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Crucial Role of Interferon-γ in Experimental Autoimmune Prostatitis

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Purpose: An autoimmune etiology is proposed in some patients with chronic nonbacterial prostatitis since they show IFN-γ secreting lymphocytes specific to prostate antigens in the periphery and increased IFN-γ in seminal plasma. We investigated the involvement of IFN-γ in an animal model of autoimmune prostatitis.

Materials and Methods: Experimental autoimmune prostatitis was studied in the no-obese diabetic and C57Bl/6 (Harlan, Zeist, The Netherlands) susceptible mouse strains, and in the IRF-1 KO and STAT-1 KO mouse strains deficient in transcription factors involved in IFN-γ signaling.

Results: Experimental autoimmune prostatitis was characterized by prostate specific IFN-γ secreting cells in the periphery and by T-helper 1 related cytokines in the target organ. Increased IFN-γ and IL-12 were observed in the prostate of autoimmune animals while IL-10 and IL-4 were decreased and unaltered, respectively. The absence of transcription factors involved in the IFN-γ signaling cascade, IRF-1 and STAT-1 made mice resistant to experimental autoimmune prostatitis. IRF-1-KO and STAT-1-KO mice immunized with prostate antigens did not show infiltration or alterations in the prostate. They did not have the typical prostate specific autoimmune response and showed decreased IFN-γ, IL-12 and IL-10, and enhanced of IL-4 in the prostate.

Conclusions: Our results argue for a crucial role of IFN-γ as a key factor in the pathogenesis of the disease. Intense research is promptly required to identify the pathogenic mechanisms underlying chronic prostatitis/chronic pelvic pain syndrome to find a more rational therapy.

Key Words: prostate, prostatitis, pain, autoimmunity, mice

Abbreviations and Acronyms

CFA = complete Freund's adjuvant
CIA = collagen induced arthritis
CP = chronic prostatitis
CPPS = chronic pelvic pain syndrome
EAE = experimental autoimmune encephalomyelitis
EAP = experimental autoimmune prostatitis
ELISA = enzyme-linked immunosorbent assay
IFN-γ = interferon-γ
IL = interleukin
IRF-1 = IFN-γ regulatory factor-1
NOD = no-obese diabetic
OD = optical density
PBS = phosphate buffered saline
PSBP = prostate steroid binding protein
STAT-1 = signal transducer and activator of transcription-1
Th = T helper

CHRONIC prostatitis/CPPS is an important worldwide health care problem since it is a highly prevalent condition that affects young and middle-aged men.[1] This chronic inflammatory syndrome is characterized by pelvic pain, irritative voiding symptoms and sexual dysfunction complaints. An inflammatory state in the absence of an invading infectious agent exists in these patients, suggesting that an autoimmune process may be involved.[2] This hypothesis is strengthened by the fact that lymphocytes in a significant percent of these patients proliferate and secrete IFN-γ in response to prostate antigens.[3,4] Increased proinflammatory cytokines and striking abnormalities in semen quality were also described in patients with CP/CPPS who had this autoimmune component.[5]

Submitted for publication June 3, 2009.
Study received Katholieke Universiteit Leuven institutional animal care committee approval.
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0022-5347/10/1833-0001/0
THE JOURNAL OF UROLOGY®

Vol. 183, 000, March 2010
Printed in U.S.A.
DOI:10.1016/j.juro.2009.11.008

Extensive study has been done of the development and characterization of rodent models of EAP that may be appropriate animal models for the human counterpart, CPPS.[6] The group at our laboratory has been a pioneer since we first described and developed an experimental model in the highly susceptible NOD mouse strain. Immunization of young male NOD mice with a mixture of prostate antigens or the purified prostate protein PSBP induced the presence of autoreactive pathogenic T cells, a humoral response, and inflammation and infiltration in the target organ.[7,8] This model shows almost all characteristics of human chronic autoimmune prostatitis, including specific lymphocytes against prostate antigens, IFN-γ in lymphocyte supernatant, and the type of infiltration and histological lesions seen in the gland.[9] Although many disease characteristics have been described, many aspects remain unknown, such as the local cytokine milieu and the putative role of IFN-γ in disease development.

We advanced the characterization of the autoimmune response by identifying Th1 related cytokines in the prostate gland of autoimmune mice. We also studied IFN-γ involvement in mice with EAP that were deficient in STAT-1 and IRF-1, 2 key transcription factors that affect the IFN-γ signaling cascade.[10–12] Our results suggest a crucial role for IFN-γ in the pathogenesis of experimental autoimmune prostatitis.

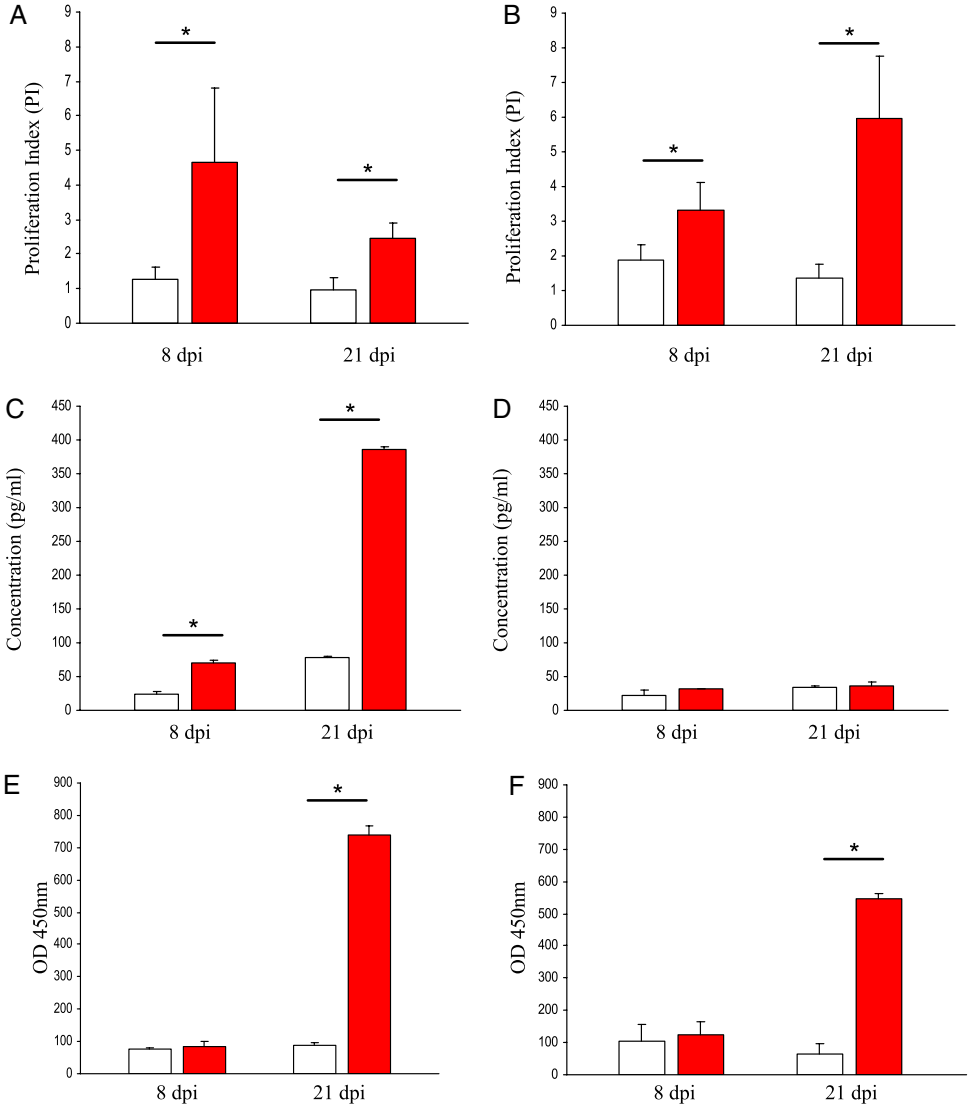

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Figure 1. Increased IFN-γ in mice with EAP. *A* and *B*, PSBP specific lymphoproliferative response in 10 CFA controls (open bars) and 20 autoimmune PSBP NOD mice (red bars) stimulated with PSBP in vitro on days 8 and 21 after immunization, expressed as proliferation index. *A*, lymph node cells. *B*, spleen mononuclear cells. *C* and *D*, specific secretion in response to PSBP in culture supernatants on days 8 and 21 after immunization quantitated by sandwich ELISA. *C*, IFN-γ. *D*, IL-4. *E* and *F*, PSBP specific humoral autoimmune response evaluated by measuring specific levels in serum samples of autoimmune PSBP and control CFA mice on days 8 and 21 after immunization by indirect ELISA. *E*, IgG1. *F*, IgG2a. Asterisk indicates $p < 0.05$.

MATERIALS AND METHODS

Mouse Strains

NOD mice were kept under semibarrier conditions. KO mice (STAT-1−/− and IRF-1−/−) had been back crossed to C57Bl/6 and were kept under specific pathogen-free conditions. C57Bl/6 mice served as WT controls. In all experiments 8 to 10-week-old mice were used. Mice were housed at the Katholieke Universiteit Leuven animal facilities. Animal breeding and experimental protocols were approved by the Katholieke Universiteit Leuven institutional animal care committee.

Antigens, Immunization and Treatments

PSBP was purified according to a previously described procedure.[7] Eight-week-old male mice were immunized on days 0 and 15 with 30 μg purified PSBP plus complete Freund's adjuvant in a final volume of 0.15 ml per animal (PSBP group) or complete Freund's adjuvant alone (controls). Mice received 4 injections intradermally at different sites, including the right foot pad (0.025 ml), the left foot pad (0.025 ml), the tail base (0.050 ml) and the shoulders (0.050 ml).

Histology and Immunohistochemistry

Prostate glands collected at day 7 after the last immunization were fixed in 4% formalin solution and processed for conventional histology. A prostatitis score was calculated for individual glands by summing the grade of each section and dividing the total number of sections examined, usually 4. Inflammation degree was assessed using a scale of 0—no inflammation, 1—focal atrophy in a portion of acini and luminal corpora amylacea, 2—atrophy and numerous vacuoles in the epithelium of most acini, hemorrhage and moderate mononuclear cell infiltration, and 3—atrophy and numerous vacuoles in the epithelium of most acini, hemorrhage and marked mononuclear cell infiltration.[13]

For immunohistochemistry the prostate from immunized and control mice were excised at day 8 or 21 after the first immunization, cryoprotected in 30% sucrose and frozen in TissueTek® OCT™. Cryostat sections (5 μm) were air dried for several hours, blocked to avoid nonspecific binding and incubated for 1 hour at 37C with a fluorescein isothiocyanate labeled monoclonal antibody against CD45RB (BD®).

Cell Mediated Immune Response

Single cell suspensions were prepared in Hanks balanced salt solution from teased spleen or pooled lymph nodes of individual mice. Cell suspensions in Dulbecco's modified Eagle's medium supplemented with GlutaMAX™, sodium pyruvate, nonessential amino acids, HEPES buffer, penicillin/streptomycin, 50 mM 2-mercaptoethanol and 10% fetal calf serum were cultured at 3×10^5 cells per well in 0.2 ml volume in flat bottomed 96-well microtiter plates. PSBP was added to a concentration of 20 μg/ml. All cell combinations were performed in quadruplicate. Plates were incubated for 4 days at 37C in water saturated 7.5% CO_2 and pulsed for the final 18 hours with 1 μCi [methyl-^{3}H] thymidine. Labeled material was automatically harvested and counted in a β-plate scanner. The response is expressed as a proliferation index calculated from cpm incorporated in antigen pulsed cultures per cpm incorporated in cultures with medium alone.

Culture Supernatant Cytokines

Supernatants from cultures prepared as described were collected after 72 hours. IFN-γ and IL-4 quantities were determined by a solid phase sandwich ELISA protocol according to manufacturer instructions. Briefly, 96-well plates were coated with primary anti-IFN-γ or anti-IL-4 capture Abs and blocked with PBS-5% bovine serum albumin. Supernatant samples were incubated 2 hours at room temperature, followed by the addition of biotinylated anti-IFN-γ detecting mAb or biotinylated anti-IL-4 detecting mAb. Plates were developed by adding avidin peroxidase and its substrate, tetramethylbenzidine. OD was measured at 450 nm in a microplate reader. IFN-γ and IL-4 amounts were extrapolated from the standard curve. Results are expressed in pg/ml.

Specific Antibody Detection

Antibodies against PSBP were titrated by conventional ELISA in Costar® multiwell plates precoated with 100 μl PSBP per well at 20 μg/ml in 0.05 M carbonate buffer, pH 9.6. After overnight incubation at 4C microwells were washed twice and blocked with PBS-1% bovine serum albumin for 2 hours, rinsed with PBS-0.05% Tween 20 and filled with 100 μl serum sample dilutions (1:50) for 1 hour at 37C. To detect specific IgG1 and IgG2a plates were incubated with biotinylated anti-mouse IgG1 and biotinylated anti-mouse IgG2a antibodies (BD) for 1 hour at 37C. Plates were developed by adding avidin peroxidase and its substrate, tetramethylbenzidine. OD was measured at 450 nm in a microplate reader (Bio-Rad).

Quantitative Real-Time RT-PCR

Quantitative RT-PCR was done for IL-12, IFN-γ, IL-17, IL-10, IL-4 and β-actin in prostate glands from control and immunized mice at days 8 and 21, as previously described.[14] Briefly, total RNA was extracted using the TRIzol® method. A constant amount of 3 μg target RNA was reverse transcribed using 100 U SuperScript® II RT at 42C for 80 minutes in the presence of 5 μmol/l oligo deoxythymidine 16 (PerkinElmer®). PCR reactions were performed in the Prism® 7700 Sequence Detector containing a GeneAmp® PCR system 9600. PCR amplifications were done in triplicate wells. Data are expressed as relative input copy number normalized to β-actin.

Prostatitis clinical score according to histological findings

	Mean ± SD Score
NOD 8 dpi:*	
CFA	0.20 ± 0.20
PSBP	1.10 ± 0.20
NOD 21 dpi:*	
CFA	0.10 ± 0.22
PSBP	2.40 ± 0.42
21 dpi:	
C57B1/6	1.60 ± 0.42
IRF-1 KO	0.15 ± 0.22*
STAT-1 KO	0.25 ± 0.25*

* $p < 0.05$.

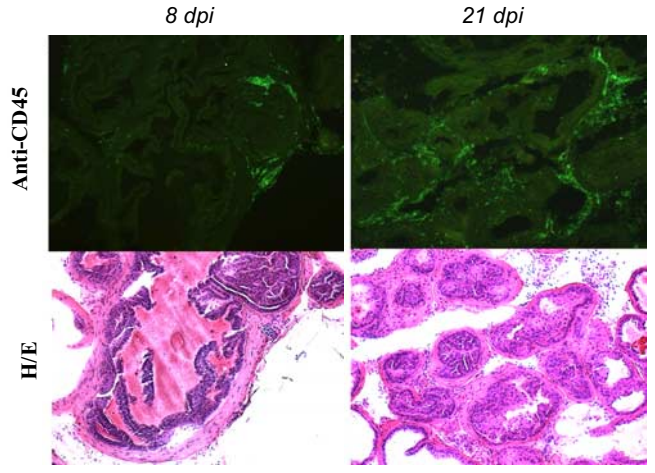

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Figure 2. Increased IFN-γ in mice with EAP. Immunofluorescence and histology of prostates of autoimmune PSBP NOD mice excised at 8 or 21 dpi. Cryostat sections were incubated for 1 hour at 37C with fluorescein isothiocyanate labeled monoclonal against CD45RB. *H/E*, H & E. Reduced from ×200.

Statistical Analysis

Statistical analysis was performed with the Wilcoxon paired and the paired t test, 1-way ANOVA followed by Dunnett's test when ANOVA showed statistical differences among experimental groups, and Fisher's exact test as appropriate with $p < 0.05$ considered statistically significant.

RESULTS

We previously reported that strong autoimmune prostatitis develops in NOD and C57Bl/6 mice since immunized mice showed a T-cell mediated response accompanied by a florid infiltrate circumscribed at the lateral and dorsal prostatic lobes.[7,8] To further characterize the autoimmune response we currently studied the specific autoimmune response at different times of the experimental protocol and quantitative analysis of intraprostatic mRNA levels of different cytokines in NOD mice. Autoimmune NOD mice showed prostate specific IFN-γ secreting cells in the periphery and Th1 related cytokines in the target organ.

No response was observed in controls but a positive proliferative response was noted in lymph node and spleen cells from immunized NOD mice at day 8 (fig. 1, *A* and *B*). The positive PSBP specific lymphoproliferative response in lymph node cells decreased as disease progressed but by day 21 it had increased in PSBP immunized mouse spleen cells. This proliferative response was accompanied by specific IFN-γ secreting cells detectable in the spleen (fig. 1, *C*) and in lymph nodes (data not shown) but no IL-4 production was detected (fig. 1, *D*). A specific humoral response was also observed in immunized mice, characterized by increased specific IgG2a and IgG1 at day 21 (fig. 1, *E* and *F*). PSBP specific IgG2a showed a higher increase than PSBP specific IgG1,

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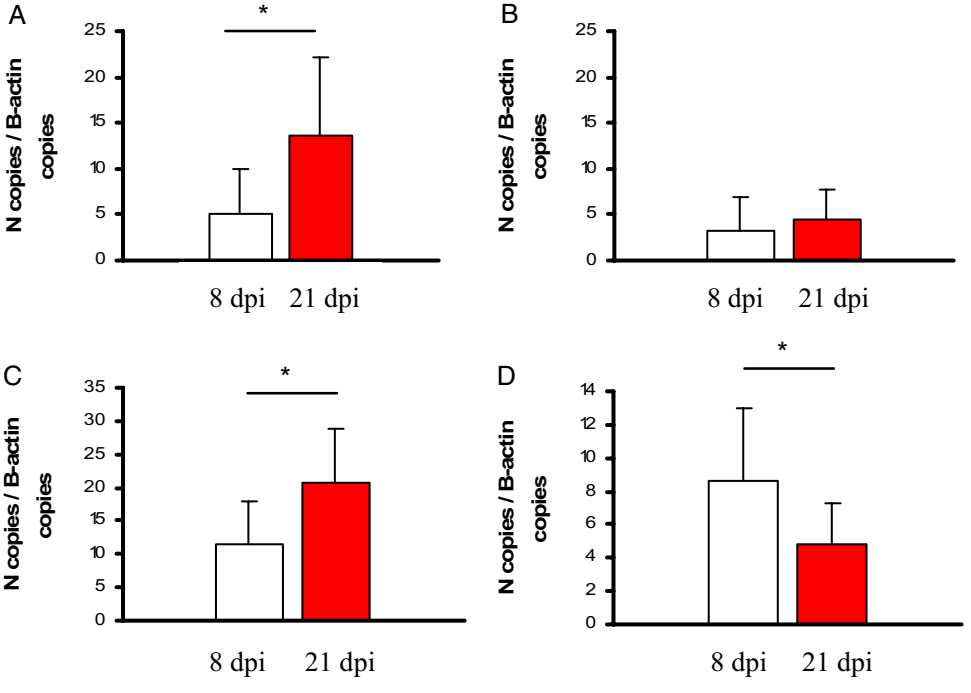

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Figure 3. Increased IFN-γ in mice with EAP. Cytokine secretion in target organ was evaluated by measuring mRNA cytokines in prostate tissue of autoimmune PSBP NOD mice at days 8 and 21 by real-time PCR. *A*, IFN-γ. *B*, IL-4. *C*, IL-12. *D*, IL-10. Asterisk indicates $p < 0.05$.

suggesting deviation to a Th1 pattern in the immune response involved in EAP (fig. 1, *E* and *F*). Histological changes in the target organ revealed minor lesions 8 days after PSBP immunization, and marked lymphomononuclear cell infiltration and atrophy of the epithelium of most acini accompanied by numerous vacuoles in the prostate gland on day 21 after immunization (see table and fig. 2). In parallel CD45+ cells infiltrating the prostate gland showed the same kinetic pattern with an increase in CD45+ infiltrating cells as disease progressed (fig. 2). When different cytokines in the target gland were analyzed, significantly increased IFN-γ and IL-12 were observed in the prostate of mice with advanced disease on day 21. Intraprostatic IL-4 showed no major change but IL-10 significantly decreased as disease progressed (fig. 3).

IFN-γ Role in Induced Autoimmune Prostatitis

C57Bl/6 (WT), IRF-1−/− and STAT-1−/− mice were immunized, and the immune response and histological changes were analyzed. C57Bl/6 mice showed high PSBP specific IFN-γ secretion at days 8 and 21 (fig. 4, *A* and *B*), and increased PSBP specific serum antibodies of IgG2a and IgG1 isotypes (fig. 2). Immunized IRF-1−/− and STAT-1−/− mice had significantly decreased PSBP specific IFN-γ secretion (fig. 4, *A* and *B*) with no detectable IL-4 production (data not shown). Analysis of specific antibodies at day 21 showed lower IgG2a ($p < 0.05$) but IgG1 levels similar to those in WT mice (fig. 4, *C* and *D*).

When prostate sections were analyzed, mononuclear cell infiltration with epithelial acini atrophy was seen in WT glands but neither infiltration nor lesions were observed in immunized mice deficient in IRF-1 and STAT-1 (see table and fig. 5). Analysis of prostate cytokines showed increased IFN-γ, IL-12 and IL-10 in PSBP immunized WT mice on day 21. Analysis of these cytokines in the prostate of PSBP immunized IRF-1−/− and STAT-1−/− mice revealed lower IFN-γ only in IRF-1−/− mice ($p < 0.05$) while STAT-1−/− mice showed no differences vs WT controls. IRF-1−/− and STAT-1−/− mice had significantly decreased IL-12 and IL-10 compared to WT mice ($p < 0.05$). While no detectable IL-4 was noted in immunized WT mice, an increase in this cytokine was observed in mice deficient in key molecules involved in the IFN-γ signaling cascade (fig. 6). Intraprostatic IL-17 was not detected at 21 dpi in any experimental group at the time point assayed (data not shown).

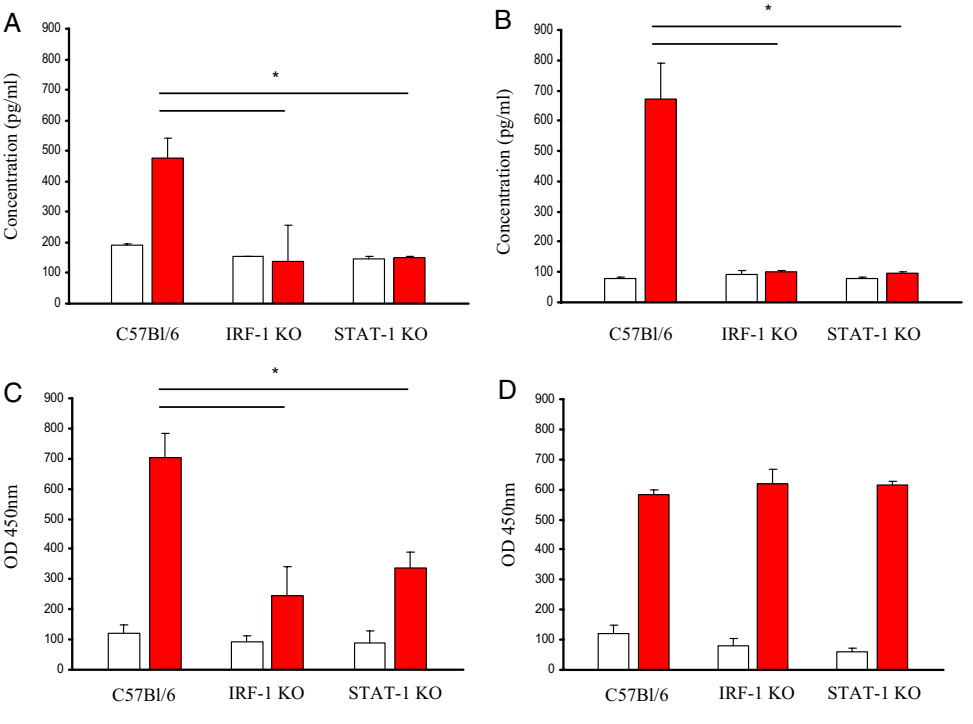

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Figure 4. IFN-γ role in mice with EAP. *A* and *B*, IFN-γ specific secretion from draining lymph node mononuclear cells of control CFA and autoimmune PSBP deficient mice, including 8 IRF-1 KO, 8 STAT-1α KO and 10 WT C57BL/6 mice, stimulated with PSBP in vitro on days 8 and 21 after immunization. IFN-γ in culture supernatant was quantitated by sandwich ELISA. *A*, 8 dpi. *B*, 21 dpi. *C* and *D*, humoral autoimmune response. PSBP specific levels on day 21 after immunization were assayed in serum samples of control CFA, and autoimmune PSBP deficient IRF1 KO, STAT1 KO and WT C57BL/6 mice by indirect ELISA. *C*, IgG1. *D*, IgG2a. Asterisk indicates $p < 0.05$.

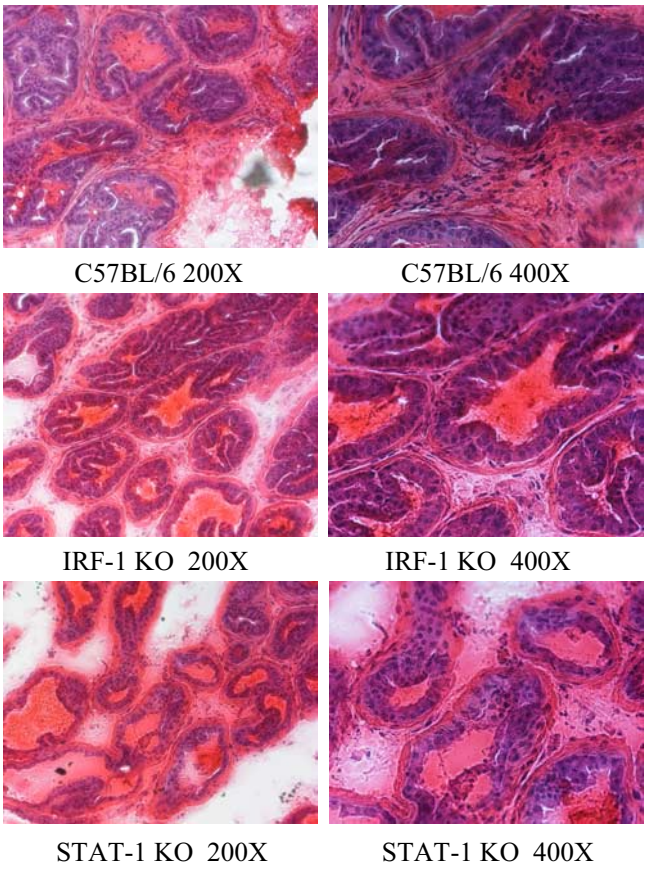

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Figure 5. IFN-γ role in mice with EAP. Microphotographs show histology of prostates of autoimmune WT C57Bl/6, IRF-1 KO and STAT-1 KO mice excised at 21 dpi.

DISCUSSION

Our results argue for a relevant role of IFN-γ in EAP, an animal model of the human disease CP/CPPS. EAP was largely characterized by prostate specific IFN-γ secreting cells in the periphery and Th1 related cytokines in the target organ. Current findings agree with data in the EAP rat model and in the human disease.[4,5,13] In accord with these previous data we observed Th1 cytokines in the target organ accompanying the course of the autoimmune response in the periphery. Increased INFγ and IL-12, and low IL-10 were noted in prostate tissue as the disease progressed, in parallel with the presence of antigen specific INF-γ secreting cells in the periphery.

The key role of IFN-γ in autoimmune prostatitis was also noted in mice deficient in IRF-1 or STAT-1. Deficiency in these transcription factors involved in the INF-γ signaling cascade made these mice resistant to EAP. IFN-γ acts mostly via the Janus kinase/STAT-1 pathway.[15] The diverse biological effects of IFN-γ rely on its actions in regulating gene transcription. The transcription factor STAT-1 is a key mediator of biological responses to IFN-γ. Upon triggering IFN-γ receptor Janus kinases are activated and phosphorylate STAT-1, which then acts at the nuclear level, binding to IFN-γ activated sequences in target gene promoters.[16] As a result, STAT-1 modulates the expression of primary response genes, including other transcription factors such as IRF-1. IRF-1 is strongly inducible after IFN-γ stimulation and participates in the transcription of many IFN-γ regulated secondary genes whose promoters contain IFN-γ stimulated regulatory elements.[17] PSBP immunized IRF-1−/− and STAT-1−/− mice did not have prostate gland infiltration and/or alterations. The typical prostate specific autoimmune response characterized by IFN-γ secreting lymphocytes could not develop. Also, they showed decreased IFN-γ, IL-12 and IL-10 in the target organ. Although intraprostatic IFN-γ did not significantly decrease in STAT-1−/− mice, positive PSBP specific IFN-γ secretion was not evident in the periphery and these animals showed no histological changes or prostate lesion. These findings suggest that, although IFN-γ is present in the cytokine milieu in the target organ, it does not have its effector function because these mice are deficient in key transcription factors involved in its signaling cascade. While IL-4 was almost undetectable in prostate samples from PSBP immunized NOD and C57Bl/6 WT mice, enhanced IL-4 levels were detected in PSBP immunized IRF-1−/− and STAT-1−/− mice. We analyzed IL-4 in culture supernatants of these animals but observed no detectable values. These results support the importance of IFN-γ in the pathogenesis of autoimmune prostatitis and confirm that EAP cannot develop in mice deficient in IRF-1 or STAT-1.

Different results were reported when animals deficient in IRF-1 or STAT-1 were analyzed for susceptibility to typical Th1 mediated autoimmune diseases such as EAE, CIA and diabetes. IRF-1 deficient mice showed decreased incidence and severity of CIA, EAE and diabetes.[18–20] In regard to EAE protection from brain inflammation in mice lacking IRF-1 was associated with Th2 type cytokines.[19] In contrast, STAT-1−/− mice were highly susceptible to EAE and had more severe, accelerated disease with atypical neuropathological features.[21] Similar to these contradictory reports were those in which IFN-γ−/− or IFN-γR−/− mice showed CIA or EAE.[22,23] Recent studies suggest greater diversification of the CD4 T-cell effector repertoire than that encompassed by the Th1/Th2 paradigm. New studies linking the cytokines IL-23 and IL-17 to immune pathogenesis previously attributed to the Th1 lineage have led to the delineation of a new effector CD4 T-cell arm referred to as Th17.[24] IL-17 deficient mice show impaired joint inflammation after type II collagen immunization.[25] Furthermore, neutralization of IL-17 decreases disease severity[26] and IL-17 over expression in joints exacerbates the dis-

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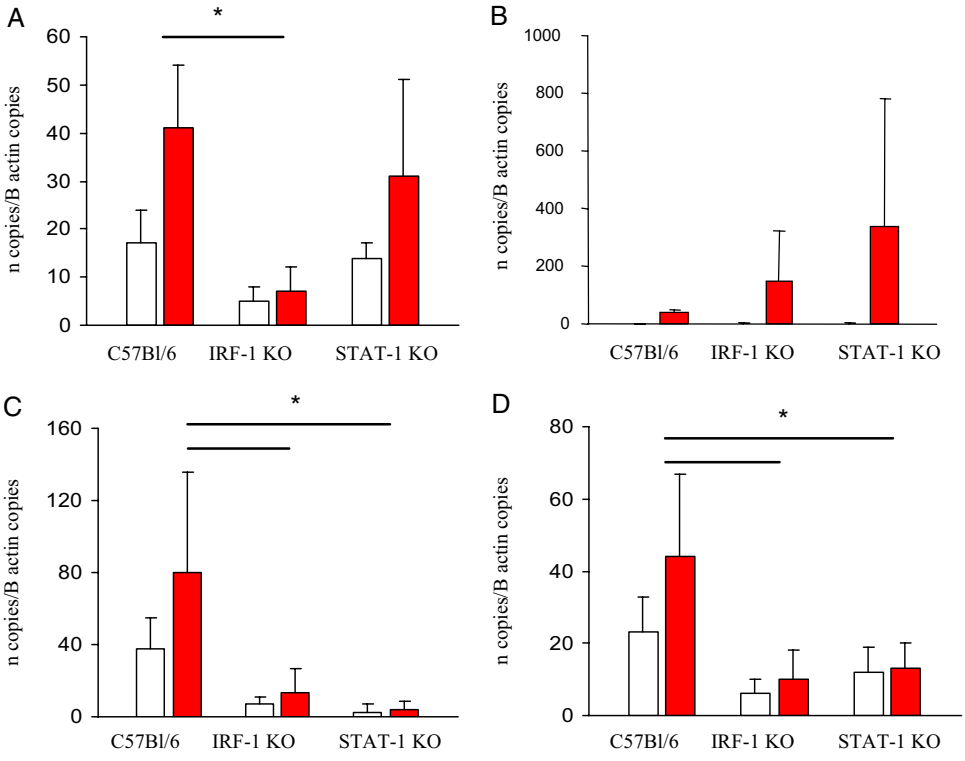

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Figure 6. IFN-γ role in mice with EAP. Cytokine secretion in target organ was evaluated by measuring mRNA cytokines in prostate tissue of control CFA and autoimmune PSBP deficient IRF1 KO, STAT1 KO and WT C57BL/6 mice by real-time PCR. *A*, IFN-γ. *B*, IL-4. *C*, IL-12. *D*, IL-10. Asterisk indicates $p < 0.05$.

ease.[27] These results link the development of self-antigen reactive IL-17 producing effector CD4 T cells but not IFN-γ producing effectors to autoimmune inflammation in these models. However, other experimental models of rheumatoid arthritis, such as proteoglycan induced autoimmune arthritis, are independent of IL-17.[28] Thus, in at least some forms of autoimmunity the IL-23/IL-17 cytokine axis and not the IL-12/IFN-γ axis is crucial for disease pathogenesis. In EAP models the exact role of Th17 cells has not yet been properly analyzed but the fact that mice deficient in 2 transcription factors crucial in the IFN-γ signaling cascade are resistant to EAP argue for a key role of IFN-γ in this model and suggest that Th17 may not have a major role in EAP. In fact, intraprostatic IL-17 was not detected in deficient or WT autoimmune animals at a time when the disease was clearly established. In accordance with our results Penna et al reported that IFN-γ deficient mice showed a significantly decreased number of infiltrates in the prostate and suggested that the T cells specific for prostate antigens are IFN-γ producing Th1 cells, which may have an essential role in disease induction.[29] However, they also reported that prostate draining lymph node T cells in NOD mice immunized with prostate homogenate produced IFN-γ and IL-17 after a polyclonal stimulus.[30] However, they did not investigate the presence of this cytokine in the target organ. More data are needed to assign any Th17 cell role in EAP cases.

CONCLUSIONS

The current results argue for a crucial role of IFN-γ as a key factor in EAP pathogenesis. Major characteristics of the autoimmune response in patients with CP/CPPS have barely been described. Intense research in human disease and its animal models is promptly required to identify the pathogenic mechanisms underlying CP/CPPS to find a more rational, effective therapy in this large patient group.

ACKNOWLEDGMENTS

Dr. David E. Levy, Molecular Oncology and Immunology Program, New York University School of Medicine, New York, New York, provided STAT-1−/− mice. Dr. W. Tak, Ontario Cancer Institute, Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, provided IRF-1−/− mice.

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