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Spontaneous and Prostatic Steroid Binding Protein Peptide-Induced Autoimmune Prostatitis in the Nonobese Diabetic Mouse

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Chronic nonbacterial prostatitis is a poorly defined syndrome of putative autoimmune origin. To further understand its pathogenesis, we have analyzed autoimmune prostatitis in the NOD mouse, a strain genetically prone to develop different organ-specific autoimmune diseases. Spontaneous development of autoimmune prostatitis in the NOD male, defined by lymphomononuclear cell infiltration in the prostate gland, is well-established by ~20 wk of age and is stably maintained afterward. Disease development is indistinguishable in NOD and NOR mice, but is markedly delayed in IFN-γ-deficient NOD mice. A T cell response to the prostate-specific autoantigen prostatic steroid-binding protein (PSBP) can be detected in NOD males before development of prostate infiltration, indicating lack of tolerance to this self Ag. The intraprostatic inflammatory infiltrate is characterized by Th1-type CD4+ T cells, which are able to transfer autoimmune prostatitis into NOD.SCID recipients. We characterize here experimental autoimmune prostatitis, detected by intraprostatic infiltrate and PSBP-specific T cell responses, induced in 6- to 8-wk-old NOD males by immunization with synthetic peptides corresponding to the CI subunit of PSBP. Three PSBP peptides induce in NOD mice vigorous T and B cell responses, paralleled by a marked lymphomononuclear cell infiltration in the prostate. Two of these peptides, PSBP21–40 and PSBP61–80, correspond to immunodominant self epitopes naturally processed in NOD mice after immunization with PSBP, whereas peptide PSBP31–111 represents a cryptic epitope. These model systems address pathogenetic mechanisms in autoimmune prostatitis and will facilitate testing and mechanistic analysis of therapeutic approaches in this condition. The Journal of Immunology, 2007, 179: 1559–1567.

Prostatitis is a common condition seen in urologic practice (1) and represents a major medical problem (2). The National Institutes of Health consensus definition and classification identifies four categories of prostatitis (3). Category I includes acute bacterial prostatitis; category II includes chronic bacterial prostatitis. Category III, also known as chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), is defined by pelvic pain in the absence of demonstrable bacterial infection, and is further subdivided into category IIIA, or inflammatory, and category IIIB or noninflammatory, based on the presence of leukocytes in expressed prostatic secretion or seminal plasma, respectively. Category IV, or asymptomatic inflammatory prostatitis, is defined by the presence of leukocytes in seminal secretions or by inflammatory infiltrates detected in histological specimens, in the absence of typical chronic pelvic pain. The prevalence rate of CP/CPPS (National Institutes of Health category III) in the general population ranges from 5 to 14.2% (4), suggesting that ~60–80% of all prostatitis could be classified as CP/CPPS.

CP/CPPS is a poorly understood syndrome but immunological, neurological, and endocrine dysfunctions have been proposed to be involved in disease development (5). Evidence indicating a predominant autoimmune component in the pathogenesis of CP/CPPS is however emerging (6), and the autoimmune nature of CP/CPPS is also supported by experimental models of prostatitis (7). These models have been quite extensively characterized, demonstrating that immunization of rats or mice with prostate gland extracts can induce T cell and Ab responses to prostate Ags, associated with histological evidence of prostate inflammation (7). The development of spontaneous prostatitis is well-documented in several rat strains (7). In addition, experimental autoimmune prostatitis (EAP) can be induced in rodents immunizing rats with male accessory glands (8–10) or of mice with prostate homogenate (11) emulsified in CFA. EAP can also be induced in different mouse strains by thymectomy at day 3 of age (12) and tissue-specific CD4+ suppressor T cells activated extrathymically by prostate Ags have been implicated in maintaining peripheral tolerance to prostatic autoantigens (13, 14).

To further understand the pathogenesis of CP/CPPS, and to provide more refined models to assess therapeutic strategies, we have studied spontaneous and autoantigen-induced autoimmune prostatitis in the NOD mouse, a strain genetically prone to develop different organ-specific autoimmune diseases, including type 1 diabetes, thyroiditis, sialilitis, ooforitis, adrenalitis, and orchitis (15). The NOD mouse is susceptible to induction of EAP by injection of mouse prostate homogenate in CFA, and this protocol induces a florid intraprostatic leukocyte infiltrate in 100% of NOD male mice (16). Subsequent work has shown that EAP can also be induced in NOD mice by injection of prostatic steroid-binding protein (PSBP) (17), a major autoantigen (also known as prostatein) in...
rodent EAP (18, 19). The NOD male mouse responds to PSBP by developing specific cellular and humoral autoimmune responses associated with inflammatory infiltrates in the prostate (17). In addition, using NOD mice lacking expression of β2-microglobulin or MHC class II β-chain, an essential role for CD4+ T cells has been shown in the development of EAP in this model (17).

In the present study, we show that NOD mice spontaneously develop autoimmune prostatitis, defined by intraprostatic lymphomononuclear cell infiltrates, and this is associated with marked autoreactive T cell responses, paralleled by a marked lymphomononuclear cell infiltrates, and this is associated with lack of tolerance to PSBP and IA-2 in NOD and NOR mice. Spleen cells (10^6/well) from NOD, BALB/c, C57BL/6, and NOR mice were cultured (left panel) without Ag (△) or with 0.3 μM purified PSBP (□). After 72 h, IFN-γ secretion was determined in culture supernatants by two-site ELISA. Results in the right panel report IFN-γ secretion in response to IA-2, expressed as percent of the anti-IA-2 IFN-γ response induced in NOD spleen cells (1664 ± 673 pg/ml, n = 5). **, p < 0.005 by Mann-Whitney U test for BALB/c and C57BL/6 vs NOD, or between BALB/c and C57BL/6.

Materials and Methods

Animals

BALB/c mice and CD rats were purchased from Charles River Laboratories. NOD.SCID, NOR, and NOR mice from The Jackson Laboratory were isolator-reared at Charles River Laboratories. Mice were kept under specific pathogen-free conditions. All animal studies have been approved by the institutional review board.

Antigens

To prepare prostate homogenate, mouse CD1 prostates were homogenized at 4°C in the presence of a protease inhibitor mixture, centrifuged at 10,000 rpm for 30 min at 4°C, and protein concentration was measured by a Bio-Rad protein assay kit.

To purify PSBP, whose sequence is identical in mice and rats, prostates from adult CD rats were homogenized in Tris-HCl (pH 7.0) and processed as described (18). Briefly, insoluble material was removed by centrifugation at 4°C for 40 min, the resultant supernatant was diluted in Tris-HCl (1:1), and then fractionated by ion exchange chromatography with a Q-Sepharose Fast Flow column. Proteins were eluted with a linear NaCl gradient (0–0.1 M NaCl) in Tris-HCl. The protein content of each fraction was evaluated by absorbance at 280 nm. Fractions containing proteins were run under non-denaturating conditions in 0–15% gradient polyacrylamide gel (SDS-PAGE) and the gels were stained with Coomassie Blue. Fractions containing two bands of 18 and 20 kDa (corresponding to both subunits of PSBP) and fractions containing a single band of 40 kDa (corresponding to native PSBP) were collected and concentrated by Centriplus concentrator. Recombinant mouse IA-2 was expressed in *Escherichia coli* as a GST fusion protein and purified by affinity chromatography on glutathione-Sepharose, followed by thrombin cleavage to recover >95% pure recombinant murine IA-2, as described (20).
buffer. After 48 h, each incubate was transferred to the corresponding well of an ELISA plate (Maxisorp; Nunc) containing prebound OX-6 Abs (10 μg/ml overnight at 4°C followed by washing). After incubation at 37°C for 2 h and washing, bound biotinylated peptide-MHC complexes were detected colorimetrically at 405 nm with streptavidin-alkaline phosphatase and p-nitrophenylphosphate. Competition curves were plotted and the peptide affinity for MHC molecules was expressed as the peptide concentration required to inhibit the binding of biotinylated peptide by 50% (IC50).

Evaluation of T cell responses

To evaluate T cell responses from unprimed 8-wk-old mice, spleen cell suspensions were cultured in flat-bottom 96-well plates (Costar) in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (Serono), 50 μM 2-ME, 2 mM L-glutamine, and 50 μg/ml gentamicin (Sigma-Aldrich) with the indicated Ag concentrations.

To evaluate T cell responses from primed mice, 8-wk-old NOD mice were immunized with Ags emulsified in CFA and the indicated Ag dose/mouse injected s.c. into the hind footpads. Nine to 10 days later, single-cell suspensions from popliteal lymph nodes were cultured in flat-bottom 96-well plates with the indicated concentrations of PSBP or overlapping PSBP peptides. Lymph node cell cultures were performed in synthetic HL-1 medium (Ventrex Laboratories), supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin (Sigma-Aldrich). After 48–72 h of culture, IFN-γ was quantitated in culture supernatants using Optieia kit (BD Pharmingen). Detection limits were 15 pg/ml.

Flow cytometric analysis of prostate-infiltrating cells

Individual prostate were digested in 1 ml of HBSS containing 3 mg/ml collagenase IV (Sigma-Aldrich), by shaking (200 rpm) at 37°C for 20 min. Cell suspensions were collected after diluting the enzyme with ice-cold HBSS containing 5% FCS and removing the aggregates by settling 2 min on ice. The cells, were washed three times, and passed over a MACS Pre-Separation Filter (Miltenyi Biotec) to remove cell aggregates and clumps. Prostate single-cell suspensions were either used directly for flow cytometric analysis, or enriched first by Percoll gradient. Briefly, after washing, cells were harvested and layered over a 55% Percoll solution (Pharmacia), followed by centrifugation at 1900 × g for 13 min at 4°C. Cells recovered from the medium-Percoll interface were collected and immediately stained for flow cytometric analysis.

Prostate-infiltrating mononuclear cells, isolated as described above, were preincubated with 400 μg/ml mouse IgG (Sigma-Aldrich) for 15 min at room temperature to block unspecific staining. Cells were then stained with anti-CD4 (RM-4-4), anti-CD8 (53-6.7), anti-CD8α (53-5.8), anti-CD11c (HL3) mAbs, labeled with either FITC, PE, or PerCP. FACS medium used throughout was PBS containing 5% FCS and 0.1% NaN3.

Alternatively, single-cell suspensions, obtained by collagenase digestion of prostate, were incubated with anti-CD4 plus anti-CD8 mAb-coated MicroBeads (Miltenyi Biotec), and applied onto MiniMACS separation columns (Miltenyi Biotec). Prostatic CD8+ cells were stimulated, immediately after isolation, with 50 ng/ml PMA and 750 ng/ml ionomycin (all from Sigma-Aldrich) as described (22). Cells were stained with FITC-labeled rat anti-mouse IFN-γ (XMG1.2), PE-labeled rat anti-mouse IL-4 (4A7), and anti-IL-4-PE mAbs. The proportions of prostate-infiltrating T cells producing IFN-γ and/or IL-4 is shown in the respective quadrants. A, Mean ± SE of intraprostatic infiltrates/cross-section from 28-wk-old NOD (n = 10) and IFN-γ-deficient NOD mice (n = 4). *, p = 0.036 by Mann-Whitney U test. B, Spleen CD4+ T cells from 24-wk-old NOD mice were adoptively transferred (4 × 106/recipient) into NOD.SCID mice (n = 7), which were analyzed 45 days later. Mean ± SE of intraprostatic infiltrates/cross-section in NOD.SCID recipients transferred with spleen CD4+ T cells and their relative serum glucose levels is reported. The bar represents normoglycemic levels.

Flow cytometric analysis of prostate-infiltrating cells

FIGURE 2. Characterization of intraprostatic cell infiltrates. Prostate cell suspensions from unmanipulated 20-wk-old NOD mice were obtained as described in Materials and Methods, and double stained for the indicated cell surface markers. The proportions of positive cells were determined by flow cytometry following lymphocyte gating. Percentages of positive cells are shown in the appropriate quadrants. A, CD4 vs CD8 staining. B, B220 vs I-Aγ staining. C, Prostate cells enriched by 55% Percoll gradient were stained for CD11c and B220. The upper box indicates CD11c+B220+ myeloid DCs, the middle box CD11clowB220+ cells resembling plasmacytoid DCs, and the lower box CD11c–B220– B cells. D, H&E and immunohistochemical stainings for CD4, CD8, B220, CD11c, and CD11b in cryostatic sections of prostate from a representative 20-wk-old NOD mouse. Original magnification, ×200.

Peptide-binding assay

I-Aγ molecules were affinity-purified from detergent lysates of 4G4.7 cells by sequential adsorption to 34.14 and 14.4.4S mAbs, and desorption from OX-6 mAb respectively, as previously described (21). Peptides were dissolved at 10 mM in DMSO and diluted into 25% DMSO/PBS for assay. The indicator peptide HELE10–23 was synthesized with two spacer residues and a biotin molecule at the NH2 terminus. Approximately 500 nM of biotinylated peptide and each test peptide were coincubated with 200 ng of MHC class II protein in U-bottom polypropylene 96-well plates (Costar Serocluster; Costar) in binding buffer at room temperature (RT). The binding buffer was 6.7 mM citric phosphate (pH 6.0) with 0.15 M NaCl, 2% Nonidet P-40, 2 mM EDTA, and the protease inhibitors as used in the lysis

FIGURE 3. The prostate-infiltrating CD4+ T cells display a Th1 phenotype and transfer disease. A, Prostate-infiltrating T cells from 24-wk-old NOD mice, purified and stimulated with PMA/ionomycin as described in Materials and Methods, were double-stained intracellularly with anti-IFN-γ-FITC and anti-IL-4-PE mAbs. The proportions of prostate-infiltrating T cells producing IFN-γ and/or IL-4 is shown in the respective quadrants. B, Mean ± SE of intraprostatic infiltrates/cross-section from 28-wk-old NOD (n = 10) and IFN-γ-deficient NOD mice (n = 4), *p = 0.036 by Mann-Whitney U test. C, Spleen CD4+ T cells from 24-wk-old NOD mice were adoptively transferred (4 × 106/recipient) into NOD.SCID mice (n = 7), which were analyzed 45 days later. Mean ± SE of intraprostatic infiltrates/cross-section in NOD.SCID recipients transferred with spleen CD4+ T cells and their relative serum glucose levels is reported. The bar represents normoglycemic levels.
IL-4 (11B11), and CyChrome-labeled anti-CD4 (L3T4). Analysis was performed with a LSR flow cytometer (BD Biosciences) equipped with CellQuest software.

**Cell transfer**

Spleen cells from 24-wk-old NOD mice, were incubated with anti-CD4 mAb and negatively selected with CD4 T cell isolation kit (Miltenyi Biotec). Purified CD4⁺ T cells were adoptively transferred (4 × 10⁶/recipient) by i.v. injection into NOD.SCID male mice. NOD.SCID recipients were analyzed 45 days after cell transfer for intraprostatic infiltrates and glycermia. Glucose levels in the tail venous blood were quantified using a EUROFlash glucometer (Lifescan).

**Immunohistochemistry**

Prostates were snap-frozen in Tissue Tek (Miles Laboratories) and stored at −80°C. Frozen section (5-μm thick) were air-dried and then fixed in acetone for 10 min. Endogenous peroxidase activity was blocked with 2% hydrogen peroxide and 0.1% sodium azide in cold TBS. Endogenous biotin was blocked by incubation with an avidin solution mixed in 1% BSA in PBS for 15 min and followed by a biotin solution mixed in 1% BSA in PBS for 15 min (Vector Laboratories). Sections were stained with H&E, or with biotinylated mAbs against CD4, CD8, B220, CD11c, or CD11b (all from BD Pharmingen), followed by streptavidin-peroxidase conjugate. 3-amino-9-ethylcarbazole (DakoCytomation) was used as chromogen, and hematoxylin as a counterstain.

**Quantification of prostate infiltrates**

Prostates were snap-frozen in Tissue Tek (Miles Laboratories), and 50 cryostatic sections/mouse, covering the entire prostate, were scored blindly by a pathologist, after staining with H&E, for the presence of intraprostatic lymphomonuclear cell infiltrates organized in nodules. Single infiltrating cells were disregarded. Data are reported as number of infiltrates per cross-section.

**Statistical analysis**

Differences between groups were evaluated using two-tailed Mann-Whitney U test or unpaired two-tailed Student’s t test, as appropriate. Differences were considered to be statistically significant at p < 0.05.

**Results**

**Spontaneous development of autoimmune prostatitis in the NOD mouse**

Autoimmune prostatitis is characterized by lymphomonuclear cell infiltrates in the prostate gland. To detect intraprostatic cell infiltrates and quantify their extent, the entire mouse prostate was cut and 50 sections/prostate, stained with H&E, were scored blindly by a pathologist. Results in Fig. 1A show that autoimmune prostatitis, defined by discrete intraprostatic lymphomonuclear cell infiltrates organized in nodules (Fig. 1B), is already present, with a low score, in the NOD mouse at 7 wk of age. The extent and number of intraprostatic lymphomonuclear cell infiltration increases with age in the NOD mouse, reaching a plateau at 28 wk of age which is stably maintained afterward (Fig. 1A). In contrast, BALB/c mice fail to show appreciable intraprostatic cell infiltrates at any age tested (Fig. 1C). Interestingly, the NOR mouse, a recombinant congenic strain in which limited regions of the NOD genome have been replaced by the C57BL/KsJ genome and is resistant to the development of insulitis and type 1 diabetes (23), develops intraprostatic infiltrates similarly to the NOD mouse (Fig. 1C). To determine whether intraprostatic inflammatory infiltrates were associated with autoreactive T cell responses to prostate Ags, we examined responsiveness to PSBP, a major autoantigen in NOD EAP (17). Splenocytes from 8-wk-old naive NOD, NOR, C57BL/6, and BALB/c mice were cultured with purified mouse PSBP or with recombinant mouse IA-2, an islet autoantigen associated with type 1 diabetes in NOD mice (20), and IFN-γ secretion was measured after 72 h of culture (Fig. 1D, left panel). Autoreactive T cell responses to PSBP can be detected in all mouse strains tested, but they are significantly higher in NOD compared with BALB/c or C57BL/6 mice.
suggesting reduced tolerance to this self Ag in the NOD mouse. Interestingly, the autoreactive response to PSBP is significantly higher in C57BL/6 compared with BALB/c mice, consistent with the observation that EAP can be induced in C57BL/6, in addition to NOD mice, but not in BALB/c mice (16). The IFN-$\gamma$ response induced by PSBP in spleen cells from naive NOD and NOR mice is similar, indicating a comparable reactivity of the two mouse strains to this prostatic autoantigen. A similar pattern of responsiveness is induced by IA-2 (Fig. 1D, right panel), although IFN-$\gamma$ production to PSBP by NOD spleen cells is 2-fold higher (3252 pg/ml, $n=6$) than in response to IA-2 (1664 pg/ml, $n=5$).

Characterization of intraprostatic cell infiltrate and disease transfer by CD4$^+$ T cells

To characterize the phenotype of prostate-infiltrating cells, pooled prostate glands from 20-wk-old naive NOD mice were treated with collagenase and intraprostatic leukocytes analyzed by cytofluorometry. Results in Fig. 2A demonstrate that $\sim$15% of prostate-infiltrating leukocytes are CD4$^+$ and only 3% CD8$^+$ T cells. B cells, defined by coexpression of B220 and I-A$^\beta$ molecules, are $\sim$33%, and a B220$^+$ I-A$^\beta$-positive cell population is also present (Fig. 2B). Following 55% Percoll gradient centrifugation and staining for CD11c and B220, reveals, in addition to 48% B220$^+$ B cells, a population of 3% CD11c$^{low}$ B220$^{low}$ dendritic cells (DCs), and a population of 9% CD11c$^{high}$ B220$^{high}$ macrophages (Fig. 2C). This pattern of prostate-infiltrating cells is confirmed by immunohistochemistry in prostate glands from 20-wk-old NOD mice (Fig. 2D), showing relatively higher numbers of CD4$^+$ compared with CD8$^+$ T cells, high numbers of B220$^+$ B cells, and scattered CD11c$^+$ DCs and CD11b$^+$ macrophages.

The prostate-infiltrating CD4$^+$ T cells display a Th1 phenotype, as detected by intracytoplasmic staining for IFN-$\gamma$ and IL-4, showing 33% Th1 cells and a negligible percentage of Th2-type cells (Fig. 3A).

Spinalic CD4$^+$ T cells from 20-wk-old NOD mice can adoptively transfer into NOD.SCID recipients both autoimmune prostatitis and type 1 diabetes, as shown by development of prostatic infiltrates and hyperglycemia in recipient mice (Fig. 3C). Therefore, prostate-infiltrating CD4$^+$ T cells developing in the unmanipulated NOD mouse display a Th1 phenotype. In addition, CD4$^+$ T cells from naive NOD mice...
mice are able to adoptively transfer autoimmune prostatitis into NOD.
SCID recipients, a typical feature of autoimmune T cells.

T cell immunogenicity of PSBP peptides in NOD mice
PSBP, a tetramer composed of two subunits, one containing disulfide-linked C1 and C3 chains and the other disulfide-linked C1 and C3 chains, is an autoantigen in rats (18) and is strongly immunogenic in NOD mice (17). As shown in Fig. 1D, splenic T cells from naive NOD mice respond to purified PSBP, indicating lack of T cell tolerance to this autoantigen. To identify immunogenic PSBP epitopes, synthetic peptides of the C1 chain, which is more immunogenic than C2 and C3 chains (18), were generated and tested. The 20-mer PSBP C1 peptides, synthesized, overlapping by 10-aa residues, and covering the entire sequence of the mature C1 protein, are shown in Table I. Peptides PSBP71-90 and PSBP81-100 were insoluble and could not be tested. NOD mice were first immunized into the hind footpads with a mixture of all soluble peptides corresponding to the mature form of the mouse PSBP C1 protein, and 10 days later popliteal lymph node cells were restimulated with the individual peptides. Results in Fig. 4A show that two peptides, PSBP21-40 and PSBP61-80, recall a strong T cell response, quantified by IFN-γ production in culture supernatants. In addition, PSBP91-111 elicited a weaker T cell response, whereas the other peptides tested were markedly less active or completely inactive. To confirm the capacity of active peptides to prime T cells, NOD mice were injected with individual peptides, and 10 days later draining lymph node cells were restimulated with the homologous peptide, as well as with all the other PSBP peptides. Results in Fig. 4B, showing immunizations with individual peptides, confirm that peptides PSBP21-40 and PSBP61-80 are strongly immunogenic in NOD mice, whereas PSBP91-111 is a weaker immunogen. The specificity of the T cell response is illustrated by the complete lack of cross-reactivity among the peptides tested (Fig. 4B). The lack of immunogenicity of PSBP31-50, PSBP41-60, and PSBP51-70 is shown in Fig. 5. These results confirm the data shown in Fig. 4 and also indicate that immunogenic epitopes are not located within the overlapping sequences of adjacent peptides, suggesting core epitopes in PSBP C1 sequences 21–30 and 71–80.

Immunodominant PSBP peptides are naturally processed in NOD mice following immunization with PSBP
To determine whether the immunogenic peptides identified were naturally processed following immunization with purified PSBP, NOD mice were immunized with PSBP into the hind footpads and 10 days later popliteal lymph node cells were restimulated with equimolar concentrations of all soluble PSBP peptides, or PSBP. Results in Fig. 6 show that PSBP21-40 and PSBP61-80 are able to restimulate a T cell response comparable to that recalled by PSBP, indicating that they represent immunodominant epitopes which are naturally processed in vivo from the native PSBP protein. Conversely, the epitope contained within the sequence PSBP91-111 represents a cryptic epitope, because this peptide binds to I-A^d, although with an affinity ~10-fold lower compared with immunodominant epitopes (Fig. 7) and is immunogenic (Figs. 4 and 5) but is unable to restimulate T cells from NOD mice primed with native PSBP (Fig. 6). Binding data (Fig. 7) also confirm that PSBP21-40 and PSBP61-80 are good binders to I-A^d, comparable to the dominant hen egg-white lysozyme (HEL) peptide HEL13-23 (21), whereas peptide PSBP41-60 fails to bind to I-A^d, thus explaining its lack of immunogenicity in NOD mice.

Autoimmune prostatitis induction by immunization with synthetic PSBP peptides
To determine the capacity of PSBP peptides to induce autoimmune prostatitis, we first injected NOD mice with CFA alone, or containing a mixture of all soluble PSBP peptides, or whole mouse prostate homogenate. Results in Fig. 8A show the presence within the prostate, 30 days after priming, of similar percentages of T and B cells following immunization with either pooled PSBP C1 peptides or mouse prostate homogenate, in both cases with higher percentages of CD4^+ compared with CD8^+ T cells. The capacity to induce intraprostatic-infiltrating cells has also been tested by histological analysis of prostate sections following immunization of NOD mice with individual peptides. Results are reported as number of infiltrates/cross-section (Fig. 8B) and representative

FIGURE 6. Immunodominant PSBP peptides are naturally processed in NOD mice following immunization with PSB. NOD mice were immunized into the hind footpads with 2.5 nM of PSBP in CFA. Ten days after immunization, popliteal lymph node cells (4 \times 10^5/well) were cultured in 96-well plates with the indicate concentration of single PSBP peptide. After 72 h, IFN-γ was determined in culture supernatants by two-site ELISA. Data are mean ± SE from five mice per group.

FIGURE 7. Binding of selected PSBP peptides to purified I-A^d molecules. Inhibition of biotinylated HEL10-23 binding to purified I-A^d molecules by unlabeled HEL10-23 or by the indicated PSBP peptides. See Materials and Methods for assay details.
H&E stainings of cryostatic prostate sections are shown in Fig. 8C. The immunodominant peptides PSBP21-40 and PSBP61-80 induce a marked intraprostatic lymphomononuclear cell infiltrate, comparable to that induced by immunization with whole prostate homogenate. Conversely, peptide PSBP41-60, which does not bind to I-A\(^{\text{g7}}\) and is not immunogenic, induces a prostatic infiltrate similar to baseline or CFA controls. The cryptic epitope included in the sequence PSBP91-111 also induces a marked cell infiltrate, comparable to that observed following immunization with immunodominant, naturally processed PSBP epitopes. Immunohistological analysis, as shown in Fig. 8D following immunization with the immunodominant peptide PSBP61-80, demonstrates in intraprostatic infiltrates abundant CD4\(^{+}\) T cells, relatively fewer CD8\(^{+}\) T cells, high numbers of B220\(^{+}\) B cells, and marked infiltration by CD11c\(^{+}\) DCs and CD11b\(^{+}\) macrophages. A similar composition of intraprostatic cell infiltrates was observed following immunization with peptides PSBP21-40 and PSBP91-111 and with whole prostate homogenate (data not shown).

**Discussion**

Results in the present article document, for the first time, the spontaneous development in the NOD mouse, a strain genetically prone to develop different autoimmune diseases, of autoimmune prostatitis, preceded by a spontaneous T cell response to the prostate-specific autoantigen PSBP. EAP, typically quite severe, can be also induced in the NOD mouse by immunization with prostate homogenate or with purified PSBP, confirming previous data (16, 17). We now report the identification of dominant and cryptic epitopes of PSBP that bind to I-A\(^{\text{g7}}\) and induce a strong CD4\(^{+}\) T cell response in the NOD mouse, followed by a marked inflammation of the prostate characterized by prominent intraprostatic lymphomononuclear cell infiltrates.

Autoreactive CD4\(^{+}\) and CD8\(^{+}\) T cells specific for prostate Ags exist in normal individuals (24) and this T cell repertoire could potentially become activated to mount an autoimmune response. Indeed, PBMC and CD4\(^{+}\) T cells from CP/CPPS patients proliferate in response to seminal plasma (25, 26) and to specific prostate Ags (27, 28), indicating expression of the autoreactive T cell repertoire in disease pathogenesis. High-titer IgG autoantibodies to prostate-associated proteins are found in patients with CP/CPPS (29), further indicating a T cell-dependent autoimmune process. In addition, CP/CPPS patients show in seminal plasma higher levels, compared with controls, of proinflammatory cytokines, like IL-1\(\beta\) and TNF-\(\alpha\), and chemokines, like IL-8 (30–35).
Further support for the autoimmune nature of CP/CPPS is provided by EAP models in rodents, demonstrating that immunization of rats or mice with prostate gland extracts can induce T cell and Ab responses to prostate Ags, associated with histological evidence of prostate inflammation (7). Insights into the pathogenesis of experimental autoimmune prostatitis have been provided by injection of mouse prostate homogenate (16, 36) or PSBP (17) in the NOD mouse. In NOD EAP, T cells specific for prostate Ag have been shown to include IFN-γ-producing Th1 cells, that have an essential role in disease induction (17). In addition, we have recently shown that prostate-draining lymph node T cells from NOD mice immunized with prostate homogenate, following TCR ligation, produce IL-17, a cytokine involved in the pathogenesis of several organ-specific autoimmune diseases (37). Therefore, EAP in the NOD mouse can be considered a Th1- and Th17-dependent autoimmune disease.

Our data show that NOD mice spontaneously develop autoimmune prostatitis with age, in addition to mounting severe EAP following immunization with prostate Ags. This situation is similar to other well-defined organ specific autoimmune pathologies in the NOD mouse, such as type 1 diabetes, sialoadenitis, adrenitis, or autoimmune thyroiditis (15, 38), supporting the concept of polyendocrine autoimmunity in this mouse strain. However, compared with insulitis, which appears at 4–5 wk of age and is florid by 8–9 wk (39), intraprostatic inflammatory infiltrates develop later and are well-established by ~20 wk of age. Intraprostatic-infiltrating cells are significantly reduced in IFN-γ-deficient NOD mice, display a Th1 phenotype, and are able to transfer prostate lymphomonomuclear cell infiltration into NOD.SCID recipients, confirming the autoimmune nature of this pathology.

Interestingly, the development of intraprostatic lymphomonuclear cell infiltration in the NOD mouse in the absence of overt antigenic stimulation, is preceded by an autoreactive T cell response to PSBP, an androgen-dependent prostate-specific protein shown to be a major autoantigen able to induce EAP in rats (18, 19), and in the NOD mouse (17, 36). Our results, showing a sizeable primary T cell response to PSBP in NOD mice, and a strong response following immunization with PSBP, are surprising considering that PSBP is a very abundant self protein, and that immunological tolerance is usually directly proportional to the expression level of self proteins (40, 41). Considering that the prostate develops, under the influence of androgens, mostly after puberty, it is conceivable that PSBP may not be available in the thymus to induce central deletion of autoreactive T cell clones. This would explain the presence of PSBP-specific T cells in the periphery of all mouse strains tested. However, autoreactive T cell responses to PSBP are significantly more pronounced in NOD and NOR mice, possibly because of defective tolerance mechanisms in mice on the NOD background (38, 42). Nevertheless, we cannot infer that the spontaneous prostatitis developing in the NOD mouse is mainly due to an autoimmune response against PSBP, but only that responsiveness to PSBP reveals lack of tolerance to a prostatic autoantigen in this mouse strain.

The identification of self Ags specifically or selectively expressed in the prostate has been spurred by the search for candidate vaccines potentially applicable to the treatment of advanced and metastatic prostate cancer, a condition difficult to treat and usually fatal (43). This approach has been further refined by identifying, using in vitro human model systems, HLA-restricted T cell epitopes from several prostatic autoantigens, including prostate-specific Ag (24, 44, 45), prostate-specific membrane Ag (24, 45–47), prostatic acid phosphatase (45, 48), and kallikrein 4 (49).

PSBP is a highly androgen-regulated protein expressed predominantly by the ventral prostate, and represents the major protein secreted into the seminal fluid (50). Although the human homologue of rodent PSBP has not yet been identified, a PSBP-like protein has been found in human seminal plasma, prostate epithelia, benign prostatic hypertrophy tissue, prostatic intraepithelial neoplasia, prostate adenocarcinoma, and the human prostate cancer cell line DU145 (51, 52). PSBP is a tetramer composed of two subunits: subunit A formed by disulfide-linked polypeptides C1 and C3, and subunit B by disulfide-linked C2 and C5. Genes encoding these polypeptides have been sequenced, and their organization and expression analyzed (53). The C1 chain is more immunogenic compared with C2 and C3 chains (18), and was thus selected for the identification of immunogenic PSBP peptides. Our data demonstrate, within the PSBP C1 chain sequence, the presence of two immunodominant epitopes presented to CD4+ NOD T cell by I-Aγ7 molecules, PSBP21–40 and PSBP61–80 bind well to I-Aγ7 molecules, induce a strong T cell response in NOD mice and are naturally processed in vivo, as shown by their capacity to re-stimulate T cell responses in lymph node cells from NOD mice primed with purified native PSBP protein.

In addition, we have also identified a cryptic epitope within the PSBP C1 sequence, included in residues PSBP91–111. This peptide binds to I-Aγ7 molecules, albeit with a lower affinity compared with immunodominant PSBP epitopes, induces a T cell response of lower magnitude compared with immunodominant PSBP epitopes, but fails to re-stimulate lymph node T cells from mice primed with native PSBP, fulfilling the criteria for a cryptic epitope (54). Under normal physiological conditions, the cryptic epitopes of a native Ag are inefficiently processed and presented, and fail to induce autoreactive T cell responses despite the availability of self-reactive T cells (54). However, under inflammatory or immunostimulatory conditions, cryptic epitopes could be presented to autoreactive T cells, inducing and/or enhancing an autoimmune response (55). Although this set of events may not apply to PSBP, due to the unavailability of this autoantigen to shape the T cell repertoire via negative selection, it is remarkable that the cryptic epitope PSBP91–111 has the same prostatitis-inducing capacity as the immunodominant PSBP epitopes included in PSBP21–40 and PSBP61–80. These results highlight the potential threat for induction of autoimmunity posed by cryptic epitopes, supporting their role in the pathogenesis of autoimmune diseases (55).

In conclusion, we have shown that NOD mice spontaneously develop autoimmune prostatitis, preceded by a T cell response to prostate autoantigens like PSBP. We have also identified, within the PSBP C1 sequence, dominant and cryptic epitopes able to induce T cell responses and autoimmune prostatitis. These model systems will permit a more detailed analysis of pathogenetic mechanisms in autoimmune prostatitis and will facilitate testing of drugs potentially able to modulate the disease course. We have recently confirmed this possibility by showing the capacity of the vitamin D receptor agonist etocalcitol to inhibit T cell responses in NOD mice immunized with PSBP21–40 restimulated with peptide or with purified native PSBP, indicating the capacity of this drug to inhibit T cell responses to naturally processed autoantigen epitopes, and notably autoantigen-specific CD4+ T cell responses in prostate-infiltrating T cells (36).

Disclosures
Giuseppe Penna, Susana Amuchastegui, Roberto Mariani, and Luciano Adorini are employees of BioXell, and have stock and/or stock option interests in the company.

References


