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ORIGINAL RESEARCH

IGF1R Promotes Th17/Treg Cell Development in Experimental Autoimmune Prostatitis

Yu Guan[1,*], Shaoyu Yue[1,*], Yiding Chen[1,*], Andong Cheng[1], Hao Li[1], Feixiang Yang[1], Yi Liu[1], Chaozhao Liang[1], Jialin Meng[1,2]

[1]Department of Urology, The First Affiliated Hospital of Anhui Medical University; Institute of Urology & Anhui Province Key Laboratory of Urological and Andrological Diseases Research and Medical Transformation, Anhui Medical University, Hefei, Anhui, 230022, People's Republic of China; [2]School of Life Sciences, Anhui Medical University, Hefei, 230032, People's Republic of China

*These authors contributed equally to this work

Correspondence: Jialin Meng; Chaozhao Liang, Email mengjialin@ahmu.edu.cn; liang_chaozhao@ahmu.edu.cn

Background: Chronic prostatitis is a common urological disorder in young and middle-aged men, characterized by frequent relapses and an unknown etiology. We investigated the potential function of insulin-like growth factor 1 (IGF1) -related ligands in chronic prostatitis in the current study.

Methods: In this study, we established the chronic experimental autoimmune prostatitis mouse model H&E staining was used to assess immune cell infiltration in prostate tissue, while RT-qPCR and Western blot analyses were performed to validate gene and protein expression differences across groups, respectively. Immunofluorescence staining was utilized to determine the spatial distribution of key proteins. Flow cytometry was conducted to analyze the proportions of immune cell populations in different experimental groups. Adeno-associated virus (AAV) was employed to knock down Igflr, and ELISA was used to measure cytokine levels in the peripheral blood of mice. Statistical significance was defined as $P < 0.05$, and all tests were conducted as two-tailed. Data analysis was performed using R software (version 4.2.2).

Results: We successful established the EAP model and discovered that the expression of IGF1R, content of IGF1-related ligands, was highest in prostate tissue and $CD4^+$ T cell subset. Furthermore, protein expression levels of IGF1R were also validated that upregulated in mouse prostate tissue. Colocalization of immunofluorescence suggested that IGF1R protein is highly expressed on $CD4^+$ T cells. Stimulation with desIGF1, a truncated analogue of IGF1, resulted in the significantly increased prostate inflammation and pain scores observed in the EAP+desIGF1 group mouse compared to other groups In vitro study further suggested that desIGF1 could increase the proportion of Th17 cells while decreasing the proportion of Treg cells. In the EAP+AAV-shIgf1r group, the knock down function of igf1r led to the alleviative prostate inflammation and response frequency of pain behavior test. We found that calcium ion associated pathways are active in EAP by bioinformatics, and further validated that PKC-β protein with significantly increased expression noted in the EAP+desIGF1 group, and decreased in the EAP+AAV-shIgf1r group. We also found that the proportion of Th17 cells increased after activation of PKC- β by flow cytometry.

Conclusion: These findings support that PKC-β associated pathways mediated by IGF1/IGF1R axis may impact Th17 cell differentiation and exacerbating prostate inflammation in EAP mouse, providing new molecular targets for the clinical therapeutic strategy.

Keywords: IGF1R, Th17/Treg cell, prostatitis

Introduction

Prostatitis is the most prevalent prostate disorder among adult males, with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), type III prostatitis by NIH-III categorization, accounting for approximately 90% to 95% of all cases. CP/CPPS always with a prolonged course and displays a high recurrence rate, significantly impacting patients' physical health, contributing to the sexual dysfunction and mental health disorders, and ultimately leading to a substantial decline in their quality of life.[1] The most prevalent symptoms are abnormal urination and painful, including dysuria

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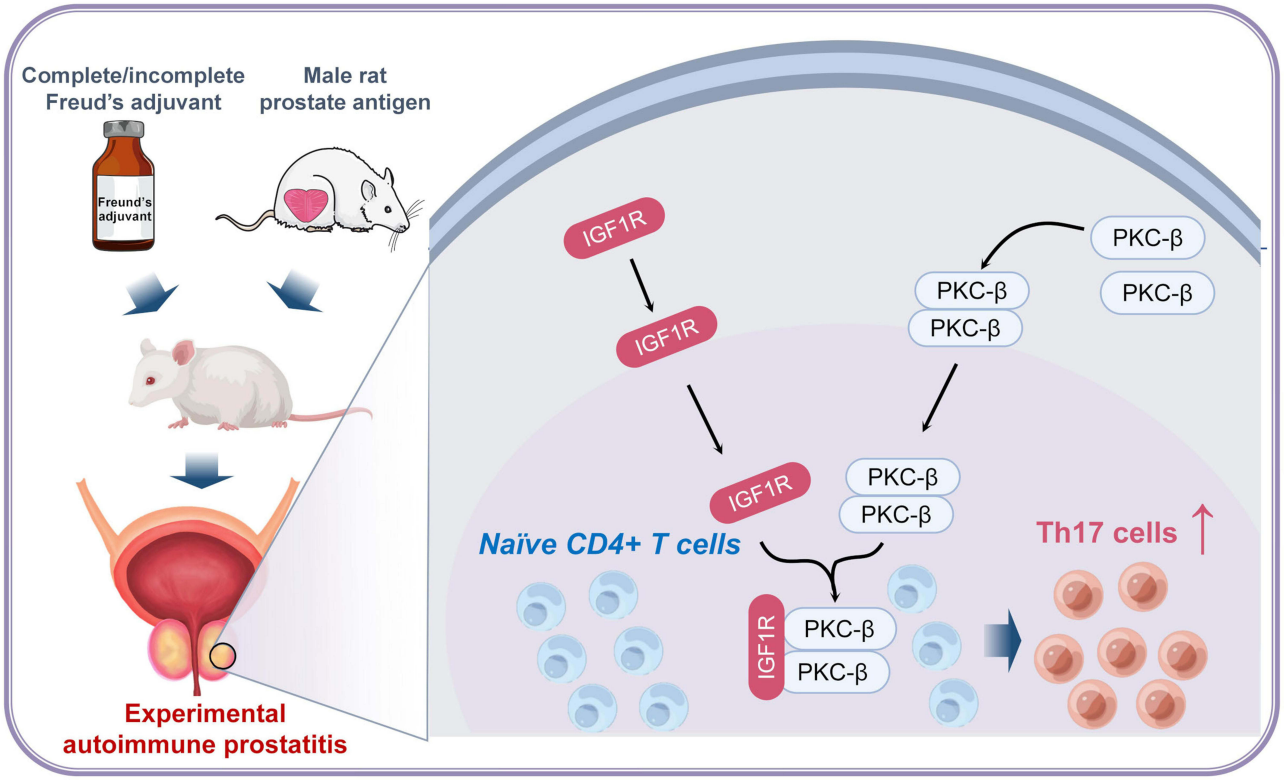
Graphical Abstract



(painful urination), nocturia, urgency, as well as perineal discomfort including suprapubic pain, penile or testicular pain during sexual intercourse or ejaculation. Recent years, there has been no universally accepted cause for the pathogenesis and progression of CP/CPPS. Several hypotheses have been proposed, including autoimmune responses, sustained inflammatory states, neuroinflammatory oxidative stress, pathogen-host specific factors, pelvic floor myalgia, and variations in systemic stress sensitivity.[2] In the previous study, our group reported that calcium signaling pathway may be an important pathway to promote the development of CP/CPPS.[3] Literature review showed that IGF1R in mouse DRG neurons can function to stimulate the increased expression of voltage-gated T-type $Ca2^{+}$ (CaV3) channels through a signal-dependent mechanism dependent on the isotramer G protein, and then activate the differentiation of $CD4^{+}$T lymphocytes, the most important of which is PKC-β.[4]

T lymphocytes—particularly $CD4^{+}$ T cells are essential to the pathophysiology of CP/CPPS.[5,6] Under the stimuli, initial $CD4^{+}$T may develop into many subpopulations, including regulatory T regulatory cell (Treg), type 1 T helper cell (Th1), type 2 T helper cell (Th2), helper T cell 17 (Th17), and other cells. The Th17 and Treg cells have opposing effects on one another, yet they work together to keep the body's immune system functioning.[7] For instance, naïve $CD4^{+}$ T cells develop into Th17 cells when interleukin IL-6 or IL-21 are present together with TGF-β; in contrast, TGF-β induces differentiation into Treg cells when proinflammatory cytokines are absent.[8]

The insulin family encompasses a diverse array of components, including insulin itself, insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2), along with their respective receptors (IR, IGF1R and IGF2R) and binding proteins. Importantly, there exist homologous sequences and structural similarities among these ligands that further underscore their intimate connection and interdependence in biological function.[9] IGF1's physiological functions encompass a wide range of activities such as stimulating cellular division, promoting cell differentiation and migration, facilitating growth processes, inhibiting cellular apoptosis, as well as regulating gene transcription and other pivotal

biological processes.[10] Apart from directly influencing cancer cell survival and proliferation, the IGF1/IGF1R axis may have significant implications in modulating the immune response within the tumor microenvironment (TME).[11] Some researcher reported that IGF1R inhibitor effectively prevents development and progression of thyroid eye disease, an autoimmune disorder. This discovery has facilitated the development of new target-based therapies by blocking pro-inflammatory cytokine receptors, lymphocytic infiltration or IGF1R.[12] Immune response and inflammatory reaction play essential roles in the progression of Diabetic kidney disease (DKD), the IGF signaling pathways and Igf1- Igf1r receptor ligand pairs might occupy essential place in the occurrence and progress of DKD.[13]

In this study, we established experimental autoimmune prostatitis (EAP) model and investigated the impact of IGF1/IGF1R on Th17/Treg balance in EAP mouse. Our findings suggest that the additional desIGF1 stimulating exacerbated prostate inflammation and led to an increase level of inflammatory components in the peripheral blood of the mouse, as well as the fraction of Th17 cells. Knocked down of Igf1r can block the above process. In addition, the key component of calcium associated pathway, PKC-β, might be the downstream of IGF1/IGF1R axis in EAP.

Material and Methods

Animals

Male non-obese diabetic mouse (NOD), aged 5 weeks and weighing 19 ± 2 grams, were obtained from the Nanjing Model Animal Center in China and housed in the Specific Pathogen Free (SPF) Laboratory Animal Room at Anhui Medical University. All procedures involving animals were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and approved by the Animal Welfare and Ethics Committee of Anhui Medical University (Approval No. LLSC20232158; LLSC20211051).

Mouse Model of EAP

In our previous study, we extensively described the establishment of an EAP mouse model. Briefly, we procured NOD mouse from the company and housed them in the SPF animal room, with 5 mouse per group. They were routinely fed for one week to acclimatize to the environment. Subsequently, the mouse in the EAP group underwent subcutaneous immunization at three sites: the caudal root (0.050 mL), bilateral foot pads (0.025 mL per side), and shoulder joint (0.050 mL). The immunizing substance was composed of a male rat prostate extract blended with complete Freund's adjuvant (CFA, Sigma - Aldrich). Two weeks after the initial immunization, the mouse were re-immunized at the same sites with equal amounts of prostate extract and incomplete Freund's emulsion adjuvant (IFA, Sigma - Aldrich). Control mouse were administered 0.9% saline instead of the prostate antigen. The mouse were sacrificed two weeks after the second immunization for subsequent experiments. Mouse were tested for pelvic pain by VonFrey (North Coast Medical, Encinitas) before sacrifice. Von Frey mechanical allodynia tests were conducted on days 0, 10, 20, 30 and 40 using filaments with strengths of: 0.04g, 0.16g, 0.40g,1.00g and 4.00g respectively for each measurement point. A positive response to pain was considered when the following three responses were present: (a) violent abdominal contractions, (b) immediate licking or grasping of the site stimulated by the filament, and (c) jumping.[14]

Hematoxylin-Eosin Staining, Immunohistochemistry and Immunofluorescence Staining

After euthanizing the mouse, the prostate was fixed in a 10% formalin solution. Subsequently, the tissue was embedded and sectioned using paraffin wax. Hematoxylin-eosin (HE) staining was employed to determine the presence of prostate inflammation. The severity of EAP was evaluated by determining the histological score. The assessment was conducted in a double-blind manner. Specifically, a separate histological score was assigned to six high-power fields, with the results presented as a mean value rounded to an integer. The detailed scoring criteria are as follows: The degree of inflammation was graded on a scale of 0–3. A score of 0 indicated no inflammation. A score of 1 signified mild yet definite perivascular cuffing by mononuclear cells. A score of 2 represented moderate perivascular cuffing with mononuclear cells. A score of 3 denoted marked perivascular cuffing, accompanied by hemorrhage and the presence of numerous mononuclear cells in the parenchyma.[15]

Immunohistochemical and Immunofluorescence staining was also conducted on the paraffin-embedded sections. The sections were first dewaxed in xylene solution and subsequently incubated in ethanol solutions of varying concentrations. Antigen repair solution containing EDTA was used, followed by quenching of endogenous peroxidase activity. Primary antibodies targeting specific proteins including: IGF1R (ab182408; 1:1000 dilution; Abcam, Cambridge, MA, USA), PKC-β (12919-1-AP;1:1000 dilution; Thermo Fisher scientific, Waltham, MA, USA), CD4 (ab133616, ab288724; 1:100 dilution; Abcam, Cambridge, MA, USA), CD3 (ab16669; 1:150 dilution; Abcam, Cambridge, MA, USA), IL-17A (ab302922, 1:500 dilution; Abcam, Cambridge, MA, USA), IL-10 (ab313401, 1:100 dilution; Abcam, Cambridge, MA, USA), IGF1R (AF6125, 1: 100 dilution; Affinity Biosciences, Jiangsu, China) were applied to detect target proteins. The sections are incubated overnight with the corresponding primary antibody at 4°C. Subsequently, the appropriate secondary antibody is used, and incubated for 2 hours in a dark environment at room temperature. Finally, digital biopsy scanner (Pannoramic MIDI, 3DHistech, Hungary) was utilized for imaging the sections.

Flow Cytometry Analysis

The ratio of Th17 and Treg cells was quantified using flow cytometry. Following euthanasia, the spleens were completely excised from the mouse and ground in phosphate buffer (PBS). The resulting suspension was treated with red blood cell lysate and washed twice with PBS solution. Subsequently, the cells were transferred to a flow tube. Two surface antibodies, FITC-CD4 (rat anti-mouse, BD, 553046, USA) and APC-CD25 (rat anti-mouse, BD, 557192, USA), were added for incubation. After washing again with PBS, phorbol 12-myristate 13-acetate (PMA), lonomycin, and monensin's medium 1640 (Gibco) from MultiSciences China were added and incubated at 37 degrees Celsius to stimulate the cells. This was followed by cell membrane permeabilization and fixation. Intracellular antibodies PE-IL17A (rat anti-mouse, BD, 559502) and PE-Foxp3 (rat anti-mouse, BD,563101) were then added for incubation at 4°C for one hour. After final cleaning steps and resuspension of the cells, the FACSCalibur flow cytometer (Beckman Coulter) was used for analysis, and data processing software was employed to analyze the obtained results.

Isolation and in vitro Differentiation of Naive $CD4^+$ T Cells

Using the method previously reported by our team, initial $CD4^+$T cells were isolated from the above-mentioned mouse spleen lymphocytes and Th17/Treg cells were differentiated in vitro. Initial enrichment of $CD4^+$T cells in splenic lymphocytes was performed using a $CD4^+$T cell isolation kit (Miltenyi, 130-104-454, Germany) and LS column (Miltenyi, 130-042-401, Germany) with an accompanying magnetic apparatus. The sorted cells were cultured in 24-well plates with 500mL of 1640 complete medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin solution) added to each well for 3–5 days. We stimulated the cells with IL-6 (40 ng/mL; Novoprotein, CG39), TGF-β1 (1 ng/mL; Novoprotein, CA59), and IL-23 (40 ng/mL; Novoprotein, CS31) to induce chemotaxis towards Th17 cells. We also used TGF-β1 (3 ng/mL; Novoprotein, CA59), IL-2 (20 ng/mL; Novoprotein, CK24), anti-IFN-γ (10 μg/mL; Bio X Cell, BE0055), and anti-IL-4 (10 μg/mL; Bio X Cell, BE0045) to promote chemotaxis into Treg cells. Added to the subsequent experiments that desIGF1 (IGF1R activator at a concentration of 10ug/mL, cat.CR39, novoprotein, China), GO6983 (PKC-β inhibitor at a concentration of 7 nM, cat. HY-13689, MedChemExpress, China), Prostratin (PKC-β activator at a concentration of 12.5 nM, cat. HY-107421, MedChemExpress, China) to study the effects of IGF1R and PKC-β on Th 17 differentiation.

Adeno-Associated Virus Infection

Adeno-associated virus was utilized as a vector for the inhibition of the *Igf1r* gene, while AAV-Igf1r and an empty vector (AAV-Control) were employed as controls. The viruses were obtained from Hanbio Biotechnology Co., LTD. (Shanghai, China, HH20230720WY-AAV01). The target sequence was *GATCCG CCACCCTGGTCATCATGGAACTAATTTCAAGAGAATTAGTTCCATGATGACCAGGGTGGTTTTTTG*. Following strict technical instructions, we transfected the AAV and confirmed inhibition through Western blotting. Subsequently, during the second immunization of the mice, AAV was administered via a single injection through the mouse tail vein at a $1*10^{11}$ vg. One week later, the same method was injected again once. An empty vector was used as a control. Two weeks post - injection in vivo, the mouse were sacrificed.

RNA Isolation and RT-qPCR

TRIzol (Invitrogen, USA) was used to extract total RNA from tissues or cells. The PrimeScript RT reagent kit with gDNA Eraser (RR047A, TaKaRa) was then used to synthesis cDNA, which was then reverse transcribed using the TB Green Premix Ex Taq II kit (RR82 WR, TaKaRa). Sangon Biotech Co., Ltd. (Shanghai) produced all of the primers (Table S1). An internal standard, mouse GAPDH enzyme, was utilized, with a volume of 20 μL in per tube. During data processing, the relative expression was applied to every sample individually.

ELISA

Using an enzyme-linked immunosorbent assay (ELISA), we were able to ascertain the amounts of cytokines, including IL-17A, IL-17F, and IL-10, in the medium's supernatants. Furthermore, ELISA was used to measure the serum concentrations of the aforementioned cytokines. The manufacturer's protocol was followed when using the respective ELISA kits (Elabscience, E-EL-M0047 for IL-17A, E-EL-M0046 for IL-10, China, and ThermoFisher scientific, BMS6020 for IL-17F, USA).

Western Blot

Protease inhibitors (Beyotime, China) together with RIPA lysate (Thermo Scientific, USA) were used to lyse the cultured tissues. After electrophoresis separation, proteins were transferred onto polyvinylidene fluoride membranes. The IGF1R antibody (ab182408; 1:1000 dilution; Abcam, Cambridge, MA, USA) was then incubated on the membranes for an additional hour at 4°C. As a loading control, β-actin (MA1-140, 1:5000 dilution; ThermoFisher Scientific, Waltham, MA, USA) was used. Following a one-hour incubation period with a matching secondary antibody (anti-rabbit or anti-mouse IgG, 1:5000, Elabscience), the membranes were once again washed in TBST. An enhanced chemiluminescence (ECL) device (ChemiScope 5600; Hengmei Technology, China) was used to observe the protein bands.

Bioinformatic Analysis

The RNA sequencing data of the EAP model (n=5) and control (n=5) were detailed in our previous study.[16] Using the "limma" package in R, we rigorously identified differentially expressed genes (DEGs). The criteria for DEG selection were set at an absolute log2 (fold-change) exceeding 2, along with an adjusted P-value below 0.05. For an extensive Gene Ontology (GO) analysis, we employed the "org.Hs.eg.db" and "msigdbr" packages. To further explore gene functionality and pathway enrichment, we utilized the "ClusterProfiler" tool.[17] Moreover, we assessed the functional potential of these DEGs using the Metascape online platform (https://metascape.org/). We employed the single-sample gene set enrichment analysis (ssGSEA) to meticulously examine the tumor infiltration dynamics of 28 distinct immunocytes, subsequently deriving an individualized infiltration score for each immunocyte subtype across every patient.[16] To examine differences between subgroups characterized by continuous variables, we performed a *T*-test. Statistical significance was defined as $P < 0.05$, and all tests were conducted as two-tailed. All analyses were performed using R software (version 4.2.2).

Statistics

The Mean ± SD is used to display all findings. The statistical analysis was carried out utilizing IBM Corp.'s SPSS 21 software (USA), employing two-tailed Student's t-tests or analysis of variance (ANOVA) to ascertain group differences. P-values less than 0.05 denoted statistical significance.

Results

Successful Establishment of EAP Mouse Model

According to the EAP mouse modeling method mentioned above, NOD mouse were selected for EAP modeling in the experimental group (n=5). Compared to the control group, mice in the EAP group showed a significant increasing prostate inflammation (Figure 1A). Results from Von Frey mechanical allodynia tests showed differences between EAP and Control groups on the tenth day after the initial immune modeling, both tingling response rate and intensity gradually

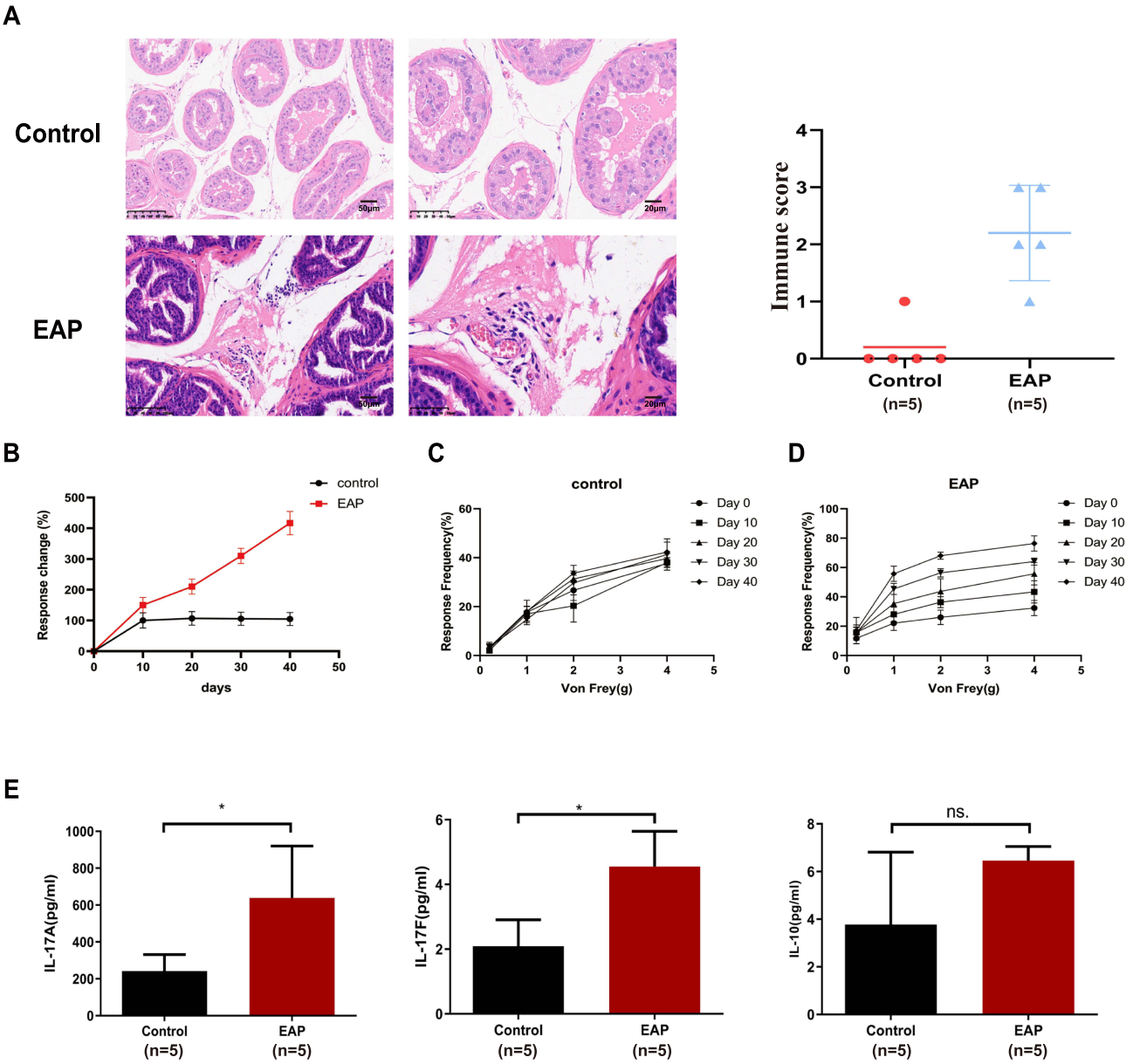


Figure 1 Successful construction of CNP mouse model. (**A**) HE staining was used to evaluate the inflammatory cell infiltration and inflammatory score in the prostate tissue of mouse (200X or 400X). X axes: group, Y axes: immune score; n=5; (**B**) Rate of change in response to the pain measurement experiment in two groups at different time points. n=5; (**C**) Control group's response frequency to the change of pain measurement degree at different time points. n=5; (**D**) EAP group's response frequency to the change of pain measurement degree at different time points. n=5; (**E**) IL-17A, IL-17F and IL-10 differential expression of cytokines in the EAP and Control groups. n=5. *: $p<0.05$; ns: no significance.

Abbreviation: EAP, experimental autoimmune prostatitis.

increased over time in the EAP group (Figure 1B–D). Expression levels of IL-17A (241.9±89.70 vs 638.9±281.7 pg/mL, $P < 0.05$) and IL-17F (2.091±0.8149 vs 4.554±1.089 pg/mL, $P < 0.05$) increased in the peripheral blood of EAP group but no significant difference was observed in IL-10 (3.779±3.040 vs 6.454±0.5951 pg/mL) (Figure 1E).

The Expression of Igf1r Was Significantly Increased in Prostate Tissue and Th17 Cells of EAP Mouse

The gene expressions of IGF1-related ligands Igf1r, Igfbp4, and Igf2r were quantified using RT-qPCR in the prostate tissues of both the EAP and the Control groups. It was observed that among several ligands, Igf1r exhibited the most significant increased expression in the EAP group (Figure 2A). Flow cytometry was employed to separation Th1, Th2,

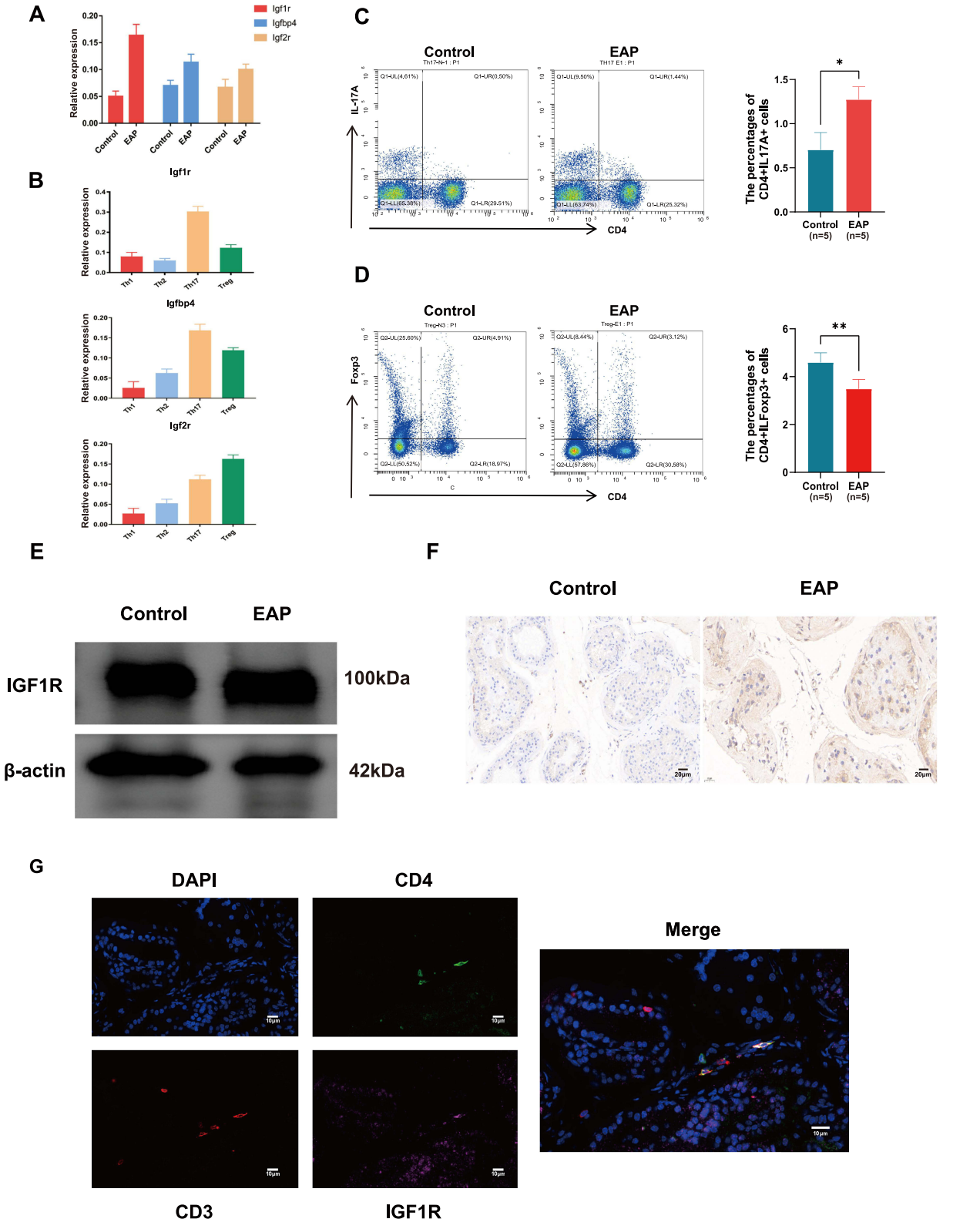


Figure 2 IGF1-related ligands and IGF1R's expression in EAP and Control groups. (**A**) The gene expressions of IGF1-related ligands Igf1r, Igfbp4, and Igf2r were quantified using qPCR in the prostate tissues of both the EAP group and the Control group (n=5); (**B**) Flow cytometry was employed to screen Th1, Th2, Th17, and Treg cells while assessing the expression levels of Igf1r, Igfbp4, and Igf2r in these cell types (n=5); (**C**) The ratio of Th17 cells in the spleen of EAP group and Control group was detected by flow cytometry (n=5). (**D**) The ratio of Treg cells in the spleen of EAP group and Control group was detected by flow cytometry (n=5). (**E**) The expression of IGF1R in the prostate tissue of two group was detected by WB; (**F**) The expression of IGF1R in the prostate of two group was detected by IHC (400X). (**G**) Immunofluorescence co-localization experiments were conducted to observe the subcellular localization of the IGF1R protein within the prostate tissue of mouse in the EAP group and to identify the cell types with high IGF1R expression (1000X). *: p<0.05, **: P<0.01.
Abbreviations: EAP, experimental autoimmune prostatitis; WB, Western blot.

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Th17, and Treg cells, and further assessing the expression levels of Igf1r, Igfbp4, and Igf2r in each cell type. The results demonstrated that both Igf1r and Igfbp4 had significantly higher expression levels in Th17 cells compared to other cell types, suggesting their potential involvement in Th17 cell differentiation. Additionally, high expressions of both Igfbp4 and Igf2r were detected in Treg cells (Figure 2B). Previous studies have indicated that the imbalance in Th17 cell differentiation plays a crucial role in promoting CP/CPPS development. In this study, we also observed a significant increase proportion of Th17 cells in EAP group (1.273±0.1447%) compared to the control group (0.7033±0.1955%) (Figure 2C), along with a notable decrease of Treg cell proportion (Control vs EAP, 4.593±0.4061% vs 3.485±0.3957%) (Figure 2D). Subsequently, the expression level of IGF1R protein was detected by WB and IHC technology, and it was confirmed that the expression level of IGF1R protein was up-regulated in EAP group (Figure 2E and F). To identify the specific cells with the high IGF1R expression, we employed immunofluorescence co-localization imaging, we found that IGF1R is expressed in both glands and stroma, especially highly expressed on $CD4^+$ T cells in stroma (Figure 2G).

The IGF1 Analogue Aggravated the Prostate Inflammation of EAP Mouse and Promoted the Transformation of Th17 Cells

To further investigate the role of IGF1R in exacerbating prostate inflammation in EAP mouse, we selected desIGF1, a truncated analogue of IGF1, to stimulate the IGF1/IGF1R axis. Stimulation with desIGF1 resulted in increased infiltration of prostatic inflammatory cells in the EAP mouse model (Figure 3A), and the inflammatory score in the EAP+desIGF1 group was significantly higher compared to other groups (Figure 3B). Furthermore, mouse in the EAP +desIGF1 group exhibited a significant increase response frequency of pain behavior test (Figure 3C), and ELISA analysis revealed a significant elevator levels of IL-17A (EAP+desIGF1 vs Control, 955.9±81.90 vs 314.5±126.8 pg/mL, $P < 0.001$) and a decreased IL-10 level (EAP+desIGF1 vs Control, 0.2587±0.03384 vs 2.799±0.8080 pg/mL, $P < 0.001$) within their peripheral blood (Figure 3D).

To assess how desIGF1 affects Th17 and Treg cell differentiation, we isolated naive $CD4^+$ T cells and cultured them ex vivo. Naive $CD4^+$ T cells were induced to differentiate into Th17 cells by adding low concentrations of TGF-β along with IL-6. Subsequent addition of desIGF1 led to the increase in the proportion of Th17 cells (EAP vs EAP+desIGF1, 1.550±0.06928 vs 1.948±0.03834%, $P < 0.05$, Figure 3E). Additionally, when high concentrations of TGF-β were used to induce differentiation into Treg cells, stimulation with desIGF1 resulted in further reduction of the proportion of Treg cells (EAP vs EAP+desIGF1, 3.762±0.2553 vs 2.582±0.2913%, $P < 0.05$, Figure 3F).

Knockdown of Igf1r Alleviated Inflammatory Phenotype and Decreased Th17 Cell Proportion

In order to assess the significance of Igf1r in the escalation of inflammation in EAP mouse, we developed an AAV virus that knocks down Igf1r. We initially assessed the knockdown efficacy of three AAV viruses in naive $CD4^+$ T cells, these viruses dramatically decreased Igf1r mRNA levels (Figure 4A) and protein levels (Figure 4B). Ultimately, we selected AAV2 for the subsequently investigations. Using animal models, we first administered desIGF1 and AAV-shIgf1r into several groups via the caudal vein. In the prostate tissue of the mouse in the EAP+desIGF1 group, we found a significant increase in inflammatory cell infiltration, whereas in the EAP+AAV-shIgf1r group, we found there was no significant inflammatory cell infiltration (Figure 4C). The quantified inflammatory score also demonstrated this trend (Figure 4D).

Simultaneously, we computed the cell distribution in the spleen of EAP mouse across all groups, and found that Th17 cells were considerably higher in EAP+desIGF1 (1.674±0.07232%) mouse than in EAP+AAV-shIgf1r (0.8280 ±0.06140%) mouse. Simultaneously, the EAP+desIGF1 (3.914±0.3477%) group and the EAP+AAV-shIgf1r (7.448 ±0.08044%) group both showed a large increase in Treg cells (Figure 4E–G). Additionally, multiple immunofluorescence labeling was carried out, and the prostate tissue of mouse in the EAP+desIGF1 group showed many cells of both CD4 red fluorescence and IL-17 green fluorescence, but the EAP+AAV-shIgf1r group did not show any appreciable positive findings (Figure 5A). We then also studied CD4 / IL-10 fluorescent staining, indicating Treg cells, and we found that the positive rate of immune cells in EAP+desIGF1 was lower compared with EAP+AAV-shIgf1r (Figure 5B). These findings

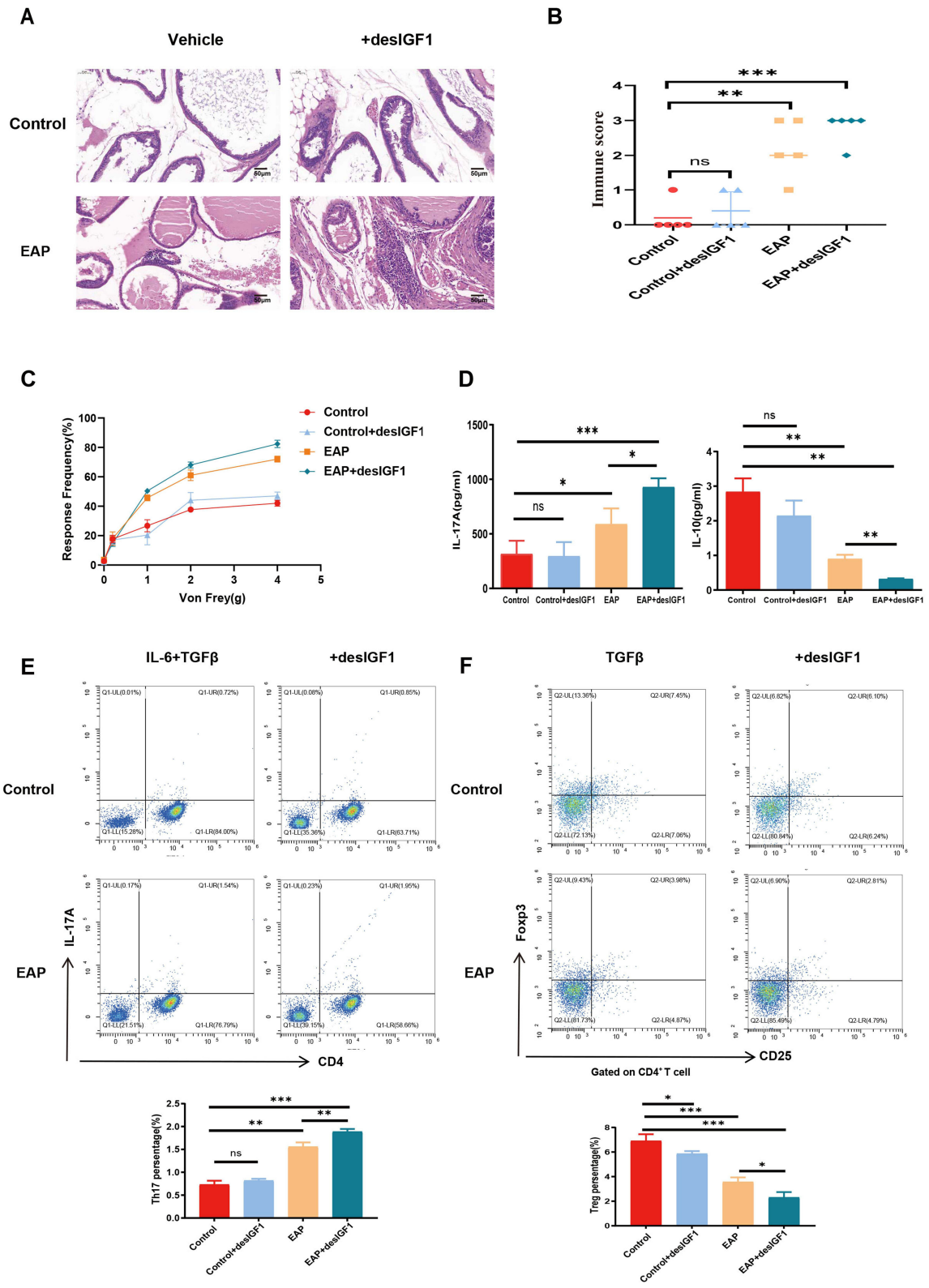

Figure 3 The IGF1R's stimulator desIGF1 aggravates the inflammatory in a mouse model of CNP. (**A**) HE staining was used to evaluate the infiltration of inflammatory cells and stimulation with desIGF1 resulted in increased infiltration of inflammatory cells in prostate tissue in four groups (200X); (**B**) Inflammation score of each group and the inflammatory score of mouse in the EAP+desIGF1 group was significantly higher compared to other groups (n=5); (**C**) The Von Frey pain measurement results of the four groups of mice are shown mouse in the EAP+desIGF1 group exhibited a significant increase in pain response; (**D**) ELISA was used to evaluate IL-17A (n=5) and IL-10 (n=5) and revealed a significant elevation of IL-17A levels and a decrease in IL-10 levels within their peripheral blood; (**E**) desIGF1 promotes the differentiation of naive $CD4^+$ T cells into Th17 cells (n=5); (**F**) desIGF1 inhibited naive $CD4^+$ T cells from differentiating into Treg cells (n=5). *p<0.05, **p<0.01, ***p<0.001.

Abbreviations: EAP, experimental autoimmune prostatitis; desIGF1, IGF1R's stimulator cytokines; ns, no significance.

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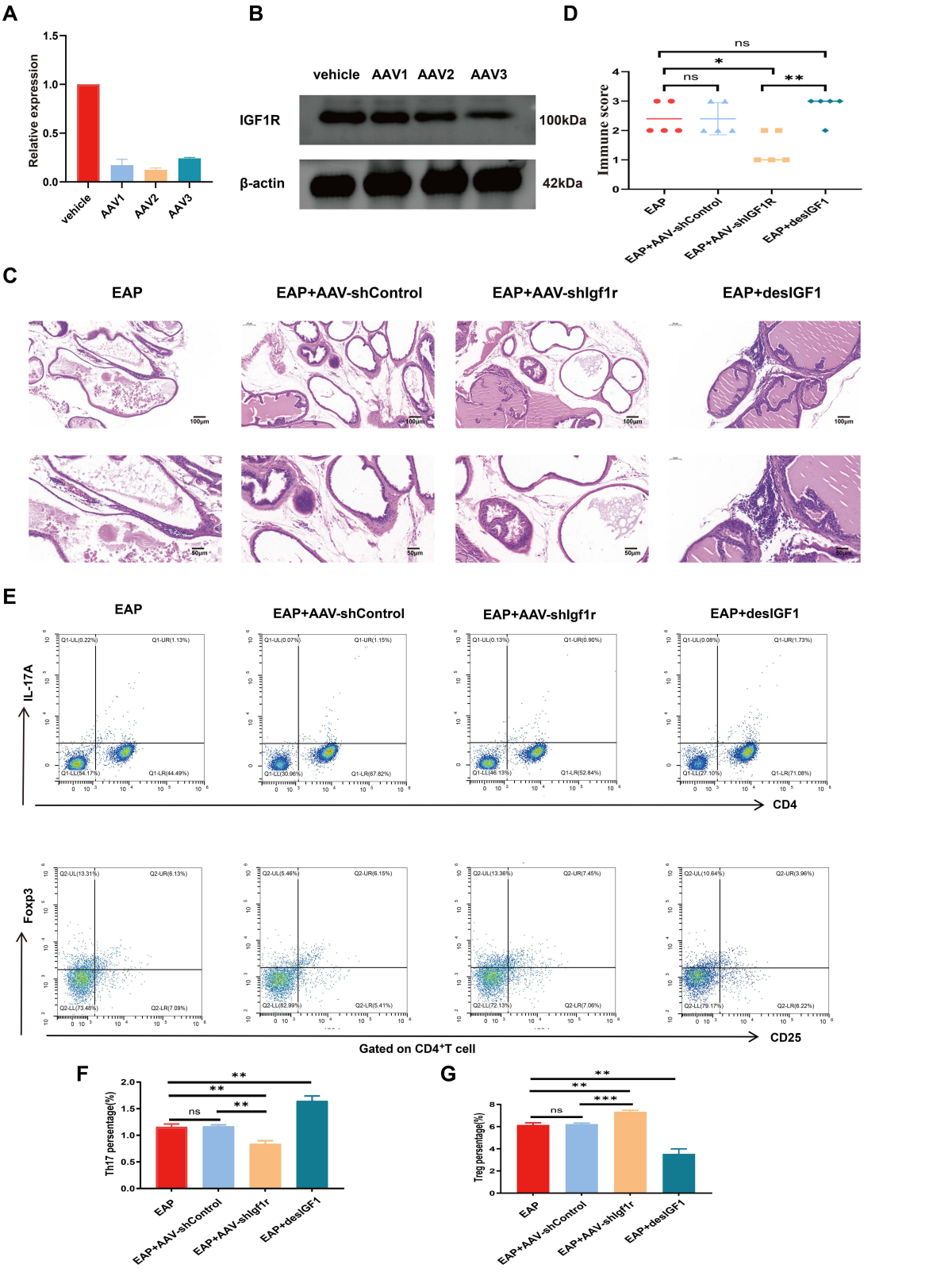

Figure 4 Knockdown of Igf1r alleviates inflammatory phenotype in EAP group. (**A**) We initially assessed the knockdown efficacy of three AAV-Igf1r in naive $CD4^+$ T cells following induction, these viruses dramatically decreased Igf1r mRNA levels; (**B**) WB to evaluate the IGF1R protein level after knockdown by AAV-igf1r; (**C**) Inflammatory cell infiltration in the prostate tissues of 4 group was detected by HE staining, we found a significant increase in inflammatory cell infiltration in EAP+desIGF1 group, whereas in the EAP+AAV-shIgf1r group had no significant inflammatory cell infiltration (n=5) (100X or 200X); (**D**) Inflammation score of each group; (**E**–**G**) desIGF1 and AAV-shIgf1r regulate the proportion and quantification of cell proportion of Th17/Treg cells (n=5); *p<0.05, **p<0.01, ***p<0.001.

Abbreviations: AAV, adeno-associated virus; desIGF1, IGF1R's stimulator cytokines; ns, no significance.

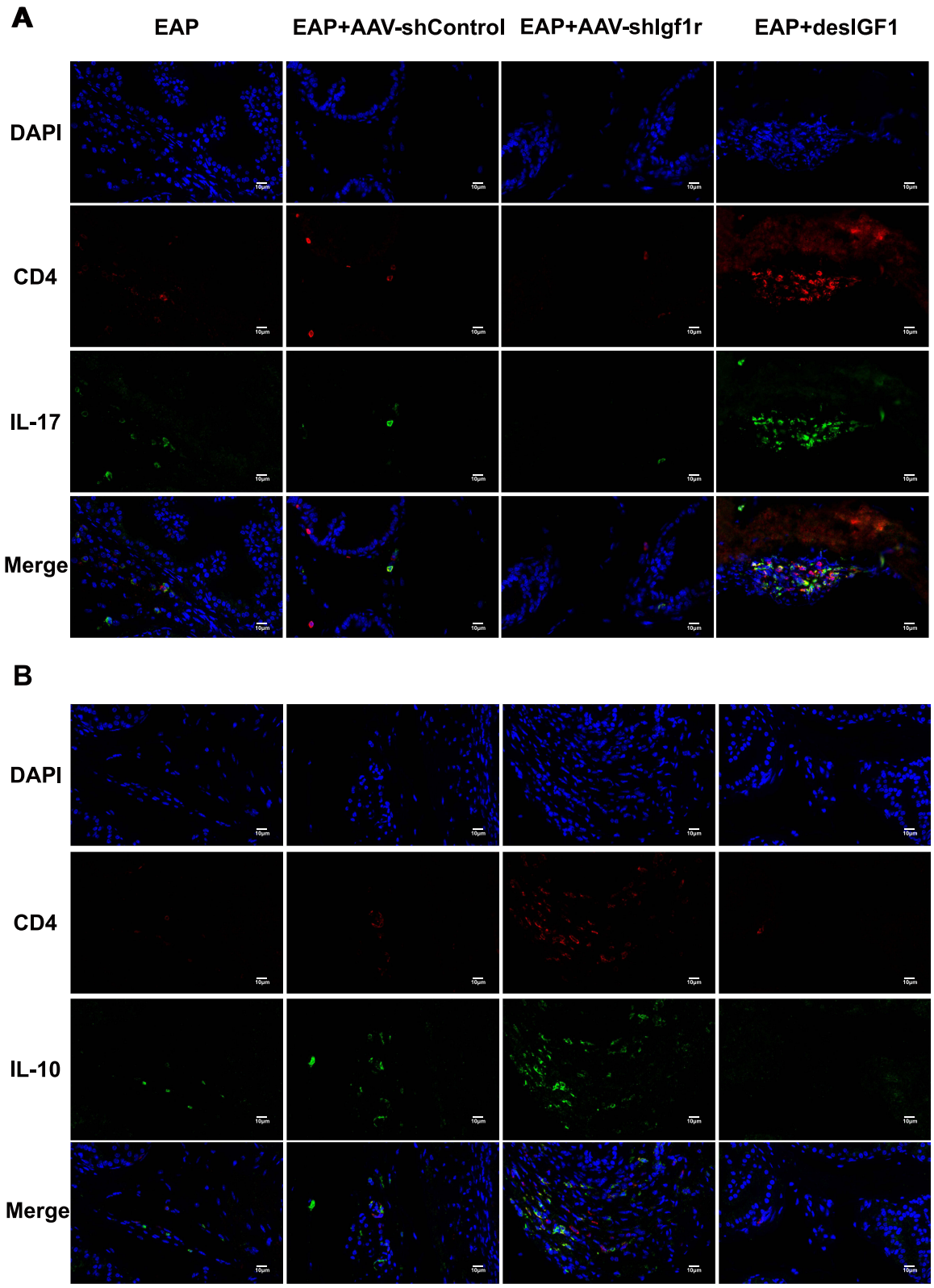

Figure 5 The multiple immunofluorescence staining expression in each groups. (**A**) the prostate tissue of mouse in the EAP+desIGF1 group showed many cells of both CD4 red fluorescence and IL-17 green fluorescence, but the EAP+AAV-shIgf1r group did not show any appreciable positive findings (1000X). (**B**) For CD4 / IL-10 fluorescent staining, and we found that the positive rate of immune cells in EAP + desIGF1 was lower compared with EAP + AAV-shIgf1r.
Abbreviations: AAV, adeno-associated virus, desIGF1, IGF1R's stimulator cytokines (1000X).

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support our theory that Igf1r is crucial for the development of Th17 cell transition and the exacerbation of inflammatory phenotype in EAP mouse.

Calcium Associated Pathways and PKC-β Might Be the Downstream of IGF1/IGF1R Axis

We compared the DEGs between EAP and Control groups, 184 DEGs were filtered, with 169 upregulated in EAP group, and 25 elevated in control group (Figure 6A). We further enriched the potential function of these genes via Metascape, and focused on the impact of regulation of neuroinflammatory response, which might lead to the neuralgia for patient (Figure 6B). After literature review, we found that IGF-1 has been implicated in the modulation of pain sensitivity in primary afferent neurons. It has been reported that IGF1R functionally enhances voltage-gated T-type Ca2+ (CaV3) channels in mouse dorsal root ganglia (DRG) neurons through a mechanism reliant on heterotrimeric guanine nucleotide-binding protein signaling, thereby elevating DRG neuron excitability and increasing pain sensitivity.[18] Therefore, with the ssGSEA, we evaluated the activation of Calcium associated pathways, and revealed that calcium signaling pathway, calcium homeostasis, transport and import pathways showed highly activated status in EAP group, as compare to normal control (Figure 6C). Subsequently, we directly compared the calcium signaling associated gene expression, resulted in the highly expression of Prkcb ($P = 0.0178$), Prkcg ($P = 0.0149$) (Figure 6D). We assessed the protein expression of PKC-β, a crucial component of the calcium signaling system, in the aforementioned animal models to confirm the function of the calcium signaling pathway. In EAP mouse, we saw inflammation in the prostate tissue due to PKC-β. The EAP+desIGF1 group showed a considerable increased inflammation and PKC-β protein level in the immunocytes, however the EAP+AAV-shIgf1r group did not show any significant improvement in results (Figure 6E). In vitro, we observed that the addition of the PKC-β activator, Prostratin, led to an increase in the proportion of Th17 cells, whereas the addition of the PKC-β inhibitor, GO6983, resulted in a decrease in the proportion of Th17 cells (Control vs Control +GO6983 vs Control+Prostratin: 5.308±0.5558 vs 3.084±0.8020 vs 7.972±1.729%)(Figure 6F).

Discussion

IGF1R is one of the most studied kinase targets in tumor research, and it is challenging to modify. According to several research findings, the signaling cascade of IGF1R and its interactions with other cell signaling pathways suggest that it could potentially serve as a promising novel target for cancer treatment.[19] The cell signaling mediated by IGF1R is a well-acknowledged element within the downstream molecular cascade network. This network encompasses other members of the IGF family, receptor tyrosine kinases (RTK), adhesion receptors, and signaling pathways mediated by G-protein-coupled receptors (GPCR). Moreover, these pathways play roles in giving rise to the side effects and constituting resistance mechanisms associated with targeted therapies. Multiple research efforts have demonstrated that the IGF1-IGF1R signaling pathway participates in the regulation of diverse tumor cell processes. It encompass cell cycle progression, apoptosis, and differentiation.[20,21] Furthermore, the signaling pathways associated with IGF1R are implicated in crucial aspects of cancer development, such as cancer metastasis, anchorage - independent growth, tumor angiogenesis, and epithelial-mesenchymal transformation.[22] Radioactivity and chemotherapeutic drug resistance can also result from elevated or activated IGF1R levels.[19] IGF1R is normally expressed on the surface of T cells. Evidently, regulating its activation is of utmost importance for governing T cell development and function. The naïve $CD45RA^+$ T cell subpopulation exhibits a higher level of IGF1R expression compared to the memory $CD45RO^+$ T cell subpopulation. This differential expression pattern suggesting that the IGF1R pathway plays a significant role in T cell generation and differentiation processes.[23] For instance, Bilbao D. et al, in an in-vivo animal model of type 1 diabetes, demonstrated that IGF1 promoted the expression of a group of genes associated with Treg cell proliferation. In in-vitro experiments, IGF1 also inhibited the progression of the disease. A distinct correlation was observed between the quality and quantity of the Treg immunosuppressive response and the activation of IGF1R.[24] It has demonstrated that the IGF1R pathway facilitates the development of Th17 cells in multiple sclerosis, yet it does not promote the differentiation of Treg cells. This effect is attributed to the activation of the AKT/mTOR pathway and the upregulated expression of the aerobic glycolysis pathway.[25] The successful establishment of the EAP model was verified through HE staining, pain assessment,

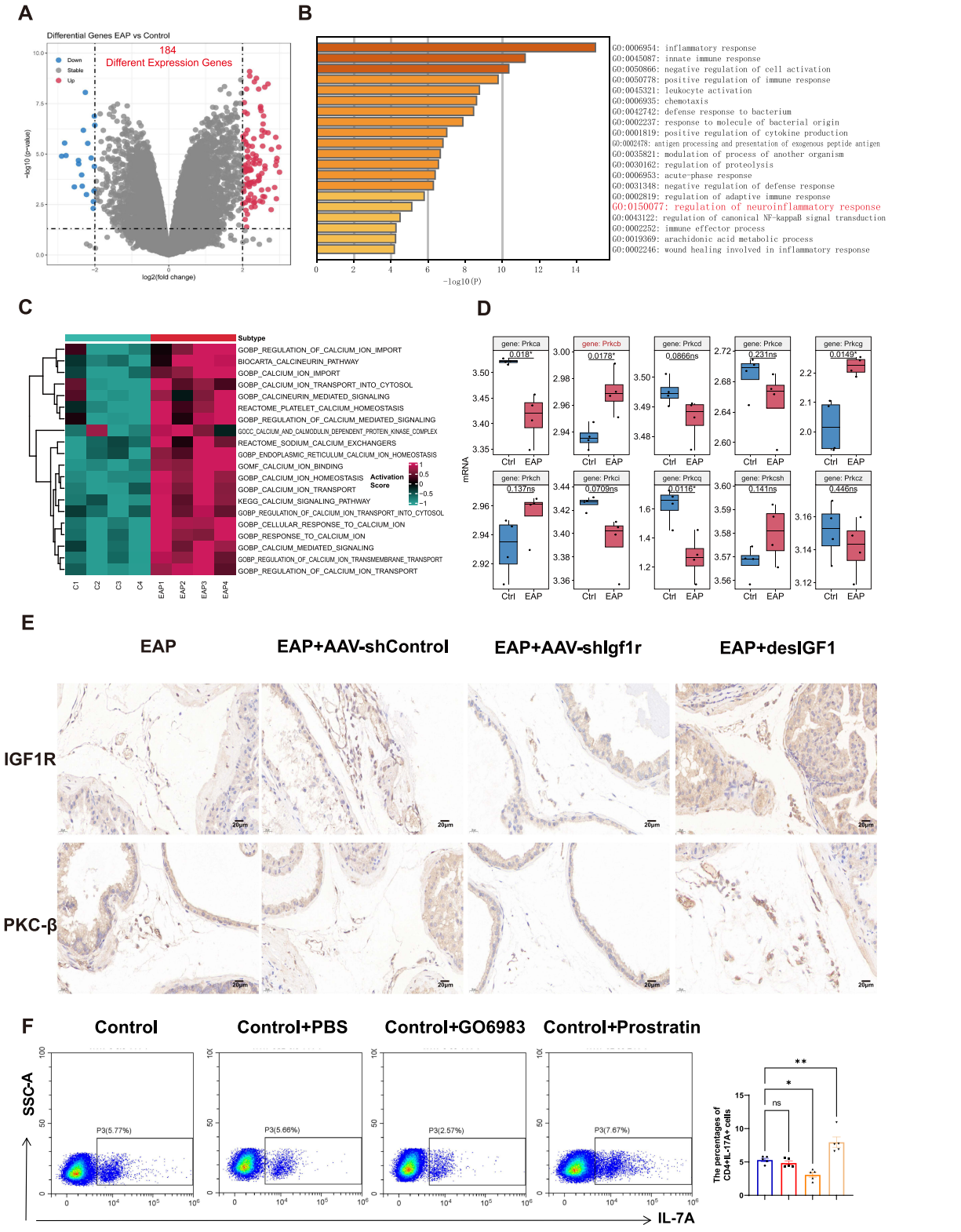

Figure 6 DEGs, signaling pathways, protein and Th17 cells ratio within different groups. (**A**) DEGs between EAP and Control groups, 184 DEGs were filtered, with 169 upregulated in EAP group, and 25 elevated in control group; (**B**) After further enriched the potential functions of these genes via Metascape. The analysis focused on the role of regulating the neuroinflammatory response, which may contribute to neuralgia in patients with CNP; (**C**) We evaluated the activation of Calcium associated pathways with the ssGSEA, and revealed that calcium signaling pathway, calcium homeostasis, transport and import pathways showed highly activated status in EAP group, as compare to normal control; (**D**) Compared the calcium signaling associated gene expression found that the highly expression of Prkcb, Prkcg; (**E**) The expression of IGF1R and PKC-β protein in each treatment group was evaluated by immunohistochemical staining (400X). (**F**) In vitro experiments, we observed that the addition of the PKC-β activator Prostratin led to an increase in the proportion of Th17 cells, whereas the addition of the PKC-β inhibitor GO6983 resulted in a decrease in the proportion of Th17 cells. *p<0.05, **P<0.01.
Abbreviations: DEGs, differentially expressed genes; Metascape, Metascape online platform; ssGSEA, single-sample gene set enrichment analysis; ns, no significance.

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and differential expression analysis of inflammatory factors in peripheral blood. Among multiple ligands, Igf1r demonstrated the most significant variation. Specifically, it showed a marked increase in both the EAP group and Th17 cells, Whereas there was a marked decline in the proportion of Treg cells. We noted that Igf1r had the highest expression level among all ligands in both EAP mouse models and Th17 cells. Additionally, the expression of IGF1R protein was upregulated in mouse prostate tissue.

CP/CPPS of the NIH-III type constitutes the vast majority of prostatitis cases, comprising 90–95% of all such cases.[26] The pathogenesis of CP/CPPS is highly complex, entailing an intricate interaction among immune, endocrine, and neuropsychiatric factors. This complexity renders it markedly different from types I and II prostatitis which are instigated by bacterial infections. However, the autoimmune etiology of CP/CPPS has emerged as a pivotal area of research within the field of urology.[27,28] Studies have demonstrated that crucial role of $CD4^+$T cells in disease development and pain symptoms,[29] with infiltration of $CD4^+$T cells[30] and macrophages[31] observed in animal models. Activated $CD4^+$T cells are capable of differentiating into diverse subtypes under the influence of the local microenvironment. Based on their cellular functions, $CD4^+$T cells can be classified into subtypes such as Th1, Th2, Th17, Treg cells and other subtypes. These distinct cell subtypes work in concert to modulate the local inflammatory response. The autoimmune-mediated damage in CP/CPPS is primarily influenced by Th17 and Th1 cells, along with their associated cytokines IL-17 and IFN-γ. In the presence of TGF-β, IL-6 assumes a pivotal role in the differentiation of $CD4^+$T cells into Th17 cells.[32,33] Recent literature indicates that monotherapy with anti-inflammatory and immunomodulatory agents proves ineffective for CP/CPPS. Nevertheless, short-term NSAIDs may be considered as an element of a multimodal treatment regimen.[34] CPPS is a common condition that substantially impairs patients' quality of life. Owing to the ambiguous and intricate, etiology of CPPS, monomodular therapy has been largely unsuccessful thus far. Consequently, the treatment paradigm for CPPS has transitioned towards a multi-modal approach integrating both pharmacological and non-pharmacological interventions.[35]

In the EAP model, stimulation with desIGF1 led to a marked elevation in the infiltration of prostatitis cells, Moreover, compared with other groups, the pain score of the mouse showed a significant increase. Upon the addition of desIGF1, in vitro culture experiments demonstrated a substantial increase in the number of Th17 cells. Concomitantly, there was a subsequent decline in the proportion of Treg cells. Mice in the EAP+desIGF1 group, following tail - vein injection of desIGF1 and AAV-shIgf1r, exhibited significant inflammatory cell infiltration within their prostate tissue. Conversely, in the EAP+AAV-shIgf1r group, no discernible inflammatory cell infiltration was detected, a finding that was in line with the quantified inflammatory scores. Based on the flow cytometry findings, in the EAP+desIGF1-treated mice, the Th17 cell population demonstrated a significantly increase. Conversely, in the EAP+AAV-shIgf1r-treated animals, there was a notable decrease in the Th17 cell population. In the prostate tissue sections of the EAP+desIGF1 group, co-localization fluorescence of CD4/IL-17 was clearly detected. On the contrary, in the EAP+AAV-shIgf1r group, no significant positive results were observed. These results lend strong support to our hypothesis that IGF1R plays a pivotal role in not only promoting the transition of Th17 cells but also exacerbating the inflammatory phenotype in CP/CPPS.

The subcellular compartment houses protein kinase C (PKC), Which is one of the eleven isoenzymes within the serine-threonine kinase family. Multiple studies have shown that overexpression of PKC-β lead to the transcription of growth-related genes.[36] Mechanosensitive ion channels are commonly used to transfer calcium, which is necessary for many different cellular functions. Studies have shown that increased extracellular stress promotes tumor development by activating the T-type Ca2þ channel Cav3.3. After calcium surge, PKC-β-dependent pathways is triggered, which phosphorylate IKK and IκB and activate and proliferate NF-κB.[37] MandalayAK's group found that specific inhibitors of PI3K, ERK, and PKC significantly affected IGF-1 stimulation of urate transport in oocytes.[38] Furthermore, PKC can be activated by multiple receptor tyrosine kinases that are associated with osteosarcoma. These kinases include members of the ERBB family, such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (Her - 2), and human epidermal growth factor receptor 4 (Her - 4) in osteosarcoma, IGF1R, fibroblast growth factor receptor (FGF), and others.[39] PKC alpha plays a pivotal role in the IGF1-signaling cascade. It is involved in the regulation of crucial signaling proteins that participate in cell - signaling processes and modulate gene expression.[40] Previous research conducted by our laboratory has demonstrated that, within the EAP group, intracellular $Ca2^+$ activated calcium/calmodulin-dependent kinase IV (CaMK4) in Th17 cells. An elevation in the cytoplasmic $Ca2^+$ concentration within Th17 cells is essential for the phosphorylation of CaMK4. Based on a mechanistic investigation,

the inhibition of CaMK4 can reduce the production of IL-17A by decreasing Akt-mTOR phosphorylation. Notably, it serves as an active regulator of Th17 differentiation.[3] We identified PKC-β-induced inflammation in prostate tissue of EAP mouse. In the EAP+desIGF1 group, the expression level significantly increased, while no significantly changed in the EAP+AAV-shIgf1r group. Subsequently, we observed that during in vitro culture, the addition of a PKC-β activator led to an increase in the proportion of Th17 cells, while the addition of a PKC -β inhibitor resulted in a decrease in the proportion of Th17 cells.

In the present study, we carried out a preliminary investigation of the expression of IGF1R in both CP/CPPS patients and EAP mouse models, along with its influence on the differentiation of Th17 cells. Our findings demonstrate a marked upregulation of IGF1R expression in EAP models, especially within Th17 cells. This strongly indicates that the IGF1/IGF1R axis may play a pivotal role in the onset and development of CP/CPPS. Furthermore, our experimental data indicate that the activation of the PKC-β promotes the differentiation of Th17 cells. Moreover, it's expression is reduced following IGF1R knockdown. Thus, we hypothesize that the activation of the PKC-β signaling pathway by IGF1R may represent an important mechanism underlying the promotion of Th17 cell differentiation. However, it is crucial to acknowledge that this study has certain limitations. In the EAP model, we predominantly utilized splenic tissue, which is an immune organ, to evaluate the immune status. Nevertheless, Th17/Treg cells are also present in other immune organs or tissues, like lymph nodes. Therefore, additional research is imperative to confirm whether comparable immune alterations transpire in these organs or tissues. Prospectively, we intend to broaden the research scope to further probe into the role of IGF1R in the pathogenesis of CP/CPPS. Our ultimate objective is to furnish more precise molecular targets for the diagnosis and treatment of CP/CPPS, thereby advancing the understanding and management of this complex condition.

Conclusion

Through the establishment of EAP model, and the application of the techniques including HE, IHC, Western blotting, flow cytometry, lentivirus transduction and other techniques, we validated the up-regulation of IGF1R expression in the prostate tissues and lymphocytes of EAP mouse. This upregulation leads to an increase in the proportion of Th17 cells and decrease in the proportion of Treg cells. Furthermore, by comparing the levels of DEG between EAP and control group, we observed that calcium signaling pathways, calcium homeostasis mechanisms, transport processes, and input pathways were significantly more activated in the EAP group than in the control group. Notably, PKC-β exhibited elevated expression levels in this context and its expression was significantly upregulated following treatment with EAP +desIGF1. PKC-β also promotes the differentiation of Th17 cells. Additionally, its expression is reduced after IGF1R knockdown. Therefore, we speculated that the activation of the PKC-β signaling pathway by IGF1R may serve as an important mechanism for promoting Th17 cell differentiation.

Data Sharing Statement

Data will be available upon reasonable request from the corresponding author.

Ethics Approval and Consent to Participate

All procedures involving animals were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and approved by the Animal Welfare and Ethics Committee of Anhui Medical University (Approval No. LLSC20232158; LLSC20211051).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest, financial or otherwise in this work.

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