Research Paper

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IL-17 is not essential for inflammation and chronic pelvic pain development in an experimental model of chronic prostatitis/chronic pelvic pain syndrome

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Abstract

Pain and inflammation in the absence of infection are hallmarks in chronic prostatitis and chronic pelvic pain syndrome (CP/CPPS) patients. The etiology of CP/CPPS is unclear, and autoimmunity has been proposed as a cause. Experimental autoimmune prostatitis (EAP) models have long been used for studying CP/CPPS. Herein, we studied prostate inflammation induction and chronic pelvic pain development in EAP using IL-12p40-KO, IL-4-KO, IL-17-KO, and wild-type (C57BL/6) mice. Prostate antigen (PAg) immunization in C57BL/6 mice induced specific Th1 and Th17 immune responses and severe prostate inflammation and cell infiltration, mainly composed of $CD4^+$ T cells and macrophages. Moreover, chronic pelvic pain was evidenced by increased allodynia responses. In immunized IL-17-KO mice, the presence of a prominent PAg-specific Th1 immune response caused similar prostate inflammation and chronic pelvic pain. Furthermore, markedly high PAg-specific Th1 immune responses, exacerbated prostate inflammation, and chronic pelvic pain were detected in immunized IL-4-KO mice. Conversely, immunized IL-12p40-KO mice developed PAg-specific Th2 immune responses, characterized by high IL-4 secretion and neither infiltration nor damage in the prostate. As observed in wild-type control animals, IL12p40-KO mice did not evidence tactile allodynia responses. Our results suggest that, as in patients, chronic pelvic pain is a consequence of prostate inflammation. After PAg immunization, a Th1-associated immune response develops and induces prostate inflammation and chronic pelvic pain. The absence of Th1 or Th2 cytokines, respectively, diminishes or enhances EAP susceptibility. In addition, IL-17 showed not to be essential for pathology induction and chronic pelvic pain development.

Keywords: Prostatitis, Chronic pelvic pain syndrome, Inflammation, Autoimmunity, T-helper cells, Pain

1. Introduction

Chronic prostatitis and chronic pelvic pain syndrome (CP/CPPS), or category III prostatitis, accounts for more than 90% of all cases of prostatitis, affecting 10% to 14% of men of all ethnic origins and being the most common urologic morbidity in men younger than 50 years.[8,50] Chronic prostatitis and chronic pelvic pain syndrome is characterized by chronic pelvic pain and signs and symptoms of prostate inflammation in the absence of infection.[27,51] Its etiology is still unknown, and effective treatments are limited. An inflammatory or autoimmune basis for CP/CPPS is a very prominent theory based on substantial evidence from studies in patients and animal models.[44,31] On the one hand, self-reactivity of T cells from CP/CPPS patients to prostatic and seminal plasma proteins has been reported.[1,3,20,28] Also, elevated levels of proinflammatory cytokines in the seminal fluid[2,29,57] and the presence of prostatic intra-acinar T-cell-rich infiltrates[18] in the absence of infection have been described in patients. Remarkably, interferon (IFN)-γ–producing Th1 cells specific to prostate antigens were detected in 34% of CP/CPPS patients,[25] suggesting a Th1 autoimmune response against the prostate as the underlying disease mechanism.[44]

On the other hand, cumulative evidence for an autoimmune basis for CP/CPPS comes from experimental autoimmune prostatitis (EAP) models that have proven to be reliable for the study of CP/CPPS.[31,54] Our laboratory has pioneered the development of EAP, a noninfectious autoimmune animal model of CP/CPPS achieved by the immunization of rats or mice with prostate antigens plus adjuvant. In fact, EAP models mirror the human disease, showing its typical characteristics: the presence of IFN-γ–secreting Th1 lymphocytes specific to prostate antigens, increased levels of cytokines in semen, chronic pelvic pain, and associated prostate tissue inflammation and lesions.[31,44,46,54] Immunized mice develop severe prostate inflammation accompanied with specific Th1 cell–mediated responses.[32] Moreover, IFN-γ–secreting Th1 cells were showed to be crucial in EAP induction.[6,32,37] Also, the expression of the Th1-associated chemokine receptors CXCR3 and CCR5 on prostate-specific effector T cells was shown to be associated with their homing and infiltration of the prostate.[6]

For many years, Th1 cells were deemed responsible for the initiation of several autoimmune diseases. Conversely, Th2 cells were believed to have regulatory properties.[52,4] The discovery of Th17 cells in 2005 revolutionized research in immunology and resolved some inadequacies of the previous Th1-Th2 concept of

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inflammatory autoimmune diseases. Since then, IL-17 produced by Th17 cells has been recognized to play a major role in the pathogenesis of autoimmune diseases that were previously considered as prototypically Th1 mediated, such as experimental autoimmune encephalomyelitis and rheumatoid arthritis, opening new therapeutic perspectives.[52,23] Nevertheless, no data about the involvement of Th17 cells in CP/CPPS have been reported to date. Moreover, the role of IL-17 produced by Th17 cells in EAP is still unclear. Some authors have reported data proposing EAP as a Th1-Th17 mixed autoimmune disease in which Th17 cells would be the main inducers of pain.[33,37,38,40] Nonetheless, conclusive evidence is lacking to support the precise role of IL-17/Th17 cells in CP/CPPS, EAP pathogenesis, and mediating chronic pelvic pain. In this context, we considered it to be most important to study the possible role of IL-17 in EAP, an animal model of CP/CPPS.

We studied the precise role of different Th cell subsets in EAP pathogenesis and chronic pelvic pain development using IL-12p40-KO mice (Th1 and Th17 cell deficient), IL-4-KO mice (Th2 cell deficient), IL-17A/F-KO mice (IL-17A/F deficient), and wild-type (C57BL/6) mice. Our results show that PAg-specific Th1 cells induce prostate tissue inflammation and, in turn, provoke chronic pelvic pain, the hallmark symptom of CP/CPPS. Conversely, IL-17A or IL-17F production of Th17 cells is not required for prostate inflammation and chronic pelvic pain development.

2. Materials and methods

2.1. Mice and antigens

Mouse strains used in this study were IL-12p40-KO,[11] IL-4-KO,[48] IL-17A/F-double KO[14] mutants, and wild-type mice, all on the C57BL/6 genetic background. IL-12p40-KO and IL-4-KO mice were kindly provided by Dr. M.S. Di Genaro (Division of Immunology, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, Argentina). All animals were housed and maintained under specific pathogen free conditions in the Animal Facility of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, and they were studied at the age of 6 to 8 weeks. All animal experiments were approved by and conducted in accordance with guidelines of the Committee for Animal Care and Use of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba in strict accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication 86-23).

The preparation of a mixture of PAg and the purification of prostatein, or prostate steroid-binding protein (PSBP), was performed as previously described.[30] Briefly, PAg extracts were prepared from Wistar rat prostate glands. Pooled glands from 50 rats were homogenized in 0.01 M phosphate-buffered saline (PBS), pH 7.2, with protease inhibitors in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 100,000*g* for 30 minutes, and the supernatant was used as PAg mixture. Protein concentration was determined using the Folin phenol reagent. The preparations were aliquoted and kept frozen at −20°C.

Then, PSBP was purified following the procedure described previously by Chen et al.[7] Briefly, ventral prostates were removed in a sterile manner from Wistar male rats (body weight, 250-300 g) and homogenized in 20 mM Tris-HCl using an Ultra-Turrax homogenizer. The cytosolic fraction was obtained after 60 minutes of centrifugation at 100,000*g* and 4°C. The resultant supernatant was applied onto a Mono-Q fast protein liquid chromatography column. Proteins were eluted with a linear 0% to 60% NaCl gradient in 20 mM Tris-HCl. The protein concentration of each fraction was evaluated by measuring its optical density (OD) at 280 nm. Each fraction was run under nondenaturing conditions in 15% polyacrylamide gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis); then, the gels were stained with Coomassie blue. Fractions containing 2 bands of 18 and 20 kDa (corresponding to both subunits of PSBP) were also run under denaturing conditions to verify their identity. The purity of the PSBP preparation was >95%, evaluated by Western blot using a mouse monoclonal antibody recognizing the C3 polypeptide (kindly given by Dr. P. Bjork, Pharmacia and Upjohn, Uppsala, Sweden), and the preparation was lipopolysaccharide-free tested by Gel clot 0.03 endotoxin units/mL sensitivity (Charles River, Laboratories International, Wilmington, NY).

2.2. Antibodies

Commercially available antibodies used in the different experiments performed and their respective manufacturer were as follows: anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD3 (145-2C11), anti-GR1 (RB6-8C5), anti-IFN-γ (XMG1.2), anti-IL-17A (TC11-18H10), anti-IL-10 (JES5-16E3), and IgG1 isotype controls were purchased from BD Biosciences (San Diego, CA). Anti-CD8a (53-6.7), anti-IL-17A (eBIO17B7), anti-IL-10 (JES5-16E3), and anti-CD45 (30-F11) were purchased from eBioscience (San Diego, CA). Anti-CD45 (30-F11), anti-CD11b (M1/70), anti-Ly6G (1A8), anti-CCR6 (29-2L17), anti-CXCR3 (CXCR3-173), anti-CCR4 (2G12) and anti-CCR5 (HM-CCR5) were purchased from BioLegend (San Diego, CA). Antibodies were allophycocyanin, Fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll-Cy5.5, Alexa Fluor 647, or PE-Cy7 conjugated and were properly combined.

2.3. Experimental autoimmune prostatitis induction and histopathologic score

Six- to 8-week-old male IL-12p40-KO, IL-4-KO, IL-17A/F-KO, and C57BL/6 mice were subcutaneously immunized in the hind footpad and in the base of the tail with PAg (300 μg/mouse, PAg groups) or saline solution (control group, WT-C) emulsified in Complete Freund's Adjuvant (Sigma-Aldrich, St Louis, MO) in a total volume of 150 μL/mouse, as previously described.[6] Mice received immunizations at days 0 and 15 and then were killed at day 24 of the experimental schedule. As previously described,[6,30] the severity of EAP was assessed by determining the histologic score, which was analyzed in a double-blind manner and computed for individual glands by summing the pathologic grade of each prostate tissue section and dividing by the total number of sections examined. The degree of inflammation was assessed using a score of 0 to 3: 0, no inflammation; 1, mild but definite perivascular cuffing with mononuclear cells; 2, moderate perivascular cuffing with mononuclear cells; 3, marked perivascular cuffing, hemorrhage, and numerous mononuclear cells in the parenchyma, in 5-μm-thick prostate tissue sections of each organ per animal that were processed by conventional hematoxylin and eosin staining. Slides were visualized in a microscope Nikon TE 2000U (Nikon, Osaka, Japan), and pictures were analyzed using the Adobe Photoshop CS6 version 13 image analysis software (Adobe Systems, Inc, San Jose, CA).

2.4. Cell culture

Single mononuclear cell suspensions were prepared in Hank's Balanced Salt Solution (Sigma-Aldrich) from spleen and prostate-draining lymph nodes of individual mice by Ficoll-Paque PREMIUM

1.084 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) centrifugation gradients. Live cells were counted by trypan blue exclusion, resuspended in Roswell Park Memorial Institute 1640-GlutaMAX medium (Life Technologies, Carlsbad, CA) supplemented with 1% penicillin or streptomycin (Life Technologies), 50 mM 2-ME (Life Technologies), and 10% fetal bovine serum (Life Technologies), and cultured in round-bottomed plates in the presence of PSBP (20 μg/mL) or medium alone. Plates were incubated at 37°C in a water-saturated 5% CO_2 atmosphere. After that, cells were processed for surface or intracellular cytokine staining and analyzed by fluorescence-activated cell sorting. Supernatants were frozen at −80°C for cytokine quantifications.

For lymphoproliferation assays, cells were cultured at 3×10^5 cells per well in 0.2-mL volume in flat-bottomed 96-well plates. Then, PSBP was added to a concentration of 20 μg/mL. All cell combinations were performed in quadruplicate. Plates were incubated for 4 days at 37°C in water-saturated 7.5% CO_2 atmosphere and pulsed for the final 18 hours with 1 μCi [methyl-3H] thymidine per well. Labeled material was automatically harvested and counted in a β-plate scanner. The response was expressed as a proliferation index calculated from counts per minute incorporated in antigen-pulsed cultures per cpm incorporated in cultures with medium alone.[30]

2.5. Prostate steroid-binding protein–specific antibodies in serum

Prostate steroid-binding protein–specific total IgG, IgG1, IgG2a, and IgG3 serum levels were titrated by conventional enzyme-linked immunosorbent assay (ELISA) using specific detection antibodies in multiwell plates (Corning Costar, Sigma-Aldrich). Plates were precoated with 50 μL per well of PSBP (20 μg/mL) in 0.05 M carbonate buffer of pH 9.6. After overnight incubation at 4°C, microwells were washed twice and blocked with 3% bovine serum albumin (Sigma-Aldrich) in PBS for 2 hours at 37°C, rinsed with PBS-Tween 20 at 0.05%, and then filled with 100 μL of serum (obtained after cardiac puncture) serial dilutions (starting at 1/50) for 1 hour at 37°C. To detect specific total IgG, IgG1, IgG2a, and IgG3, plates were again washed and incubated with HRP-conjugated rat anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2a (BD Biosciences) or with horseradish peroxidase-conjugated rat anti-mouse IgG3 (BioLegend) for 1 hour at 37°C. Plates were thoroughly washed, and the reaction was developed with BD OptEIA TMB Substrate Reagent Set (BD Biosciences). Serum reactivity was expressed as optic density measured at 450 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA).

2.6. Cytokine quantification

Cytokine secretion of cell suspensions was assessed after antigen stimulation at a cell density of 1.5×10^6 cells/mL. Cell suspension from spleen or prostate-draining lymph nodes were stimulated during 72 hours with PSBP (20 μg/mL). Concentrations of IFN-γ, IL-17A, IL-4, IL-10, and transforming growth factor (TGF)-β in culture supernatants were measured by ELISA using paired antibodies or specific kits (eBioscience for IL-17A, IL-4, and TGF-β; and BD Biosciences for IFN-γ and IL-10) according to standard protocols and following the manufacturer's instructions.

2.7. Flow cytometry

Cells were in vitro stimulated with PSBP, and then, staining for cell surface markers and intracellular cytokines was performed as previously described.[6] In other experiments, after in vitro stimulation with PSBP (20 μg/mL) for 72 hours, cells were incubated for 5 hours with 50 nM phorbol myristate acetate and 0.5 μg/mL ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences). Cell-surface staining of different molecules and chemokine receptors was performed followed by intracellular staining of different cytokines using the BD CytoFix/CytoPerm and Perm/Wash kit (BD Biosciences) according to the manufacturer's instructions. Cells were incubated with APC-labeled antibodies to IL-17A (eBioscience), IL-4 (eBioscience), CCR5 (BioLegend), or CCR6 (BioLegend) and with PE-labeled antibodies to IFN-γ (BD Biosciences), IL-10 (BD Biosciences), CXCR3 (BioLegend), or CCR4 (BioLegend). Cells were acquired on FACSCanto II flow cytometer (BD Bioscience) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.8. Analysis of prostate-infiltrating leukocytes

Prostate-infiltrating leukocyte analysis was performed as previously described.[6] Freshly harvested prostate tissue samples were mechanically disrupted and enzymatically digested in RPMI 1640 medium containing 1 mg/mL collagenase D (Roche, Basel, Switzerland) and DNase I (Sigma-Aldrich) for 45 minutes at 37°C. After digestion, suspensions were filtered through 75-μm and 40-μm cell strainers (BD Biosciences), and single-cell suspensions were washed twice in 10% fetal bovine serum, 2 mM EDTA, and 50 mM 2-mercaptoethanol supplemented RPMI 1640 medium. Live lymphocyte counts were deduced from the acquisition of a fixed number of 10-μm latex beads (Beckman Coulter, Brea, CA) mixed with a known volume of unstained cell suspension in propidium iodide (BD Biosciences). Analyses were performed on a FACSCanto II using DIVA software (BD Biosciences), allowing the exclusion of dead cells (propidium iodide positive) inside the indicated gates. After that, cells were stained with different antibodies for flow cytometry analysis. Cells were acquired using a FACSCanto II, and data were analyzed using FlowJo software (Tree Star).

2.9. Chronic pelvic pain assessment by behavioral testing

Chronic pelvic pain development was assessed by behavioral testing, as previously described.[40,46] Behavior testing was based on the concept of cutaneous hyperalgesia resulting from referred visceral pain.[16,17] An irritable focus in visceral tissues reduces cutaneous pain thresholds, allowing for an exaggerated response to normally nonpainful stimuli (allodynia). Mice under study were tested for allodynia before immunization (baseline, day 0) and at 7, 14, and 24 days after immunization. As previously described,[40,46] tests were performed in individual acrylic glass chambers with a stainless steel wire grid floor (mouse acclimation period of 20 minutes before testing). Referred hyperalgesia and tactile allodynia were tested using von Frey filaments with forces of 0.04, 0.16, 0.4, 1, and 4 g (Bioseb, Chaville, France). Each filament was applied for 1 to 2 seconds with an interstimulus interval of 5 seconds for a total 10 times, and the filaments were tested in ascending order of force. Stimulation was confined to the lower abdominal area in the general vicinity of the prostate, and care was taken to stimulate different areas within this region to avoid desensitization or "wind-up" effects. An investigator blinded to the treated group graded responses in animals. Three types of behaviors were graded as positive responses to filament stimulation: (1) sharp retraction of the abdomen, (2) immediate licking or scratching of the area of filament stimulation, or (3) jumping. Response frequency was calculated as the percentage of positive response, and data were reported as the mean percentage of response frequency ± SEM.

2.10. Statistics

Statistical analysis was performed using 1-way or 2-way analysis of variance with Bonferroni post hoc test analysis. Mean ± SEM are represented in the graphs. Statistical tests were performed using the GraphPad Prism 5.0 software. The P value of < 0.05 was considered significant in all analyses.

3. Results

3.1. IFN-γ–secreting Th1 cells mediate autoimmune prostatitis induction

To analyze the precise role of cytokines produced by different Th cell subsets in the pathogenesis of EAP and chronic pelvic pain development, we took advantage of cytokine-deficient mouse strains, such as IL-12p40-KO mice (unable to mount normal Th1 and Th17 responses), IL-4-KO mice (unable to mount Th2 response), IL-17A/F-KO mice (IL-17A/F deficient), and of their wild-type counterparts (B6 mice). Mice from all strains were immunized, and 24 days later, we analyzed the induction of humoral and cellular immune responses against prostatein or PSBP (the major autoantigen described in our model) and the presence of tissue alterations and damage in the target organ (**Fig. 1**). Positive proliferative cell responses to PSBP were detected either from prostate-draining lymph nodes or spleen mononuclear cells from PAg-immunized B6 mice when compared with control animals (WT-PAg vs WT-C; **Fig. 1A**). These prostate-specific proliferative responses were characterized by the secretion of high levels of IFN-γ and IL17, and low levels of IL-10 (WT-PAg vs WT-C; **Fig. 1B**), showing the induction of specific Th1 and Th17 cells upon immunization. Secretion levels of IL-4, or TGF-β in culture supernatants from WT-PAg animals showed no differences when compared with those from WT-C mice (**Fig. 1B**). Although nonpathogenic autoantibodies typically parallel the type and kinetics of the cell autoimmune response induced in EAP. As expected, T-cell responses were accompanied by specific humoral IgG responses in WT-PAg mice (data not shown). Furthermore, significant histologic alterations were detected when prostate histologic sections from WT-PAg mice were analyzed. Remarkably, the peripheral autoimmune response detected in WT-PAg mice was capable of inducing prostate tissue inflammation and damage, consisting of mononuclear cell infiltration, hemorrhage, edema, and tissue disorganization (WT-PAg vs WT-C; **Fig. 1C**).

IL-12p40-KO and IL-4-KO mice also exhibited positive proliferative PSBP-specific T-cell responses after PAg immunization (**Fig. 1A**). As expected, elevated levels of specific IL-4 and IL-10, and no IFN-γ or IL-17 secretion, were detected in prostate-draining lymph node or spleen cells from IL-12p40-KO mice (**Figs 1A and B**). When assaying the antibody response in these mice, it mirrored the pattern of the Th2 cell response induced, being mainly composed of high levels of PSBP-specific IgG1 (data not shown). However, the analysis of prostate tissue sections from these mice showed no significant histologic changes when compared with WT-C mice, revealing no pathology induction (**Fig. 1C**). Although PAg-immunized IL-12p40-KO induced cellular and humoral immune responses, associated to a Th2 pattern, these cells were not capable of infiltrating and causing inflammation in the target organ.

On the contrary, the prostate-specific T-cell immune response induced in IL-4-KO mice was characterized by the secretion of markedly increased levels of IFN-γ and low levels of IL-17 and IL-10 (**Fig. 1B**). As expected, IL-4 was not detectable. Antibody analyses in these mice revealed the prevalence of high levels of PSBP-specific IgG2a, showing the induction of a definite enhanced prostate-specific Th1 immune response. This response was certainly capable of causing inflammation and cell infiltration in prostate tissue, as evidenced by the high prostate histopathologic scores and tissue lesions observed in these mice (**Fig. 1C**). Finally, PAg-immunized IL-17-KO mice also induced prostate-specific immune responses, which were mainly characterized by IFN-γ–secreting Th1 cells. Antigen-specific secretion levels of IL-4, IL-10, and TGF-β showed no statistically significant differences when compared with those observed in control animals. As expected, no IL-17 secretion was observed in culture supernatants from these animals (**Fig. 1B**). Histologic analysis of the prostate also revealed severe histopathologic scores resulting from marked cell infiltration and tissue inflammation and damage in PAg-immunized IL-17-KO mice (**Fig. 1C**). The analysis of PSBP-specific TGF-β secretion showed no statistically significant differences among the different animal strains under study, suggesting no main regulatory T-cell differences involved among them (**Fig. 1B**).

In order to confirm the particular T-cell pattern of the prostate-specific immune responses induced upon immunization in different animal groups under study, we additionally performed intracellular cytokine staining of prostate-draining lymph node or spleen cells that were in vitro stimulated with PSBP for 72 hours. **Figure 2** shows results obtained from prostate-draining lymph node cell assays and comparable results were obtained when analyzing spleen mononuclear cells (data not shown). As can be seen in the results depicted on dot plots (gated on $CD4^+$ T cells) and quantification graphs in **Figure 2A and B**, immunized B6 mice (WT-PAg) showed the induction of increased frequencies of IFN-γ–secreting or IL-17–secreting T cells and lower frequencies of IL-10-secreting T cells when compared with control wild-type animals (WT-C). The analysis of intracellular cytokine staining confirmed the induction of prostate-specific Th1 and Th17 immune responses in immunized wild-type C57BL/6 mice. Besides, the presence of increased IL-4–secreting T cells was detected in PAg-immunized IL-12p40-KO mice showing once again the Th2 nature of the immune response involved. On the contrary, immunized IL-4-KO mice showed highly elevated frequencies of IFN-γ–secreting T cells, low frequencies of IL-10-secreting T cells, and similar levels of IL-17–secreting T cells when compared with WT-PAg animals, showing the induction of a clearly biased Th1 response (**Figs. 2A and B**). In agreement with results presented in **Figure 1**, high frequencies of specific IFN-γ–secreting T cells and lower quantities of specific IL-10–secreting T cells were observed in PAg-immunized IL-17-KO mice when compared with control wild-type mice (WT-C), confirming the induction of a clear Th1 immune response.

Altogether, these results show that prostate inflammation and tissue damage is induced in strains able to mount prostate-specific Th1 immune responses.

3.2. CXCR3 and CCR5 are expressed on pathogenic PAg-specific T cells

T-cell differentiation is accompanied by the expression of chemokine receptors responsible for T-cell subset recruitment to and extravasation at inflammation sites.[22] The expression of chemokine receptors has been associated with different Th subsets.[5] It has been shown that CXCR3 and CCR5 are preferentially expressed on Th1 cells, whereas CCR3, CCR4, and CCR8 are expressed on Th2 cells.[47] CCR6 has been identified as expressed distinguishably by the Th17 subset,

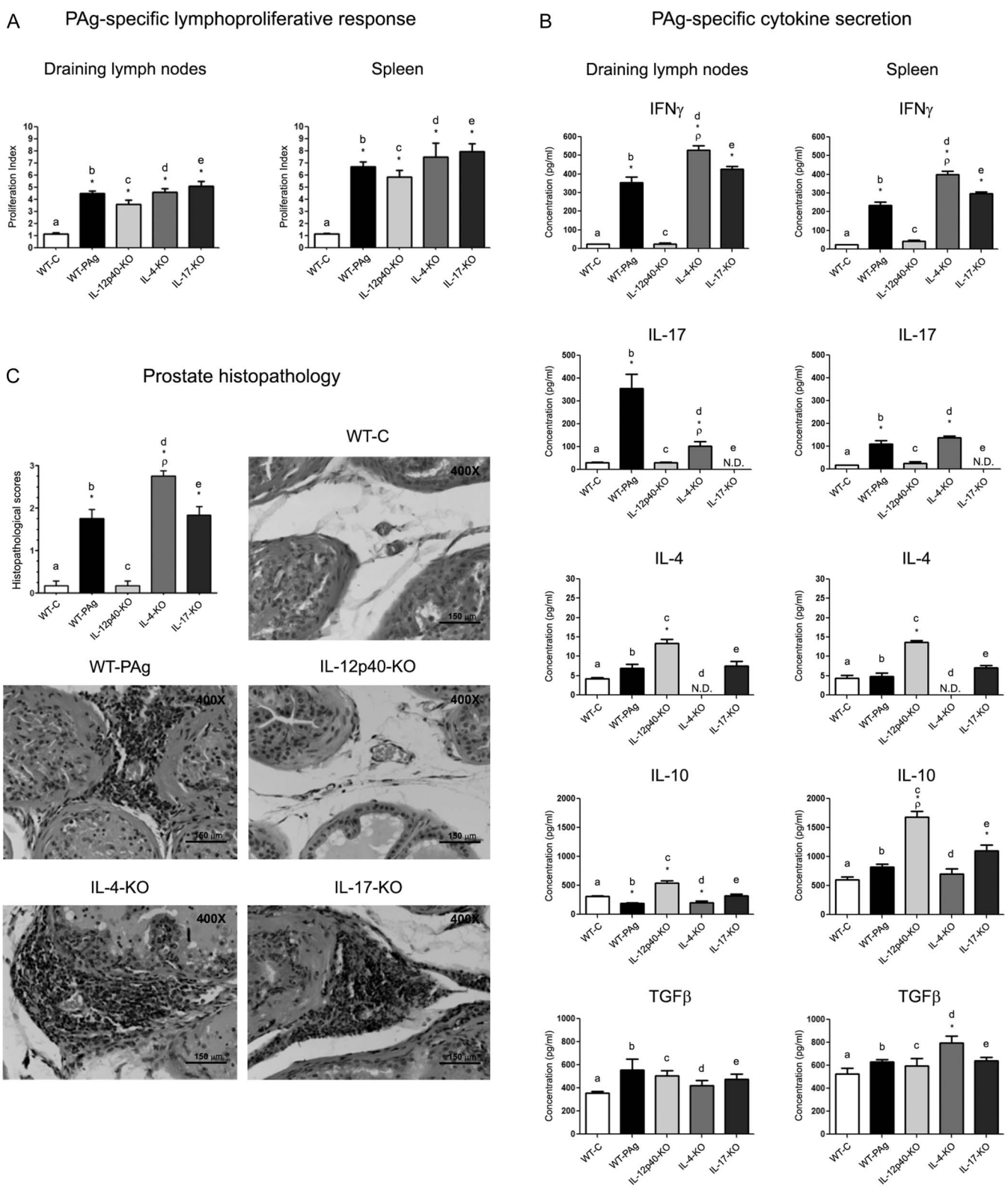

Figure 1. EAP is only induced in strains able to develop prostate-specific Th1 immune responses. IL-12p40-KO, IL-4-KO, IL-17A/F-KO, or C57BL/6 mice were immunized with PAg emulsified in Complete Freund's Adjuvant (CFA) (PAg groups: WT-PAg, IL-12p40-KO, IL-4-KO, and IL-17-KO mice) or saline solution plus CFA alone (control animals, WT-C). Mice were euthanized at day 24 after immunization and sera, lymphoid organs and the prostate glands were obtained. Prostate-draining lymph node or spleen mononuclear cells were cultured for 72 hours in the presence of PSBP or medium. (A) PSBP-specific lymphoproliferative responses from prostate-draining lymph node or spleen mononuclear cells were expressed as proliferation index. (B) Cytokine secretion in culture supernatants: IFN-γ, IL-17A, IL-4, IL-10, and TGF-β levels measured by sandwich enzyme-linked immunosorbent assay in culture supernatants. (C) Prostate histopathologic scores and representative hematoxylin and eosin–stained histologic assays in prostate tissue sections from mice under study (original magnification, ×400). Data are shown as mean ± SEM; n = 6 per group and are representative of 4 independent experiments. Lanes with asterisk are significantly different from lane a (WT-C); lanes with ρ are significantly different from lanes with asterisk. Statistical analysis was performed using 1-way analysis of variance with Bonferroni post hoc test analysis. $^*P < 0.05$.

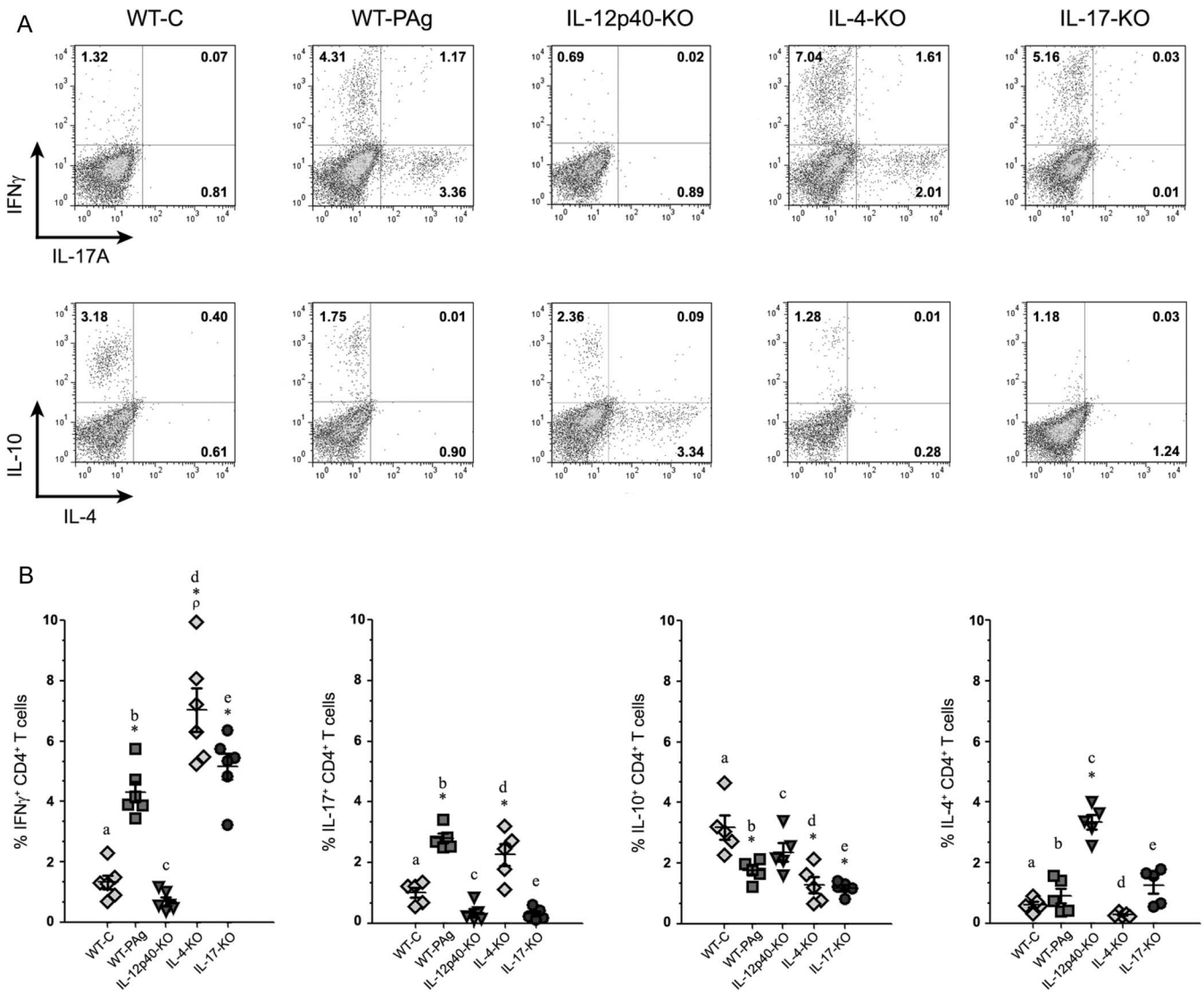

Figure 2. Prostate-specific Th1 immune responses underlie EAP induction. IL-12p40-KO, IL-4-KO, IL-17A/F-KO, or C57BL/6 mice were immunized with PAg emulsified in CFA (PAg groups: WT-PAg, IL-12p40-KO, IL-4-KO, and IL-17-KO mice) or saline solution plus CFA alone (control animals, WT-C), euthanized at day 24 after immunization, and lymphoid organs were obtained. Prostate-draining lymph node cells were cultured for 72 hours in the presence of PSBP or medium. (A) Representative flow cytometry dot plots of intracellular staining for IFN-γ, IL-17A, IL-10, and IL-4 was performed on prostate-draining lymph node cells stimulated with PSBP for 72 hours. Shown data are cells gated in the $CD4^+$ T-cell population. (B) Percentages of IFN-γ^+ $CD4^+$, IL-$17A^+$ $CD4^+$, IL-10^+ $CD4^+$, and IL-4^+ $CD4^+$ prostate-draining lymph node T cells from animals under study. Numbers indicate percentage among gated $CD4^+$ T cells. Numbers indicate the percentage of cells in each quadrant. Data are shown as mean ± SEM; n = 6 per group and are representative of 4 independent experiments. Lanes with asterisk are significantly different from lane a (WT-C); lanes with ρ are significantly different from lanes with asterisk. Statistical analyses were performed using 1-way analysis of variance with Bonferroni post hoc test analysis. $^*P < 0.05$.

although there is also evidence that Th17 cells may express CCR4, CCR2, and CCR9.[47] Furthermore, we recently have shown that CXCR3 is essential for conferring prostate-reactive Th1 cells the capacity to home to and infiltrate prostate tissue during EAP induction in NOD mice.[6]

We then investigated if chemokine receptor expression on autoreactive T cells was in accordance with the type of immune response involved and also with prostate tissue cell infiltration and inflammation. Thus, we analyzed the expression of the main chemokine receptors associated with the different Th phenotypes. The expression of CXCR3, CCR5, CCR4, and CCR6 was assayed in antigen-stimulated prostate-draining lymph node T cells from the different animal groups under study. To do that, we cultured prostate-draining lymph node or spleen mononuclear cells in the presence of PSBP or medium alone. T cells from all animal groups cultured in the presence of medium showed very low levels of the expression of chemokine receptors (data not shown). However, a high frequency of $CXCR3^+$ $CCR5^+$ cells with important quantities of $CCR6^+$ cells were detected when analyzing T cells from immunized C57BL/6 mice (WT-PAg), when compared with T cells from control C57BL/6 mice (WT-C) (**Figs 3A and B**). In agreement with the polarization of the immune response toward Th2 observed in IL-12p40-KO mice, T cells from these mice cultured in the presence of PSBP were mainly $CCR4^+$ simple positive, showing no expression of CXCR3, CCR5, or CCR6 (**Figs. 3A and B**). On the contrary, in accordance with the previously observed strong Th1 response induced, PSBP-stimulated T cells from immunized IL-4-KO mice expressed much higher levels of CXCR3 and comparable levels of CCR5 and CCR6 when compared with T cells from immunized wild-type mice (WT-PAg; **Figs. 3A and B**). Besides, PSBP-stimulated T cells from IL-17-KO mice expressed similar levels of CXCR3 and CCR5 to those observed in T cells from

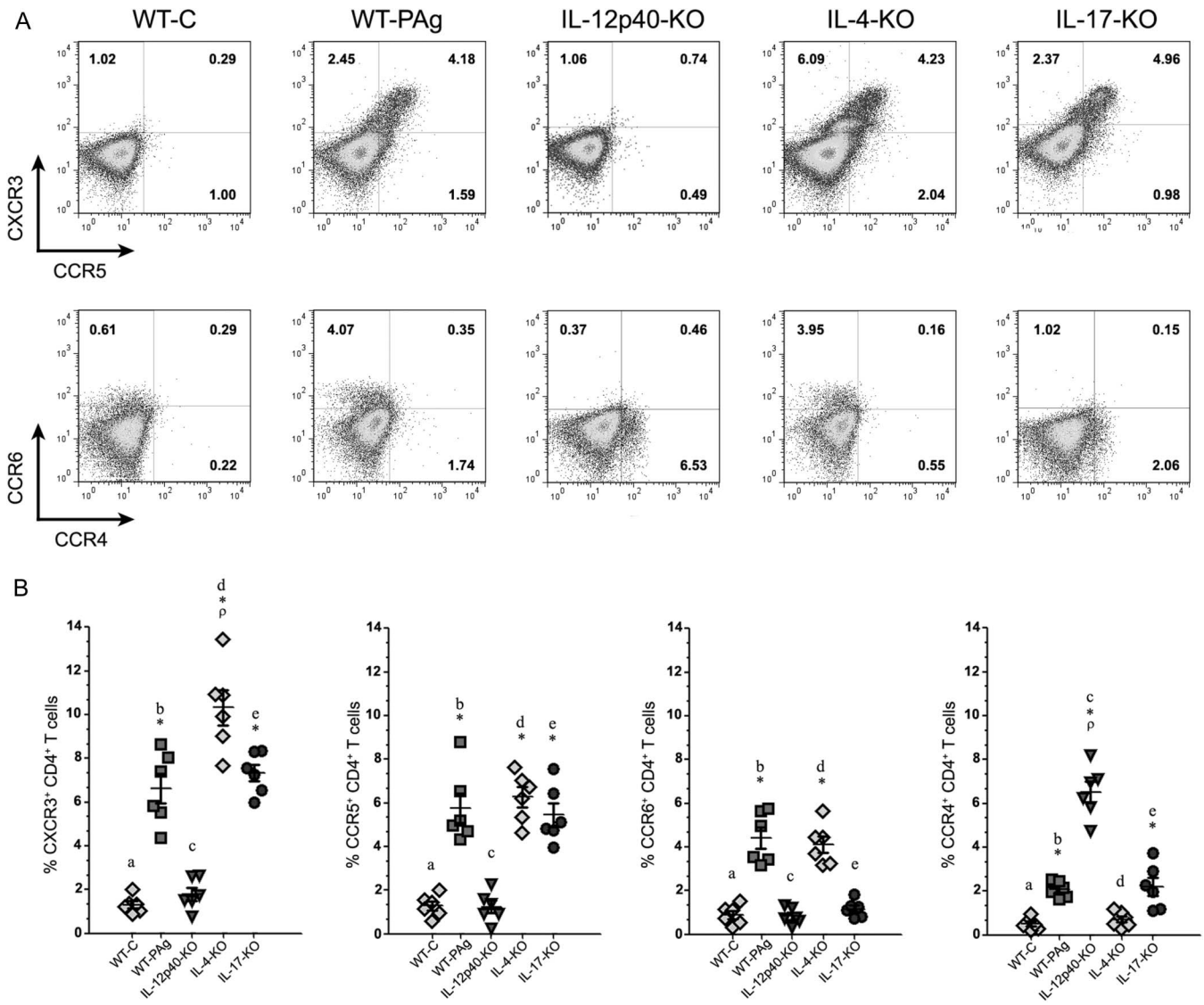

Figure 3. Prostate-specific T cells from PAg-immunized wild-type, IL-12p40-KO, IL-4-KO, and IL-17-KO mice and from control wild-type mice express different patterns of chemokine receptors. IL-12p40-KO, IL-4-KO, IL-17A/F-KO, or C57BL/6 mice were immunized with PAg emulsified in CFA (PAg groups: WT-PAg, IL-12p40-KO, IL-4-KO, and IL-17-KO mice) or saline solution plus CFA alone (control animals, WT-C), euthanized at day 24 after immunization, and lymphoid organs were obtained. Spleen or prostate-draining lymph node mononuclear cells were cultured for 72 hours in the presence of PSBP or medium; then, surface staining for chemokine receptors was performed. (A) Representative flow cytometry dot plots of surface staining for CXCR3 vs CCR5 and for CCR6 vs CCR4 on prostate-draining lymph node mononuclear cells stimulated with PSBP for 72 hours. (B) Percentages of $CXCR3^+$ $CD4^+$, $CCR5^+$ $CD4^+$, $CCR6^+$ $CD4^+$, and $CCR4^+$ $CD4^+$ prostate-draining lymph node T cells from animals under study. Staining analysis performed in spleen mononuclear T cells showed comparable results. Numbers indicate percentage among gated $CD4^+$ T cells. Numbers indicate the percentage of cells in each quadrant. Data are shown as mean ± SEM; n = 6 per group and are representative of 4 independent experiments. Lanes with asterisk are significantly different from lane a (WT-C); lanes with ρ are significantly different from lanes with asterisk. Statistical analyses were performed using 1-way analysis of variance with Bonferroni post hoc test analysis. $^*P < 0.05$.

immunized wild-type mice (WT-PAg), and no expression of CCR6 was detected, as expected (**Figs. 3A and B**).

These results showed that PAg-immunized animals, which are capable of inducing peripheral specific Th1 cells and expressing associated chemokine receptors, develop cell infiltration and inflammation in the prostate gland. The expression of Th1-associated chemokine receptors would be the feature that confers on autoreactive T cells the capacity to migrate to and infiltrate the prostate gland.[6]

3.3. Analysis of the leukocyte infiltration in mice under study

In EAP developed in NOD and C57BL/6 mice, prostate leukocyte infiltration has been shown to start increasing at 8 days post immunization (DPI) and to reach maximal and sustained levels from 21 to 30 DPI.[6,32,37,38,41,42] Infiltrating leukocytes locally secrete Th1-associated cytokines and chemokines that in turn recruit more leukocytes enhancing inflammation and pathology.[6,32,37] We next analyzed the leukocyte infiltration in prostate tissue and the development of chronic pelvic pain in the different animal groups under study.

As shown in flow cytometry dot plots and quantification graphs depicted in **Figure 4A and B**, control C57BL/6 mice (WT-C) showed scarce amounts of leukocyte cells in prostate tissue at 24 DPI, with the presence of $CD3^+$ T cells with a prevalence of $CD8^+$ over $CD4^+$ T lymphocytes, and granulocytes, in accordance with the known normal prostate leukocyte resident populations.[25] In contrast, immunized C57BL/6 mice (WT-PAg) showed intense

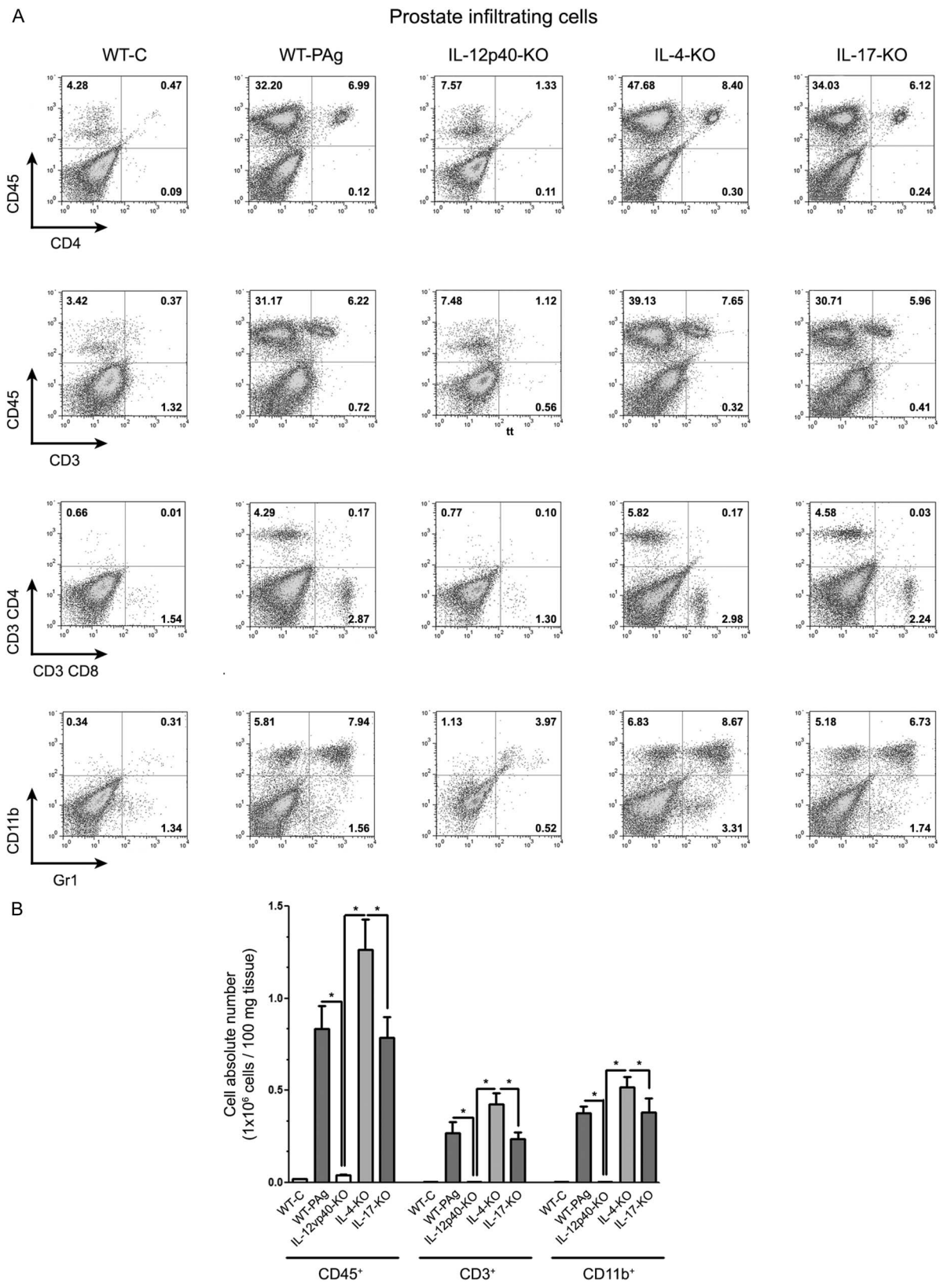

Figure 4. Prostate infiltration is only induced in strains able to mount prostate-specific Th1 immune responses. IL-12p40-KO, IL-4-KO, IL-17A/F-KO, or C57BL/6 mice were immunized with PAg emulsified in CFA (PAg groups: WT-PAg, IL-12p40-KO, IL-4-KO, and IL-17-KO mice) or saline solution plus CFA alone (control animals, WT-C), euthanized at day 24 after immunization. The prostates were collected at day 24 after PAg immunization and processed for flow cytometry analysis. Live cells were counted, and flow cytometry was preformed to evaluate leukocyte infiltration in the glands. (A) Presence of leukocytes in the prostate from animals under study at day 24 after PAg immunization. Representative flow cytometry dot plots of the analysis of different leukocyte subpopulations: $CD45^+$ vs $CD4^+$ cells, $CD45^+$ vs $CD3^+$ cells, $CD3^+$ $CD4^+$ vs $CD3^+$ $CD8^+$ cells, and $CD11b^+$ vs $Gr1^+$ cells. (B) Absolute number of $CD45^+$, $CD3^+$, and $CD11b^+$ cells in the prostate gland from mice under study. Numbers indicate the percentage of cells in each quadrant. Data are shown as mean ± SEM; n = 6 per group and are representative of 4 independent experiments. Statistical analyses were performed using 1-way analysis of variance with Bonferroni post hoc test analysis. *$P < 0.05$.

$CD45^+$ leukocyte infiltration of prostatic tissue that was mainly composed of $CD4^+$ T lymphocytes, macrophages, and granulocytes. Immunized IL-12p40-KO mice showed mild to absent leukocyte infiltration, and the characterization of the present cell populations mirrored that observed in control wild-type mice (IL-12p40-KO vs WT-C; **Figs 4A and B**). On the contrary, immunized IL-4-KO mice showed more intense and florid prostate leukocyte infiltration, with the prevalence of $CD4^+$ over $CD8^+$ T lymphocytes, and abundant macrophages and granulocytes, as observed in immunized wild-type mice (IL-4-KO vs WT-PAg; **Figs 4A and B**). In agreement with what was observed in prostate histologic tissue sections, the analysis of the prostate leukocyte infiltration in immunized IL-17-KO mice showed no significant differences from that found in immunized wild-type mice (IL-17-KO vs WT-PAg; **Figs 4A and B**).

3.4. Th1-induced prostate leukocyte infiltration is accompanied by chronic pelvic pain development

In order to assess if the development of chronic pelvic pain was a consequence of prostate inflammation, mice from the different experimental groups were tested at baseline and over time for EAP induction of suprapubic allodynia, a consequence of referred hyperalgesia and a characteristic of visceral pain[13,16,17] (**Fig. 5A**). While control wild-type mice (WT-C) showed no changes in tactile allodynia over time, significant increases were detected at 14 and 24 DPI in immunized wild-type mice, evidencing chronic pelvic pain development (WT-PAg, **Fig. 5A**). Similar results were observed in immunized IL-17-KO and IL-4-KO mice (**Fig. 5A**). On the contrary, and similar to what shown by control wild-type animals (WT-C), no increase in tactile allodynia responses were observed in IL-12p40-KO mice, showing no chronic pelvic pain development over time. When comparing tactile allodynia responses detected in all animal groups at 24 DPI (**Fig. 5B**), significant elevations were clearly observed in immunized wild-type (WT-PAg), IL-17-KO, and IL-4-KO mice showing the development of chronic pelvic pain and evidencing that IL-17 is not essential for chronic pelvic pain development. On the contrary, immunized IL-12p40-KO and control wild-type (WT-C) mice showed no increases from baseline in the tactile allodynia responses (**Fig. 5B**). All these results mirrored the induction of prostate leukocyte infiltration, tissue inflammation, and damage observed on histologic examinations and flow cytometry analyses of the prostates from animals under study. Moreover, the kinetics of chronic pelvic pain development in susceptible animal groups correlated with the previously reported kinetics of prostate tissue infiltration and inflammation in EAP using different animal strains.[6,32,37,38]

In summary, these results show that PAg-specific Th1 immune responses underlying EAP induce prostate tissue inflammation and, in turn, provoke chronic pelvic pain, the hallmark symptom of CP/CPPS. Also, IL-17 is neither crucial for prostate cell inflammation nor essential for chronic pelvic pain development.

4. Discussion

Chronic prostatitis and chronic pelvic pain syndrome is a syndrome that currently represents an important health care problem not only because of its considerable incidence in young men but also because of its unknown etiology.[8,27,44,50,51] Due to the latter, most medical treatments are empiric and ineffective,[15,44] and despite treatment, patients report diminished quality of life, alongside stable reports of chronic pain, disability, depression, anxiety, and catastrophizing over a 2-year period.[21] Noteworthy, cumulative evidence points to the possibility that this syndrome is a consequence of dysregulated inflammation in the form of autoimmunity directed against prostate antigens. Several human studies have reported self-reactive T-cell responses against prostate antigens, such as prostate specific antigen, prostatic acid phosphatase, and others from seminal plasma.[1,3,20,28,31,44,51] In fact, the presence of IFN-γ–secreting lymphocytes to prostate antigens has been reported in an important fraction of CP/CPPS patients.[28] Also, the analysis of expressed prostatic secretions (EPS) from men with CP/CPPS showed increased numbers of granulocytes, macrophages, T lymphocytes, and B lymphocytes.[18,24,35] In addition, high levels of inflammatory cytokines and chemokines in seminal plasma, or EPS, have been detected in CP/CPPS patients, suggesting an active inflammatory process of the male genital tract in the absence of infection.[2,10,18,19,26,29,36,39,45] Th17 cells have been implicated in disease pathogenesis in patients with different autoimmune diseases.[52] Although some authors have speculated that IL-17 would be involved in CP/CPPS, particularly mediating chronic pelvic pain development,[34] they failed in identifying IL-17 in clinical samples from CP/CPPS patients.[33] To our knowledge, no reports in humans have been published about the role of IL-17 produced by Th17 cells in CP/CPPS.

Models of EAP have provided further support for the autoimmune nature of CP/CPPS.[31,54] They have been widely used to study CP/CPPS and have provided important data about the immune mechanisms underlying disease induction, development, and pathologic consequences.[31,54] Findings from EAP animal models have mirrored most findings in patients, thus improving the understanding of the human disease.[44] Immunization with prostate gland homogenates,[30,42] or purified prostate proteins such as PSBP,[30,41] induces PAg-specific Th1 cell and antibody responses associated with histologic evidence of prostate inflammation and the induction of chronic pelvic pain.[6,30,32,37] Moreover, IFN-γ-KO mice or mice deficient in transcription factors involved in the IFN-γ–signaling cascade, such as IRF1 and STAT-1, have been shown to be resistant to autoimmune prostatitis induction, supporting the Th1 nature of the pathogenic immune response involved.[6,32,37] However, some authors have argued for a role of Th17 cells in EAP induction and, especially, for IL-17 in the development of chronic pelvic pain.[33,34,37,38,40] They found elevated levels of IFN-γ and IL-17 in the inflamed prostate tissue of mice with prostatitis and also ex vivo when prostate-draining lymph node T cells from these mice were in vitro stimulated.[33,37,38,40] Nevertheless, it has been shown that the immunization of BALB/c mice, a strain resistant to EAP induction, also generates elevated peripheral prostate-specific IL-17-secreting T-cell responses, although these responses are unable to infiltrate the prostate and cause the disease.[6] In addition, PAg-immunized NOD-IFN-γ-KO mice have been shown to have markedly increased frequencies of prostate-specific Th17 cells in prostate-draining lymph node and spleen cells. However, these mice have also proven to be resistant to EAP induction.[6] On the contrary, using NOD mice infected with a strain of uropathogenic *Escherichia coli* (CP-1), Quick et al suggested that the infection elicited a Th1/Th17 chronic inflammatory response that infiltrated the prostate and mediated chronic pelvic pain. Also, they proposed that IL-17 produced by Th17 cells would be the main inducer of pain.[40] Although authors emphasized the autoimmune nature of the involved immune response, no certain evidence of any prostate-specific autoimmune response was shown in their study.[34,40] In a recent report, Murphy et al[34] provided some controversial evidence, indicating that IL-17 would be crucial for the induction but not for the maintenance of pelvic pain in EAP in C57BL/6 mice. These authors showed that prophylactic treatment with a single injection

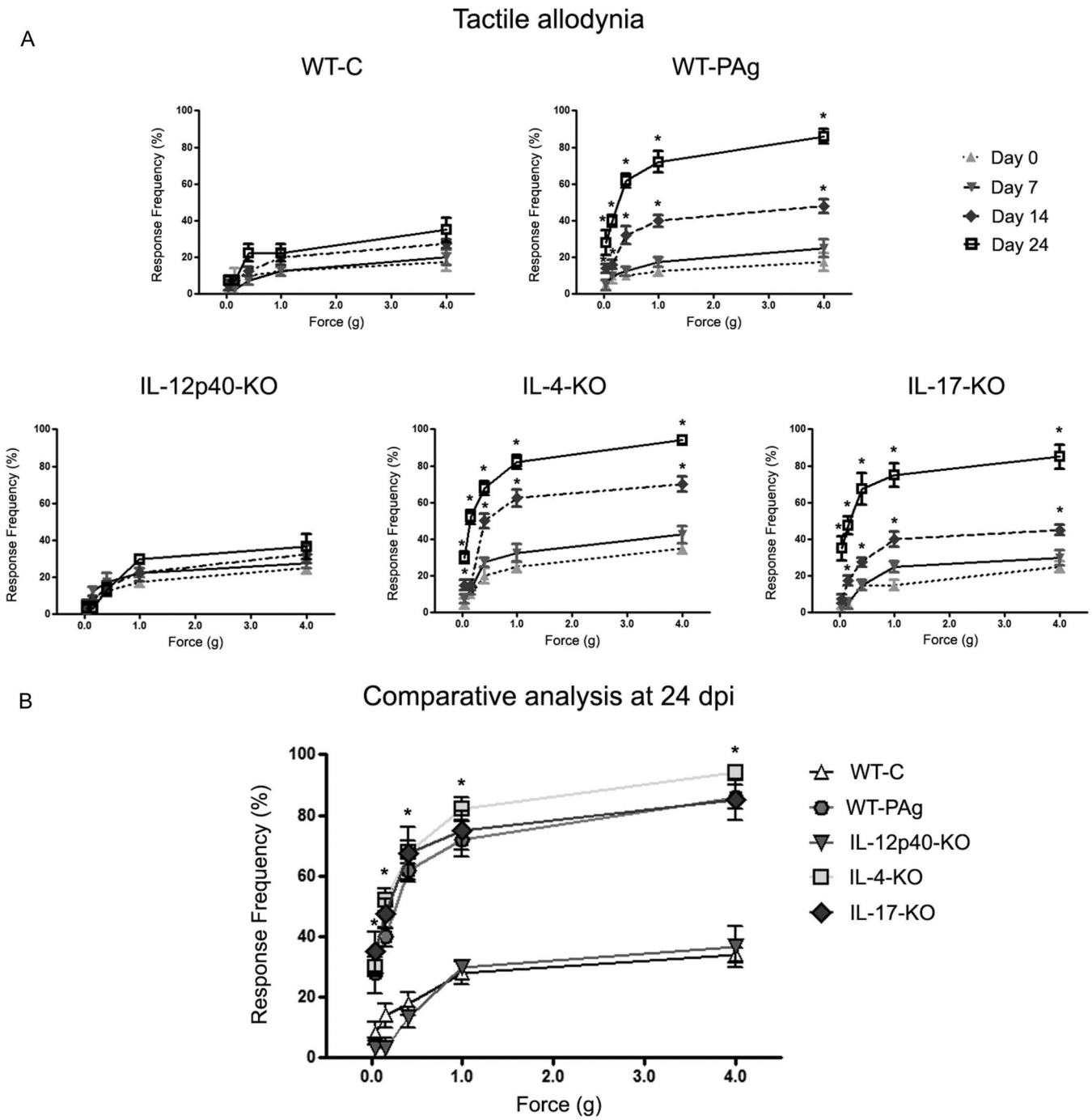

Figure 5. Chronic pelvic pain is developed by animals able to mount prostate-specific Th1 immune responses. IL-12p40-KO, IL-4-KO, IL-17A/F-KO, or C57BL/6 mice were immunized with PAg emulsified in CFA (PAg groups: WT-PAg, IL-12p40-KO, IL-4-KO, and IL-17-KO mice) or saline solution plus CFA alone (control animals, WT-C), and referred visceral hyperalgesia was measured over time of EAP induction as responses to mechanical stimulation of the pelvic region and hind paw using von Frey filaments of 5 calibrated forces. Data are shown as the mean percentage of response frequency ± SEM (eg, 5 responses of 10 = 50%) before (baseline, day 0) or at 7, 14, and 24 days after PAg immunization. (A) Tactile allodynia responses to pelvic stimulation of every experimental group under study over different time points of EAP induction. (B) Comparative analysis of tactile allodynia responses to pelvic stimulation of all animal groups under study at 24 days after PAg immunization. Shown data (n = 6 per group) are representative of 4 independent experiments. Statistical analyses were performed using 2-way analysis of variance with Bonferroni post hoc test analysis. *$P < 0.05$.

of an IL-17–blocking antibody 1 day prior to EAP induction and then weekly 1 day prior to pain assessment was sufficient to abolish pelvic pain induction. The authors surprisingly did not show any data about prostate tissue inflammation or cell infiltration in order to definitively assess if pelvic pain was related to prostate inflammation. Moreover, the authors remarkably showed that they failed in preventing or ameliorating chronic pelvic pain when administering a therapeutic treatment consisting of the same IL-17–blocking antibody on day 10 after EAP induction.[34] Taking all these data into account, conclusive evidence of an actual involvement of IL-17 in CP/CPPS, or in EAP, and in mediating chronic pelvic pain is currently lacking.

In the present work, we analyzed EAP induction and chronic pelvic pain development in IL-12p40-KO, IL-4-KO, IL-17A/F-KO,

and C57BL/6 mice. In agreement with previous results,[32] we showed that upon PAg immunization, wild-type (C57BL/6) mice induced prostate-specific Th1 and Th17 immune responses that caused prostate tissue inflammation. Moreover, these mice evidenced significantly increased tactile allodynia responses as the disease progressed, showing that chronic pelvic pain development was a consequence of prostate inflammation. Besides and as expected, PAg immunization of IL-12p40-KO and IL-4-KO mice induced prostate-specific biased Th2 and enhanced Th1 immune responses, respectively. Noteworthy, the Th2 biased prostate-specific immune response was incapable of causing prostate inflammation, rendering IL-12p40-KO mice refractory to prostatitis induction and chronic pelvic pain development. On the contrary, chronic pelvic pain was developed by immunized IL-4-KO mice, which developed strong Th1 immune responses and evidenced marked histologic cell infiltration and enhanced inflammation in prostate tissue. Interestingly, immunized IL-17A/F-KO mice induced a prostate-specific immune response that was also characterized by the secretion of high levels of IFN-γ. Strikingly, even in the absence of IL-17, that specific immune response (characterized by high IFN-γ secretion) was capable of causing prostate inflammation and certainly induced chronic pelvic pain at extents comparable to those observed in immunized wild-type mice. Therefore, if IL-17–secreting cells are playing any role in EAP induction and chronic pelvic pain development, it might be secondary or once the disease is already established. These results are in contrast to what has been postulated by other authors.[33,34] The dissimilarities between our results and the results from other studies could be explained by the different experimental settings or animal models used. To our knowledge, this is the first investigation that has studied EAP and chronic pelvic pain development in IL-17A/F-KO mice and that has provided solid and conclusive evidence, showing that IL-17 is not essential for EAP induction and chronic pelvic pain development. However, we must state that the use of IL-17A/F-KO mice may also represent a limitation of our study. The possibility of compensatory changes in constitutive IL-17A/F-KO mice may account, at least in part, for the discrepancy of our results with those previous reports, arguing for a role of IL-17 in the development of chronic pelvic pain. Using conditional IL-17A/F-KO mice would help to elucidate this issue. Also, our experiments using IL-17A/F-KO mice do not formally establish that other cytokines produced by ROR-γ^+ "wannabe Th17 cells," such as Granulocyte-macrophage colony-stimulating factor or IL-22 are not required for EAP induction. However, we have previously reported that the deficiency of IL-17 was not compensated for by the increased expression of IL-22 in knockout cells.[14]

Herein, it was confirmed that chronic pelvic pain developed in parallel with prostatitis induction and prostate tissue leukocyte infiltration. The presence of chronic pelvic pain was demonstrated only in those mice that induced prostate-specific Th1 immune responses and subsequent prostate tissue leukocyte infiltration and local inflammation after PAg immunization. It was recently shown that Th1 cells, induced upon PAg immunization, express associated chemokine receptors, such as CXCR3 and CCR5, and migrate to and infiltrate the prostate gland.[6] Once there, these lymphocytes induce the local secretion of several cytokines and chemokines, including the ligands for CXCR3 and CCR5, which in turn recruit more leukocytes augmenting tissue cell infiltration and enhancing prostate inflammation.[6,32,37] The development of chronic pelvic pain could be associated with a particular mediator or cell type present in the infiltrates. Mast cells are currently suggested as the main mediator and effector cells in disease progression from initiation to breaking of tolerance, neuronal activation, and, eventually, sensitization.[49,55] Mast cells contribute to rapidly occurring neuronal peripheral sensitization, which is mediated by neurotrophin nerve growth factor (NGF).[53] They are known to express TrkA receptors on their cell membrane, and therefore, NGF binding might cause degranulation, establishing a feedback mechanism that would promote sensitization mechanisms.[34] It has been shown that CP/CPPS patients have elevated levels of mast cell tryptase-β, carboxypeptidase A3, and NGF in EPS and urine and that those NGF levels directly correlated with pain severity.[12,45,56] These results suggest that NGF and mast cells secretion products are potential mediators involved in peripheral pain sensitization mechanisms in CP/CPPS. Although in the present report, we have not focused in the analysis of mast cell infiltration of the prostate, the presence of markedly increased numbers of mast cells in prostate cell infiltrates in EAP was already reported by our group.[9,43] Moreover, these mast cells evidenced an activated state because most of them have shown to be degranulated[9,43] and having secreted pain inducer molecules, such as tryptase-β and NGF.[12,45] All these data support the notion that within the Th1-induced leukocyte infiltrates, mast cells might be actors in the consequent development of chronic pelvic pain, a hallmark of patients with CP/CPPS.

In summary, we provided compelling data showing that IFN-γ–secreting Th1 lymphocytes are the driver cells of autoimmune prostatitis pathogenesis and chronic pelvic pain induction. In addition, our results clearly show that IL-17A or IL-17F production of Th17 cells is neither crucial for prostate inflammation induction nor essential for chronic pelvic pain development. These results represent a significant contribution to the current knowledge of the pathogenesis of autoimmune prostatitis and chronic pelvic pain development after prostate inflammation. Also, they have significant implications for delineating future studies in CP/CPPS patients in order to find more rational and effective therapies in this large patient population, which is an unmet clinical need.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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R. D. Motrich and V. E. Rivero planned the study. R. D. Motrich, M. L. Breser, L. R. Sánchez, and G. J. Godoy performed the experimental work. I. Prinz provided IL-17A/F-KO mice, reagents and advice. R. D. Motrich wrote and V. E. Rivero revised the manuscript, with contributions from all co-authors.

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