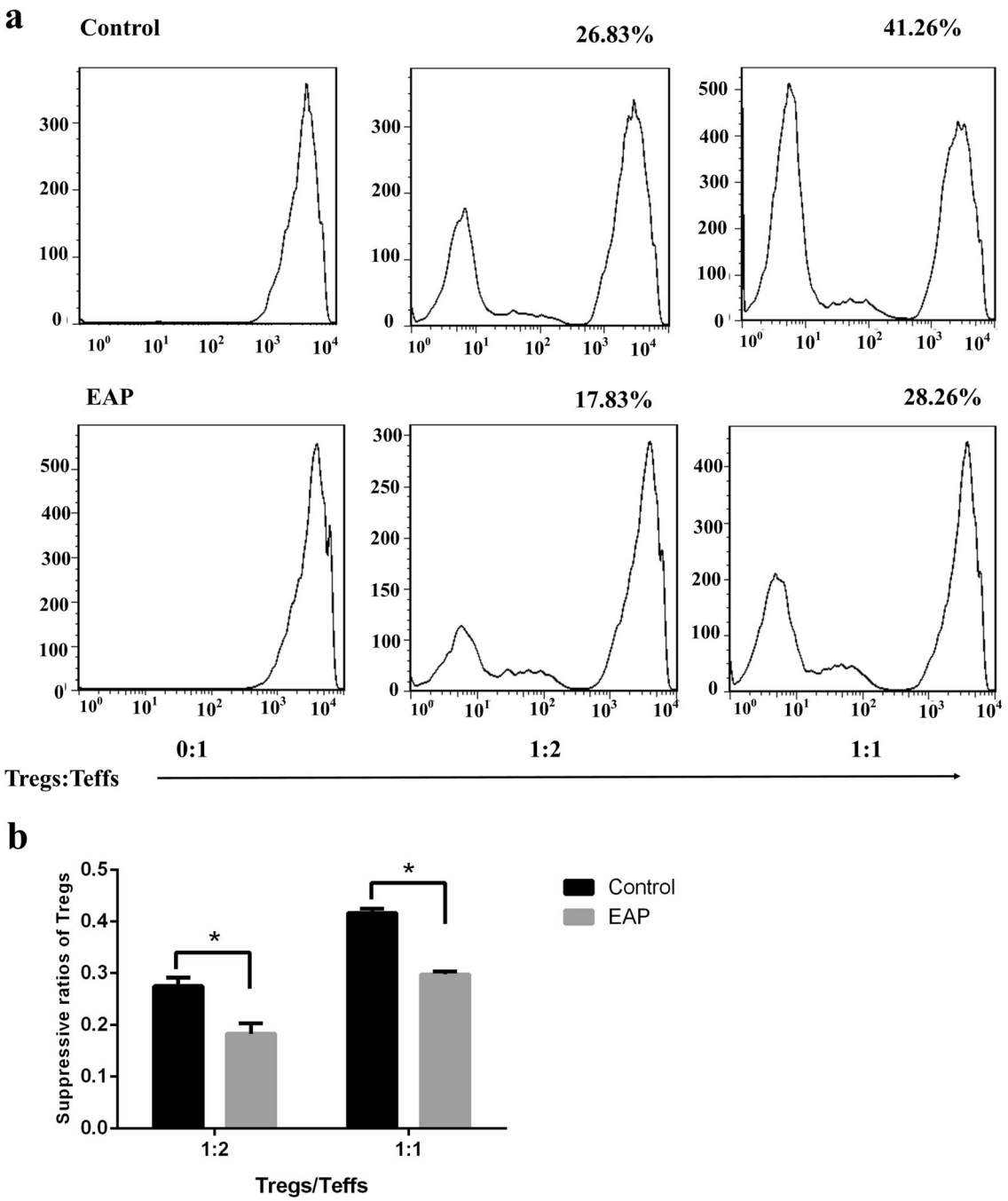

Fig. 3. The suppressive function of Tregs on Teffs was impaired in EAP mice. a CFSE-labeled Teffs were co-cultured with Tregs at different ratios in control, EAP groups. And the proliferation of Teffs was detected by flow cytometry in the FITC channel. The Treg suppressive ratio was computed by the formula: [1 − (PI of Tregs:Teffs-x:2)]/(PI of Tregs:Teffs-0:8) × 100%. The percentages represented the suppressive function of different ratios of Tregs. b The vertical axis represents relative suppression ratio which was also calculated using the same formula. Data are representative of 3 independent experiments, $*P < 0.05$.

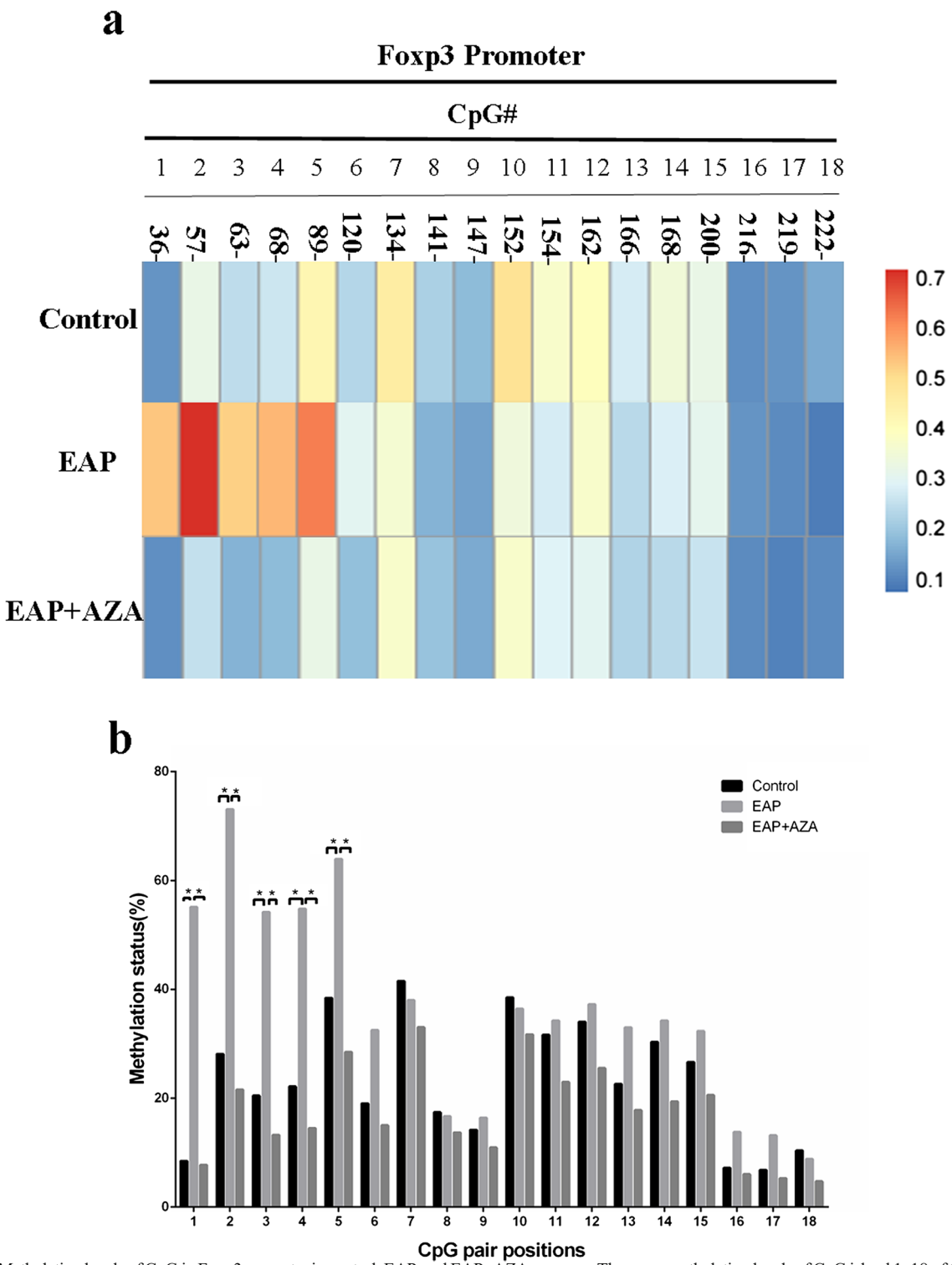

Fig. 4. Methylation levels of CpG in Foxp3 promoter in control, EAP, and EAP+AZA groups. a The mean methylation levels of CpG island 1–18 of the three groups were showed in the form. b The methylation levels of Foxp3 promoter in Tregs obtained from EAP group were compared with control and EAP+AZA groups according to data in (a). $n = 3$ mice per group. $^{*}P < 0.05$.

promoter was increased in EAP mice. So, we speculated that the impaired suppressive function of Treg in EAP mice might due to the hypermethylation of Foxp3 promoter. To verify this hypothesis, we used DNA methylation inhibitior, AZA, to alter the status of CpG island within Foxp3 promoter, and access the effect of AZA on suppressive function of Treg. Methylation percentage of CpG island in Foxp3 promoter was significantly reduced in EAP+AZA compared with EAP (Fig. 4). We found that the pathological alterations were significantly improved in the prostate tissues of mice in EAP+AZA compared with the EAP group (Fig. 5a). Moreover, the EAP+AZA group reduced the level of serum IFN-γ and increased the level of serum TGF-β compared with the EAP group (Fig. 5b). To measure the percentage of Tregs in the three groups, we detected the percentage of Tregs by flow cytometry. We found that the percentage of Tregs in the EAP+AZA group was significantly lower than that of the EAP group (Fig. 5c). On the other hand, Foxp3 protein expression in Treg cells from EAP+AZA group was increased significantly compared with the EAP group (Fig. 5d). The data suggested that AZA injection decreased the methylation of Foxp3 promoter of Treg cells, increased the expression of Foxp3, and alleviated prostate inflammation of EAP.

Tregs Isolated from EAP+AZA Mice Had Better Suppressive Function Compared with Tregs from EAP Mice *In Vitro* and *In Vivo*

To investigate the suppressive function of Tregs in the three groups described above, we firstly performed co-culture studies that Tregs co-cultured with Teffs obtained from the three groups at different ratios (Tregs:Teffs = 0:1, 1:2, 1:1) with the number of Teffs being held constant at 1.0×10^5 cells/well. Tregs isolated from the EAP+AZA group had better suppressive function at different ratios compared with Tregs isolated from EAP (Fig. 6).

Secondly, Treg cells (3×10^5) were isolated from the EAP and EAP+AZA groups of mice, and then were adoptively transferred to EAP mice on day 35. aTreg was isolated from the EAP group and bTreg was isolated from the EAP+AZA group. We found that, by injection of Treg cells, inflammatory cell infiltrations within the prostate were ameliorated, especially $CD4^+$T cell and $CD8^+$T cell, while EAP+bTreg improved much more than EAP+aTreg (Fig. 7). Meanwhile, significantly increased expression of Foxp3 was observed in prostate tissues of EAP+aTreg and EAP+bTreg compared with the EAP group. And EAP+bTreg increased much more than EAP+aTreg (Fig. 7). The results indicated that Treg cells which were adoptively transferred into EAP mice could play an anti-inflammatory role *in vivo*. But the anti-inflammatory effect of aTreg rescue did not show such improvement as bTreg. Collectively, we concluded that the immunosuppressive function of Tregs was impaired by the abnormal hypermethylation of Foxp3 promoter in EAP.

DISCUSSIONS

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) affects adult males of all ages which is characterized by pelvic pain and irritative voiding symptoms, without evidence of urinary tract infection. Due to the unknown etiology of CP/CPPS, most medical treatments are empiric and not effective on it. Cumulative evidence obtained from both human disease and animal models indicated that the abnormal immune function may trigger chronic inflammation in the form of autoimmunity against prostate [36–38]. Tregs actively took part in the prevention of autoimmunity and excessive inflammation responses. Breser et al. [18] highlighted that regulatory T cells play an important role in inhibiting excessive inflammation and controlling the development of prostatitis in EAP. But the molecular mechanisms underlying the modulation of Treg remain unclear in EAP and CPPS.

The transcription factor Foxp3 is essential to the differentiation and function of Tregs. And it is a well-characterized marker of Tregs [27, 28, 39]. Despite the important role of Foxp3 in Tregs, many questions remain in regard to the mechanisms by which Foxp3 regulates the function of Tregs. Recently, several researches demonstrated that, by epigenetic modification of some regulator regions within the Foxp3 locus, such as promoter leads to the change of its suppressive phenotype [31, 32]. Some studies showed that Tregs might change the suppressive function by the methylation of Foxp3 promoter in some autoimmune diseases, such as biliary atresia [40]. However, until now, the methylation level of Foxp3 promoter has not been reported in CP/CPPS.

In our study, we found congestion and edema of prostatic stroma, and a large number of inflammatory cell infiltration around the prostatic glandular cavity in the EAP group. As for the infiltrated inflammation cells, Quick ML's studies suggested that the immune infiltrated cells were predominantly composed of $CD4^+$ and $CD8^+$ T cells [41]. This proved that the EAP model was reliable for the study of CP/CPPS. Defective Treg function is associated with some autoimmune and inflammatory diseases. In order to confirm the change of the suppressive function of Treg in the EAP model, we test some cytokines secreted by Treg cells. Expected, the cytokines of TGF-β secreted by Treg were

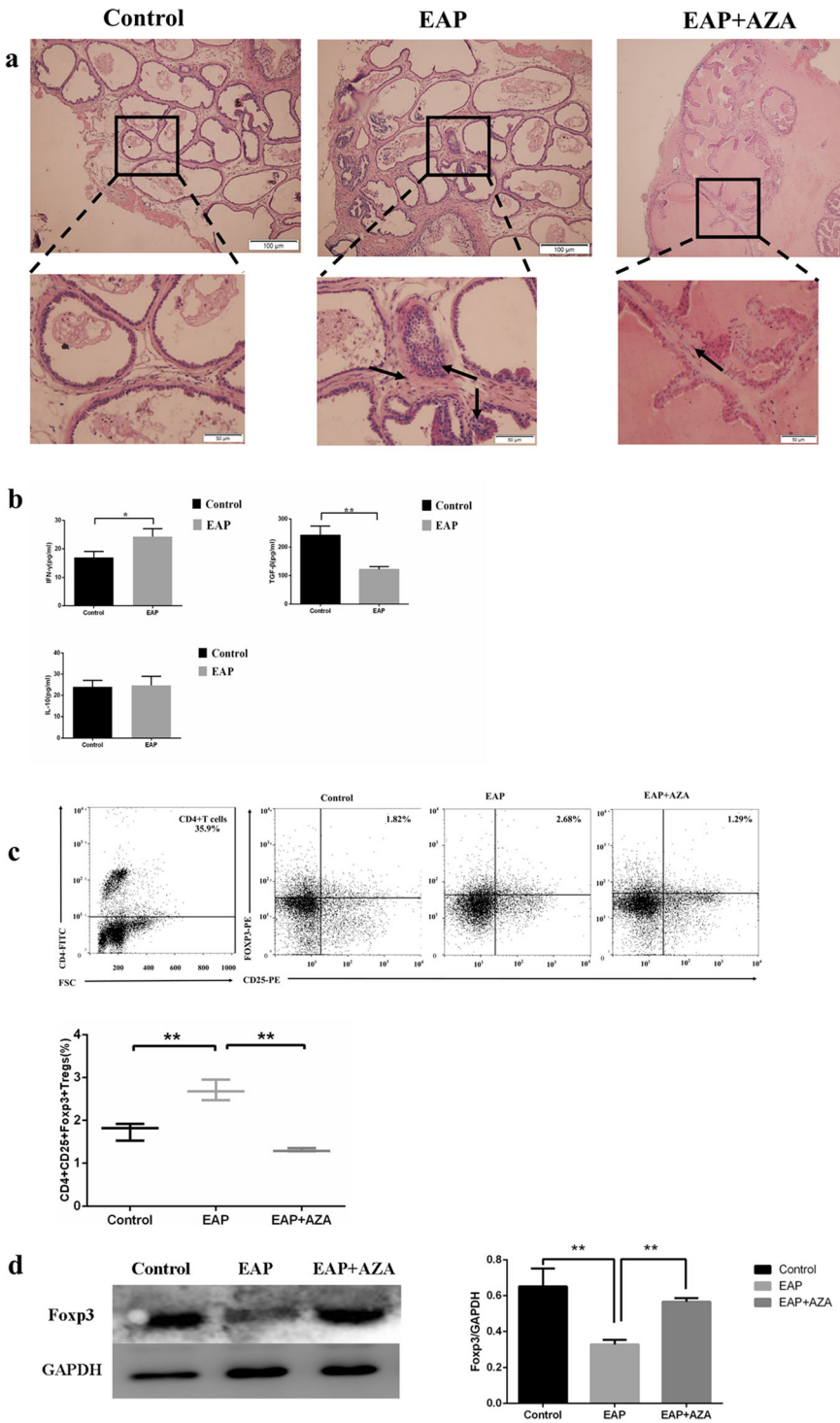

Fig. 5. AZA injected to EAP mice alleviates prostate inflammation, increases the expression of FOXP3. a Histological alterations in prostate tissue of mice from each group (magnification × 100, bar = 100 μm; magnification × 400, bar = 50 μm). b Quantitative analysis of IFN-γ, TGF-β, IL-10 in serum of mice from each group determined by ELISA and reported as mean ± SD. $*P < 0.05$, $**P < 0.01$. c The frequency of $CD4^+CD25^+Foxp3^+$ cells in splenic lymphocytes from each group, respectively. $**P < 0.01$. d The expression of FOXP3 proteins in Tregs isolated from splenic lymphocytes of each group determined by Western blotting. GAPDH was used as the control. $n = 3$ mice per group, $*P < 0.05$, $**P < 0.01$.

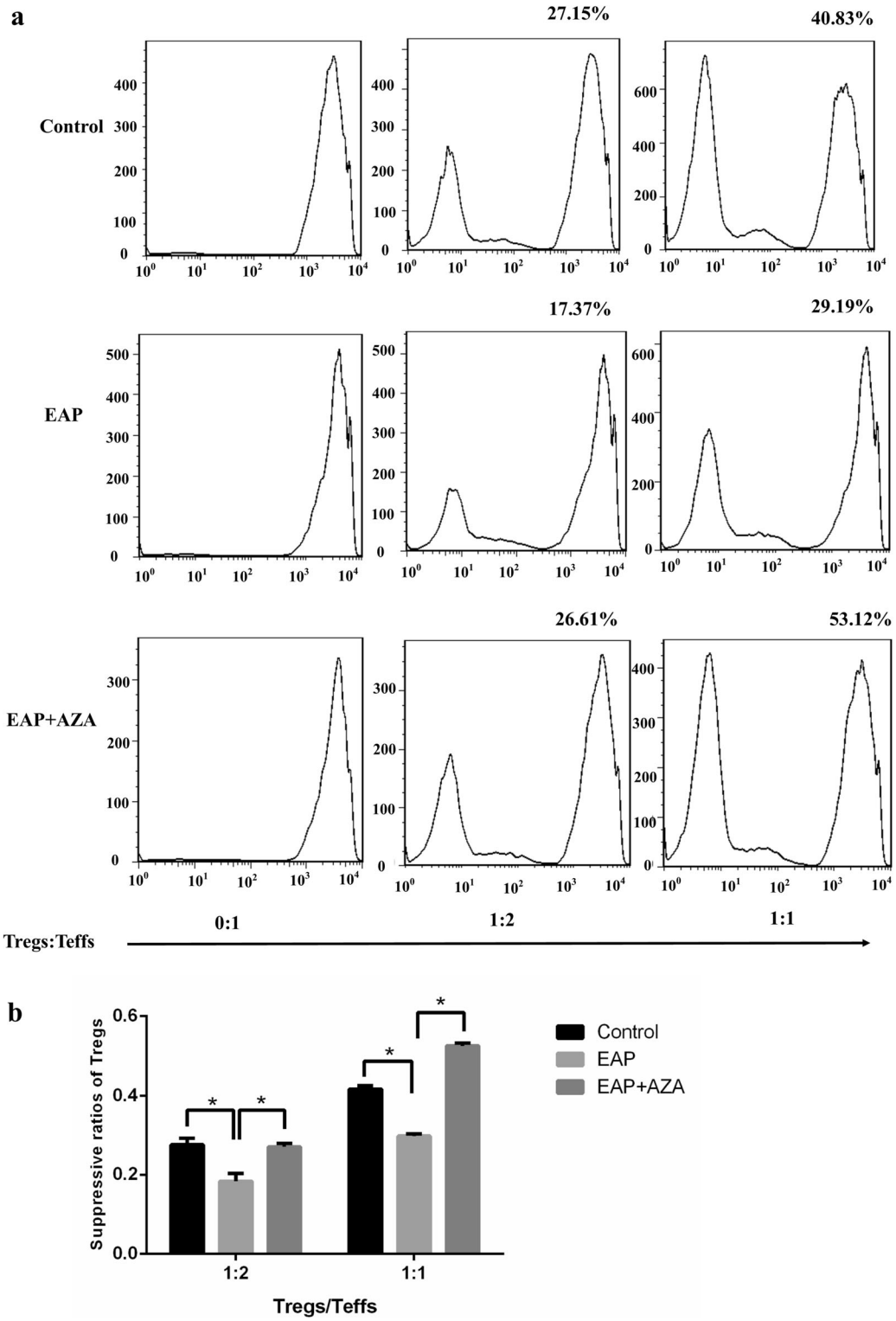

Fig. 6. The immunosuppressive effect of Tregs on Teffs was improved by AZA injection. a Representative image of immunosuppressive effect of Tregs on CFSE-labeled Teffs in each group. The Treg suppressive ratio was computed by the formula: [1 − (PI of Tregs:Teffs-x:2)]/(PI of Tregs:Teffs-0:8) × 100%. b Treg suppressive ratio which was also calculated using the same formula. $n = 3$ mice per group, $^{*}P < 0.05$, $^{**}P < 0.01$.

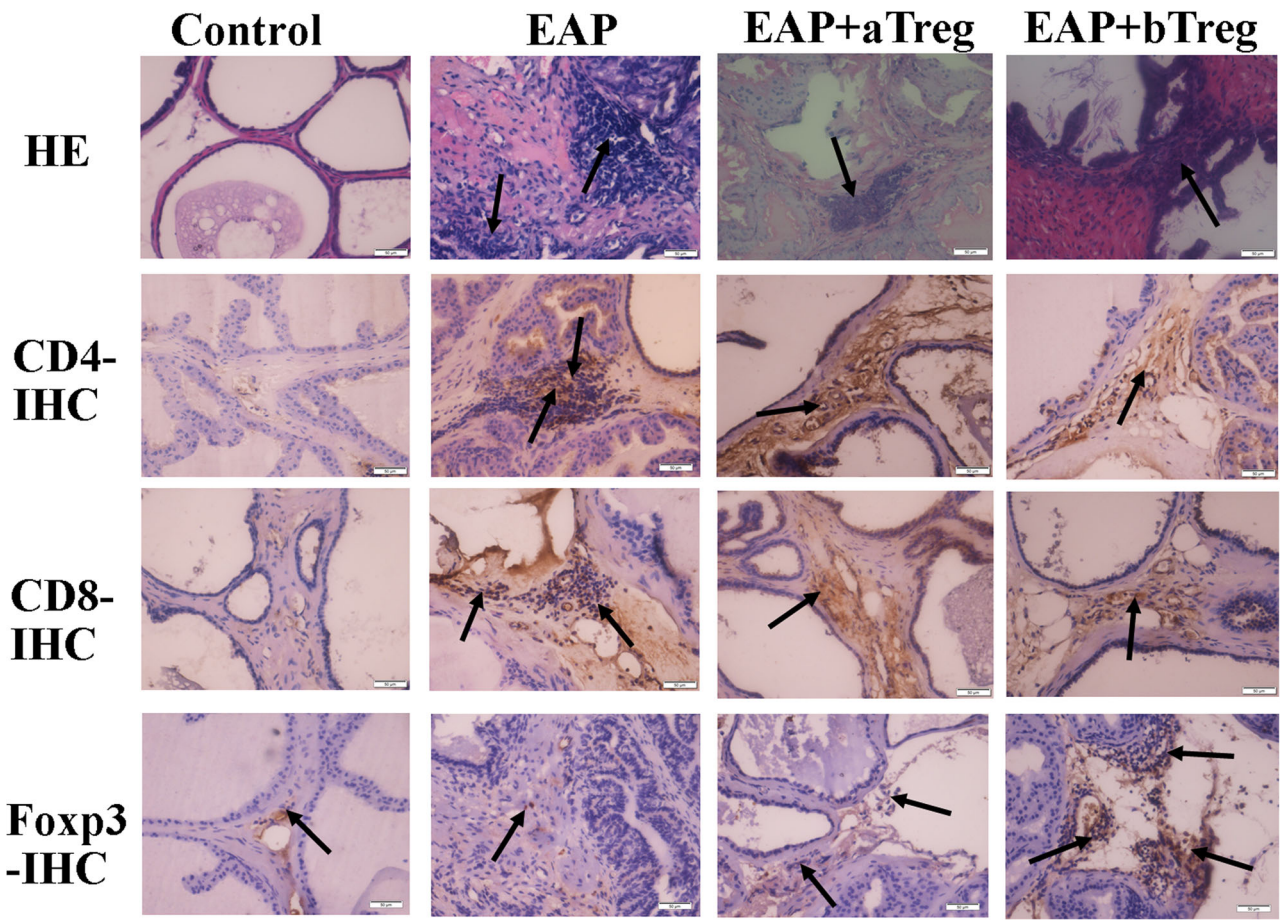

Fig. 7. Treg cells from splenic lymphocytes of EAP+AZA group had better suppressive capability *in vivo*. Representative HE staining and IHC image of prostate tissue from each group (magnification × 400, bar = 50 μm). Arrows in HE represent areas of leukocytic infiltrate in the prostate. Arrows in IHC represent stained CD4, CD8, Foxp3. aTreg were isolated from EAP group. bTreg were isolated from EAP+AZA group. HE is short for hematoxylin and eosin. IHC is short for immunohistochemistry. $n = 3$ mice per group.

observed to decrease in the EAP model group compared with the control group. As the immune negative regulation cytokines, TGF-β, were decreased in EAP mice, we tested the Treg percentage of the EAP model group and control group by flow cytometry analysis. Unexpectedly, we found the Treg percentage of the EAP model group was increased than that of the control group. In order to explore further the suppressive function of Tregs in the EAP group, we then examined the ability of Tregs to suppress Teffs ($CD4^+CD25^-$T cells) by conducting *in vitro* co-culture studies. We found that the suppressive function of Tregs was impaired in the EAP group. This result set us to think why the immune suppressive function of Treg cell was decreased in EAP while the percentage of Tregs in splenic lymphocytes was increased.

Recently, many evidence indicated that aberrant increased methylation status of Foxp3 promoter of Treg cells leads to impaired Treg suppressive function. So, we speculated that the impaired suppression function of Treg may be influenced by hypermethylation of Foxp3 promoter. So, we detected methylation level of Foxp3 promoter of control and EAP mice. We found that the methylation levels of CpG islands in Foxp3 promoter of the EAP model group were elevated compared with those of the control group.

In order to confirm whether it was the elevation of the Foxp3 promoter methylation that caused the impaired suppression function of Treg, we performed intraperitoneal injection of AZA in the EAP group and verified that the methylation percentage of CpG in the Foxp3 promoter region was significantly reduced in the EAP+AZA group compared with the EAP group. Methylation of gene promoter is closely related to gene silencing and results in low expression of the gene [42]. We found the expression of Foxp3 was increased in EAP+AZA group compared with

the EAP group. And the suppressive function of Treg cells was resumed, and inflammation in prostate tissue was obviously reduced by AZA. Treg cells isolated from EAP+AZA group showed much more anti-inflammation function than that from the EAP group.

In summary, our results suggested that the suppressive function of Treg was decreased in EAP mice, and the molecular mechanism may due to hypermethylation status of CpG islands within Foxp3 promoter. Hypermethylation status of Foxp3 promoter leads to low expression of Foxp3, which results in the impaired suppressive function of Tregs in EAP. Our data indicated that enhancing the expression of Foxp3 by reducing the methylation of Foxp3 promoter may serve as a potential therapeutic target for CP/CPPS.

CONCLUSIONS

The results suggest that aberrant increased methylation of Foxp3 promoter of Tregs impairs the suppressive function of Tregs, exacerbating autoimmune inflammatory injury in EAP.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were approved by the Institutional Animal Care and Use Committee of Anhui Medical University.

Conflict of Interest. The authors declare that they have no conflict of interest.

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