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Commensal bacterial modulation of the host immune response to ameliorate pain in a murine model of chronic prostatitis

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Abstract

The human commensal microflora plays an essential role in modulating the immune response to control homeostasis. *Staphylococcus epidermidis*, a commensal bacteria most commonly associated with the skin exerts such effects locally, modulating local immune responses during inflammation and preventing superinfection by pathogens such as *Staphylococcus aureus*. While the prostate is considered by many to be sterile, multiple investigations have shown that small numbers of gram-positive bacterial species such as *S. epidermidis* can be isolated from the expressed prostatic secretions (EPS) of both healthy and diseased men. Chronic pelvic pain syndrome (CPPS) is a complex syndrome with symptoms including pain and lower urinary tract dysfunction (LUTs). It has an unknown etiology and limited effective treatments but is associated with modulation of prostate immune responses. CPPS can be modeled using murine experimental prostatitis (EAP) where CD4+ve IL17A+ve T-cells have been shown to play a critical role in disease orchestration and development of pelvic tactile allodynia. Here we report that intra-urethral instillation of a specific *S. epidermidis* strain (designated NPI (non pain-inducing)), isolated from the EPS of a healthy human male, into EAP treated mice reduced the pelvic tactile allodynia responses and the increased CD4+ve IL17A+ve T-cell numbers associated with EAP. Furthermore, a cell wall constituent of NPI, lipotechoic acid (LTA), specifically recapitulates these effects and mediates increased expression of CTLA4-like ligands PDL1 and PDL2 on prostatic CD11b+ve antigen presenting cells. These results identify a new potential therapeutic role for commensal *S. epidermidis* NPI LTA in the treatment of prostatitis-associated pain.

Introduction

Staphylococcus epidermidis is a commensal bacteria of the skin [7] that has a role in mediating local immune homeostasis [11]. During local immune activation it can dampen inflammation signals [27] and prevent pathogenic super infection by species such as *Staphylococcus aureus* [1; 2]. It can also act as a facultative pathogen forming biofilms on surgically implanted materials [5; 21; 49]. Recently a more distinct method of immune interaction via specific sub-dermal dendritic cells has been described [26] involving activation of the IL1 and IL17 signaling pathways. The cell wall lipotechoic acid (LTA) is an

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immunogenic pathogen associated molecular pattern (PAMP) and signals via TLR2 [16; 29]. TLR2 mediates signaling responses by forming homo or hetero dimers with TLR1 and/or TLR6 [20]. Resulting downstream signaling is determined by which dimer is engaged and which signaling intermediaries are utilized.

Chronic pelvic pain syndrome (CPPS) is a disorder characterized by pelvic pain and frequently lower urinary tract symptoms (LUTs). CPPS is classically not associated with bacterial infection but prostate specific bacterial strains have been isolated from the expressed prostatic secretions (EPS) of both CPPS patients and controls. We previously described the ability of a patient derived gram-negative uropathogenic *E. coli* (UPEC) bacteria to establish pelvic tactile allodynia in male NOD/ShiLtJ mice by initiating a Th17 mediated prostate immune response [32]. These effects were restricted to the genetically susceptible NOD mice compared to C57BL/6 mice. Pelvic tactile allodynia and immune activation persisted even after bacterial clearance [32; 37]. We hypothesized that in contrast to pathogenic bacterial species, colonization of the human prostate by commensal bacteria may play a favorable role in local immune modulation, pelvic tactile allodynia reduction and/or protection against pathogenic infection.

Aged male NOD mice develop spontaneous prostatitis with incidences matching the pancreatitis observed in female mice (40–60%) [10; 18; 34]. Induction of prostatitis can be hastened in these mice by intraurethral infection with a specific strain of *E. coli* isolated from human EPS (CP-1 model) [37]. Increases in CD4+ve T-cell derived IL17A is necessary for initiation of pelvic tactile allodynia in the EAP murine model [32; 39]. Analysis of human EPS samples demonstrated that IL7, a cytokine involved in maintenance of activated CD4 T-cells [25], is increased in CPPS patients and correlated with pain severity. These lines of evidence suggest that the interplay between bacteria and the local immune system may have specific roles in determining referred allodynia. Here we report that a specific strain of the commensal flora from the normal human prostate can ameliorate pelvic tactile allodynia in a murine model of autoimmune prostatitis.

Materials and Methods

Ethics Statement

Human expressed prostatic secretion (EPS) and voided bladder (VB) 1, 2 and 3 samples were collected from the urology clinic at Northwestern Memorial Hospital by Dr. A. J. Schaeffer approved under the Northwestern IRB protocol number STU00030121 and each participant consented in writing. Animal studies were conducted under protocols 2013–3015 and 2013–2068 approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University. IACUC at Northwestern is AAALAC accredited. Northwestern University has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (A3283-01). Northwestern University conducts its reviews in accordance with United States Public Health Service (USPHS) regulations and applicable federal and local laws. The composition of the IACUC meets the requirements of the USPHS policy and the Animal Welfare Act Regulations.

Animal use

All animal procedures and protocols were approved prior to study commencement and monitored by IACUC at CCM Northwestern. Male 5–7 week old C57BL/6 (B6), NOD/ShiLtJ (NOD) and B6.129P2-II10^{tm1Ggn} (IL10KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME) as required. For all experiments, 4 to 5 animals were used per group (unless otherwise stated). Experiments were performed in duplicate (N=2) with no exclusion of animals. No randomization was performed between mice, as all comparisons were made directly between genetically identical commercially obtained animals. All experiments were conducted in male mice, as the disease condition being studied is restricted to men. EAP was induced in specific mice by subcutaneous injection of rat prostate homogenate at a 1:1 ratio with TiterMax ® adjuvant behind the right shoulder of the animals. Following this, referred allodynia responses were measured by Von Frey force filament behavioral testing for pelvic tactile allodynia, as described [31]. Testing was performed prior to EAP induction (baseline) and then every 7 days unless otherwise stated. Results were calculated as a percentage change above baseline and for each Von Frey filament of increasing force per group individually, values were averaged across experimental groups. Behavioral testing was performed following treatments (with bacteria or LTA) in a blinded manner with no knowledge of treated versus untreated groups. For these treatment experiments pelvic tactile allodynia responses were displayed using day 21 pretreatment values and all testing values subsequent to this. After 28 days of EAP (unless otherwise stated) mice were sacrificed and relevant tissues removed. Prostate and bladder tissues were dissociated using collagenase/DNase buffer (1640-RPMI (Corning) containing 10% FCS (Hyclone), 0.1ul/ml DNase (Thermo-scientific) and 1ug/ml collagenase D (Roche) and shaking for 2 hours at 37°C. Tissues were then made into single cell suspensions by passing through 0.2µm mesh filter membranes and washing with 2% FCS (HyClone) in PBS (Gibco). For splenic and iliac lymph node tissues single cell suspensions were generated without collagenase treatment.

Bacterial isolation, growth and instillation

Human EPS and VB 1, 2 and 3 samples were collected from the urology clinic at Northwestern Memorial Hospital by Dr. A. J. Schaeffer, under the IRB protocol number STU00030121. The NPI-*S. epidermidis* strain was isolated from both the VB3 (post-massage urine) and EPS of a healthy control sample, cultured and characterized by antibiotic resistance exclusion. The 7244-*S. epidermidis* strain was isolated from both the VB3 and EPS of a CPPS patient with ongoing disease and pain. A bacterial isolate was deemed “prostate-specific” if it was detected at at least one log-fold change higher level in both the VB3 and EPS compared to the VB1 and VB2 from the sample subject. Both NPI and 7244 met these criteria. The bacterial isolates were grown in Luria Broth prior to instillation or infection, as previously described for UPEC strains [37]. Briefly bacteria were grown in 3ml at 37°C overnight followed by transfer of 100µl of this culture to 40ml of LB and grown overnight for 37°C without shaking. Following this, the bacteria was pelleted and re-suspended in ice cold sterile PBS, the solution was then made up to an OD 420 value of 1.00, corresponding to 2×10^{10} bacteria per ml. 10µl of the NPI or the 7244 strain preparation was introduced into anesthetized male mice by catheterization. This correlates with 2×10^8 bacteria per infection per mouse. Both bacterial strains will be made available to researchers

upon request, in accordance with NIH rules and regulations regarding sharing of information and materials derived from NIH-funded research.

LTA isolation and treatment

The NPI strain was shipped on dry ice to a commercial biofermentation lab (University of Maryland, Rockville, MD) grown and the LTA extracted from the frozen cell paste by phenol extraction, followed by purification by multiple rounds of HPLC at FinoBiolabs (Rockville MD). Purified LTA was returned (3.011mg from a 10L culture) as a desiccated powder. Upon receipt of the purified product, the LTA, designated SELTA (*S. epidermidis* LTA), was reconstituted using DNA grade water (Gibco) to a concentration of 1mg/ml, aliquoted and stored at -80°C , until use. The working solution was generated by diluting the stock 1:100 in sterile PBS following which 10 μl (100ng) was intra-urethrally instilled via catheterization per mouse per treatment. This was calculated based on an estimation of the LTA content of the dry weight of the 2×10^8 bacteria normally used in infections. A second batch of SELTA (II) was prepared and reconstituted in the same manner to ensure that SELTA itself was functional. *S. aureus* LTA (SALTA) and *Bacillus subtilis* LTA (BSLTA) were purchased from InvivoGenTM and administered in equal doses to SELTA above. In experiments comparing the four LTAs (SELTA (I and II), SALTA and BSLTA) 10 μl of sterile PBS was used as a vehicle control. Mice were treated under anesthesia in a similar manner to the infection protocol.

In vivo antibody treatment

Blockade of CD25 and PD1 was performed, by intraperitoneal injection with 100 μg anti-CD25 (PC61) (BioLegend 102002) or 100 μg anti-PD1 (BioCell BE0146) or relevant Rat IgG2a isotype control antibody (BioCell BE0089) at day 19 post-EAP treatment and repeated every two days with pain/tactile allodynia assessed every alternate day until day 28.

QRTPCR

RNA isolation was performed from respective tissues (collagenase treated prostate and bladder homogenates) using TrizolTM (Invitrogen). RNA concentration was measured using a NanoDropTM spectrophotometer (Thermo Scientific) and normalized to 1 μg per cDNA synthesis reaction. cDNA was synthesized using qScript Supermix (Quanta) according to manufacturers instructions. QRTPCR was performed using perfeCTa qPCR Supermix (Quanta) with 50ng of template per 25 μl reaction and run on the CFX ConnectTM (Biorad) platform. Primers were designed using the online NIH curated primer blast tool. A full list of primer sequences is below. ddCT calculations were performed in ExcelTM, mouse genes were normalized first against the L19 housekeeping gene and finally against expression of CD4. QRTPCR analyses was performed using the $2^{-\Delta\Delta\text{Ct}}$ in ExcelTM to generate fold-change differences against expression levels in naïve mice. Samples were run in biological triplicate (3 mice per group) and technical duplicate. Multiple genes were assayed on different 96-well plates ensuring that each plate and gene had relevant internal controls and L19 expression levels. RNA content constraints prohibited duplicates of individual plates to be performed.

Mouse Sequences

Gene	Forward	Reverse
L19	CAACTCCCGCCAGCAGAT	CCGGGAATGGACAGTCACA
CD4	GCTCAAGGAGACCACCATGTG	GCGAAGGCGAACCTCCTC
Tbet	TGTTCCCAGCCGTTTCTACC	GCTCGGAATCCGCTTCATA
IFNγ	ACACTGCATCTTGGCTTTGC	CTTCAATGACTGTGCCGTGG
GATA3	AAGCTCAGTATCCGCTGACG	GATACCTCTGCACCGTAGCC
IL4	CCATATCCACGGATGCGACA	CGTTGCTGTGAGGACGTTTG
RoryT	AGGGCCTACAATGCCAACAA	CAGCTCCACACCACCGTATT
IL17A	TCTCCACCGCAATGAAGACC	TTCCCTCCGCATTGACACA
FoxP3	CACCCAGGAAAGACACAACC	GCAAGAGCTCTTGTCCATTGA
IL10	AAGGGTTACTTGGGTTGCCA	CCTGGGGCATCACTTCTACC

Flow cytometry analyses

Flow cytometry was performed on single cell suspensions using these mouse antibodies; PerCp-CD4, Alexa-488-CD4, Alexa-647-FoxP3, PE-IL17A, FITC-CD25, PE-PDL1 (Biolegend), PE-FoxP3, PC-IL17, FITC-CD25, APC-CD11b, FITC-PDL2, PE-PDL1 (eBiosciences). Flow cytometry was run on an Accuri benchtop C6 cytometer and analysed using FlowJo™ software. For all analyses unless otherwise stated, samples were gated on lymphocyte populations based on size, as assessed by SSC and FSC, followed by gating for CD4 positivity. Intracellular staining was performed by fixation and permeabilization using eBioscience Fix-Perm Intracellular staining buffers (Cat. Num 8222-49 and 8333-56). Staining was performed for 1 hour at room temperature followed by washing in FACS buffer (2% FCS (Hyclone), in PBS (Gibco)) Analyses were performed using FlowJo™ and data statistically tested using GraphPad Prism™ software, with tests described in respective figure legends.

Immunofluorescent staining

10% formalin-fixed tissues were mounted and embedded in paraffin and sectioned (5 μ m) onto slides. These were then rehydrated in xylene (VWR), followed by increasing dilutions of ethanol (100%, 75%, 50% and 25%). Antigen retrieval was performed by boiling for 25mins in 10mM Sodium Citrate Buffer at a pH of 6.0. Sections were then transferred to PBS (Gibco), excess liquid removed and staining performed using rabbit anti-mouse PDL1 (AbCam ab58810) or anti-mouse PDL2 (AbCam ab21239). Secondary staining with donkey alexa-488 conjugated anti-rabbit (Invitrogen) was then performed. Sections were mounted in anti-fade mounting media containing DAPI (Invitrogen) and imaged using a Leica DMLA microscope.

Bacterial counts in tissue using the Colony Formation Assay

Prostate and bladder tissues were aseptically excised from C57BL/6 mice, 1, 3 or 7 days following NPI instillation or 7244 infection. Tissues were homogenized, dissociated and

filtered as described above. Prostate and bladder digestions were serially diluted in sterile PBS. 25µl of each dilution (1:10, 1:100 and 1:1000) were plated in duplicate on LB agar and grown overnight at 37°C with relevant controls. Colonies were then counted and colony forming units (CFU)/ml quantified in Excel. Statistics and graphing were performed in GraphPad™ Prism using two-way ANOVA.

Statistical Analyses

Statistical analyses were performed using GraphPad™ Prism software. Flow cytometric samples were compared using one-way ANOVA with Tukey's multiple comparison test across samples. Statistical analyses on QRTPCR samples could not be performed as averaging across biological replicates (n=3 mice per group) was performed prior to fold change calculation and CD4 normalization, resulting in one value for each mouse group similar to methodology used in microarray data. Statistical significance was calculated between tactile allodynia responses of mouse groups using a two-way ANOVA followed by Tukey's multiple comparison test performing pairwise analysis between mouse groups at each timepoint (day) of testing. Percentage response change of tactile allodynia testing following treatment (with bacterial instillation and/or LTA) was performed using two-way ANOVA followed by Tukey's multiple comparison comparing "within each column compare rows" or a two-way ANOVA followed by Sidak's multiple comparison comparing "each cell mean with other cell mean in that column". Differences between CFUs were analyzed using a two-way ANOVA, across days and between strains. All datasets were treated as normal as assessed by median to mean agreement. P values less than 0.05 were considered significant and explanations of which symbol/s correspond to which group comparison are given in the respective figure legend of each figure.

Results

NOD mice do not develop pelvic tactile allodynia in response to *S. epidermidis* instillation

A strain of *S. epidermidis*, NPI, was isolated from the expressed prostatic secretion (EPS) of a healthy male subject who reported no pelvic pain symptoms or symptoms related to prostate dysfunction. We sought to understand the consequences of NPI instillation in mice genetically predisposed to the development of pelvic tactile allodynia through altered T-cell immune responses. We initially administered the NPI strain intraurethrally into NOD mice as previously described [32; 37] and analyzed tactile allodynia responses every 7 days for 28 days and immune activation by QRTPCR on prostate tissues excised at day 28. Pelvic tactile allodynia responses, as assessed by behavioral testing, described previously [31], showed no increase in NPI treated mice compared to naïve counterparts (Figure 1a). Examination of responses from each individual filament also showed no differences between naïve and NPI instilled mice (Supplementary Figure 1). Analyses of gene expression for markers associated with CD4 T-cell subsets (Th1: T-bet and IFN γ , Th2: GATA3 and IL4, Th17: ROR γ T and IL17A) demonstrated no observable difference with bacterial instillation (Figure 1b). Together these data show that long-term instillation with NPI does not induce sustained T-cell activation and fails to induce pelvic tactile allodynia in NOD mice. These results are consistent with the nonpathogenic/commensal nature of this specific *S. epidermidis* NPI strain in the healthy human subject from whom it was isolated.

Intra-urethral NPI instillation in EAP mice reduces referred pelvic pain

We next examined what effect instillation of NPI would have on pelvic tactile allodynia responses during ongoing inflammation using the xenogeneic EAP model of prostatitis. EAP was induced in C57BL/6 mice, as previously described [35; 39], and symptom development monitored by behavioral testing. At day 21, when prostatitis induction is deemed chronic as characterized by the presence of chronic tactile allodynia and alterations in immune response, NPI was intraurethrally instilled. Increased tactile allodynia responses between naïve and EAP treated mice were observed at days 7, 14 and 21 (Figure 2a). Pelvic tactile allodynia responses were significantly blunted in the NPI treated group, compared to EAP alone, following instillation with the bacteria. Von Frey filament testing demonstrated an amelioration of tactile allodynia responses of up to 50% compared to non-treated mice with EAP (Figure 2b). These findings were consistent for every Von Frey filament analyzed (Supplementary Figure 2). The effect of NPI instillation on prostatic immune responses was examined by flow cytometric analysis of CD4+ve T-cells. Increased levels of CD4+ve IL17A+ve T-cells were observed upon induction of EAP in prostate tissues, (Figure 2c), as previously reported [25], and this increase was lost upon treatment with NPI for 7 days. We also observed a reversal of increased IL17A expressing CD4 T-cells in the iliac lymph nodes (Figure 2d) of EAP mice following NPI instillation, where levels returned to those similar to naïve mice.

NPI's effects on tactile allodynia are strain specific but not dependent on levels of prostate bacterial colonization

To examine whether the effects on tactile allodynia and immune modulation were specific to the commensal NPI strain, we directly compared the ability of NPI to reduce tactile allodynia with that of another *S. epidermidis* strain (7244) isolated from the EPS of a CPPS patient with active disease. Mice were given EAP for 28 days, following which, mice were instilled with NPI or infected with the 7244 strain. Behavioral testing revealed a significant decrease in tactile allodynia responses at 7 days post-instillation with NPI but no significant modulation of these responses in the 7244 infected group (Figure 3a) for any of the Von Frey filaments (Supplementary Figure 3). Colony formation assays were performed on prostate and bladder samples at days 1, 3 and 7 post-instillation/infection and demonstrated no significant difference between strains to colonize either tissue (Figure 3b). These data suggest that bacterial colonization does not account for reduced responses observed upon instillation with NPI while also demonstrating the specificity of this strain to reduce tactile allodynia in EAP.

LTA is the immunogenic component of NPI

The strain specific nature of NPI to counteract EAP induced pelvic tactile allodynia response led us to consider the exact bacterial component responsible. LTA is a major constituent of the gram-positive cell wall and studies on the immune consequences of *S. epidermidis* colonization have shown that LTA acts as an immunogenic factor [14; 33]. To examine whether LTA was driving our observed responses in the prostates of EAP mice, we isolated LTA from the NPI strain by HPLC and intra-urethrally instilled this compound into naïve and EAP mice at day 21. In agreement with the NPI instillation data, behavioral testing

revealed that LTA treatment significantly decreased tactile allodynia responses in treated EAP mice compared to their untreated EAP-only counterparts (Figure 4a) for all filaments tested (Supplementary Figure 4a). Figure 4b, using day 21 as a baseline, demonstrates a tactile allodynia response reduction of up to 50% in the LTA treated group, a similar decrease to that observed upon NPI instillation. Flow cytometric analysis of prostate single cell suspensions for CD4 T-cells revealed decreased levels of IL17A expression in mice treated with LTA for 7-days, compared to the EAP-only group (Figure 4c). LTA treatment also resulted in decreased IL17A producing T-cells in the iliac lymph nodes (Figure 4d). In order to investigate the specificity of the LTA isolated from our NPI strain we compared the ability of other LTA molecules to modulate tactile allodynia responses in EAP mice. Treatment of mice with EAP at day 28, with commercially available LTA isolated from *S. aureus* (SALTA) or from *B. subtilis* (BSLTA) did not ameliorate tactile allodynia responses when compared to NPI LTA (SELTA), after 7 days (Figure 4e). A second batch of SELTA, SELTA II, isolated independently, demonstrated reduced tactile allodynia in EAP mice after 7 days, indicating the reproducibility of the compound to modulate these responses when compared to PBS vehicle control. These differences were observed for every filament used in testing (Supplementary Figure 4b). Taken together, these data indicate that instillation with NPI modulates pelvic tactile allodynia responses in EAP mice via the LTA constituent of its cell wall. More importantly, we also demonstrate the specificity of these responses to NPI and SELTA when compared to other LTA molecules from different bacterial strains. An analysis of whether these differences are due to structural differences between LTA molecules is currently ongoing.

SELTA increases expression of CD274 and CD273 (PDL1 and 2) on CD11b+ve cells

We have demonstrated that SELTA reduces pelvic tactile allodynia in EAP mice in a specific manner and that this is concomitant with decreased CD4+ve IL17 expressing T-cells in the prostates of these mice. LTA molecules have been shown to signal via TLR2, which is expressed on a wide variety of cells, including macrophages and dendritic cells [3; 41]. TLR2 signaling has been shown to negatively regulate T-cell activation and co-stimulation via expression of the ligands PDL1 and PDL2 [12; 45]. To examine whether SELTA mediates reduction in IL17A expressing T-cells and pelvic tactile allodynia in EAP mice through this pathway we performed flow cytometry on single cell suspensions of prostate tissues in EAP mice following 7 days treatment with SELTA. Figure 5(a & b) demonstrates a significant increase in the number of CD11b monocytes expressing PDL1 and PDL2 between SELTA treated mice and their naïve and EAP-only counterparts. Data from iliac lymph node and bladder tissues revealed that these increases were prostate tissue specific (Supplementary figure 5(a–d). Immunofluorescent staining for PDL1 and PDL2 on prostate sections from naïve, EAP and EAP & SELTA treated mice (Figure 5(c & d)) demonstrated increased expression of PDL1 and of PDL2, to a lesser degree, upon SELTA treatment. The cognate receptor for the PDL1/2 ligands, PD-1, is expressed on the surface of the CD4 T-cell [42; 47]. Flow cytometric analysis revealed a slight increase in PD-1 expression upon LTA treatment in EAP mice (Figure 5e) that was also prostate specific (Supplementary Figure 5(e & f)). These data suggest that SELTA potentially exploits the negative feedback loop induced by engagement of TLR2 to dampen CD4+ve T-effector cell activation by inhibiting T-cell co-stimulation.

Modulations in referred allodynia are dependent on IL10, PD1 and CD25 expressing cells

Given the increased level of PDL1 and PDL2 expression associated with LTA treatment we hypothesized that IL10 secreted by CD11b+ve cells may serve to dampen pelvic tactile allodynia responses referred from the prostate. Such increases in IL10 secretion could also account for the decreased levels of IL17 expressing CD4+ve T-cells observed when EAP mice are treated with either NPI or SELTA. To interrogate this, EAP was induced in IL10KO mice and NPI instillation was performed at day 21 as described above. Figure 6(a & b) demonstrates that IL10KO mice develop pelvic tactile allodynia in response to EAP as in C57BL/6 animals but that these responses were not ameliorated by instillation with the NPI bacteria, for all filaments tested (Supplementary Figure 6a). These data also demonstrate that NPI instillation alone for 7 days had no significant impact on pelvic tactile allodynia responses in the absence of EAP in the context of IL10 deficiency (Figure 6b). IL10 secretion from antigen presenting cells (APCs) has been linked to PDL1 and PDL2 expression [12; 45]. In order to further determine the role of these ligands in mitigating pelvic tactile allodynia and to investigate the source of IL10, we performed an antibody blocking experiment directed against both the PD1 receptor and CD25, a surface marker of IL10 producing T-regulatory cells. Figure 6c shows pelvic tactile allodynia responses at day 21 for C57BL/6 EAP mice with/without antibody treatment and for 7 days following SELTA treatment. Here we demonstrate that antibody blockade of PD1 and/or CD25 prevented SELTA treatment from ameliorating pelvic tactile allodynia further implicating PDL1/2 in mediating these events. In concert with the results from IL10KO mice these data also highlight the role of IL10 production and CD25 expressing cells in modulating pelvic tactile allodynia in response to NPI and SELTA treatment. Flow cytometric analyses of CD4+CD25+FoxP3+ve T-regulatory cells in response to NPI instillation and SELTA treatment in both prostate and iliac lymph node tissues revealed a trend towards increased numbers of these cells (Supplementary Figures 7 & 8). These data further point to the role of IL10 and modulation of T-cell immunity in amelioration of pelvic tactile allodynia responses.

Discussion

CPPS is a debilitating syndrome with a wide range of symptoms, the severity of which varies significantly between patients. Lack of understanding of the complex etiology of the disorder has hampered effective treatment development. The data presented here describe for the first time the ability of a commensal bacterial strain from the human prostate to ameliorate pelvic tactile allodynia in a autoimmune murine model of CPPS. These data suggest that the bacterial flora of the prostate may facilitate resetting of the immune milieu of diseased prostates and thus counteract EAP induced pelvic tactile allodynia responses.

Our previous work has shown that development of pelvic tactile allodynia responses in the CP-1 infection model is dependent on host genetic background and specific to certain bacterial strains [37; 38]. Compared to C57BL/6 mice, NOD mice were shown to be susceptible to induction of pelvic tactile allodynia by CP1 infection [37; 38]. NOD mice have also been shown to develop spontaneous prostatitis with age [8; 10; 18]. Data presented here demonstrated that NPI instillation does not induce referred allodynia in this

background. Furthermore, it did not show increases in expression of CD4 T-cell markers associated with activation of Th1, Th2, or Th17 immune responses that are associated with CP1 instillation. Taken together, these data demonstrate the “commensal” properties of the NPI strain, indicating that the local immune response can tolerate chronic colonization with the bacteria, even in a susceptible genetic background. The data also further strengthens our hypothesis that a combination of host genetics and immune response with specific bacterial colonization could contribute to development of CPPS.

The EAP autoimmune model of CPPS induces pelvic tactile allodynia in NOD and C57BL/6 mice and we have previously demonstrated that this is mediated, at least in part, by increased levels of CD4⁺ve T-cells expressing IL17 [25]. Instillation of NPI into C57BL/6 mice with EAP reduces both pelvic tactile allodynia responses and associated increases in IL17 expressing cells at the level of the prostate and iliac lymph nodes. The effect of bacterial instillation on referred allodynia was detectable as early as one day post treatment and remained for seven days. While modulation of T-cell immunity was concomitant with reduction in pelvic tactile allodynia, we cannot rule out other mechanisms of action of the bacteria. *S. epidermidis* species have been shown to prevent super-infection [1] of the skin via modulation of the local innate immune response [2] and additional studies have focused on the direct interaction between bacteria and neurons [6; 22]. These suggest that bacterial components can directly interact with neural pathways to influence pain in acute infection, in the absence of classical PAMP (TLR) activation [6; 22]. Whether NPI interacts at the neural level to reduce referred pain in EAP is a mechanism we are currently investigating. Furthermore, reduction of IL17 alone in the EAP model has been shown to be ineffective at reducing pelvic tactile allodynia response so it is likely that the bacteria has additional effects on prostate function [25].

There are numerous studies that have detected bacteria and bacterial components in EPS and post – prostate massage voided bladder samples of patients [4; 13; 19; 28; 46]. The exact nature of this flora, whether it is transient or permanent and its role in diseases of the male urogenital tract remain to be investigated. *S. epidermidis* species are commonly found in these analyses, detectable in the male urogenital tract of both healthy and diseased men [48]. We demonstrate that the effects of NPI instillation are highly specific compared to another *S. epidermidis* strain isolated from a patient with CPPS. This bacterium, designated 7244, showed no capacity to reduce pelvic tactile allodynia in EAP treated C57BL/6 mice but could colonize the prostate as effectively as NPI. Interestingly, infection with 7244 did not increase referred pain responses above EAP induced levels. This observation could be due to the inability of 7244 to induce allodynia by itself in an immunocompetent host or that behavioral responses were maximized by EAP induction. Preliminary data from our lab suggests the former, whereby 7244 can induce pelvic tactile allodynia in NOD mice but not in the C57BL/6 background. These data highlight the necessity for a robust profiling of the flora of the male urogenital tract and also the highly specific effects of commensal versus pathogenic bacteria on host responses in specific genetic contexts.

The LTA component of the gram-positive cell wall is highly immunogenic and has been shown to activate members of the TLR family of receptors [16; 20; 29; 33; 36; 44]. The specificity of SELTA to reduce pelvic tactile allodynia compared to SALTA and BSLTA is

most likely dependent on differences in their superstructure, which have been shown to vary significantly between bacterial strains, resulting in unique downstream signaling effects and receptor utilization [20; 41]. While we demonstrated that SELTA increased expression of the ligands PDL1 and PDL2 on the surface of CD11b+ve monocytes, the specific signaling cascade resulting in increased expression remains to be investigated. Other groups have shown that TLR2 engagement by LTA can increase expression of these ligands and also enhance IL10 secretion [3; 9; 12; 45] and that LTA can directly impact inflammation in neural tissues [14].

IL10 secretion and FoxP3 expressing T-regulatory cells have been shown in multiple studies and disease models to dampen the effects of CD4+ve IL17 expressing activated T-cells [15; 17; 23; 24; 40; 50]. Our observation that IL10 is necessary for the effect of NPI instillation on pelvic tactile allodynia, using IL10 knockout mice, correlates neatly with the observed decreases of IL17 in these mice. However, our supplementary data describing the trend of increased FoxP3 expressing T-regs in response to NPI and LTA is not robust enough to clearly define this cell type as the only source of IL10. Blockade of either CD25 (used here as a surrogate marker for T-regulatory cells) or the PD1 receptor, by antibody treatment, prevents SELTA's amelioration of the pelvic tactile allodynia induced by EAP in C57BL/6 mice. This data further highlights the PD1-PDL1/2 pathway as essential to SELTA's activity, while also further implicating the role of T-regulatory cells in mediating these effects [30]. Our working hypothesis is that NPI and SELTA through engagement of TLR2 increase PDL1/2 expression. The increased ligands serve to directly repress activated CD4+ve T-cells, including Th17 cells, via PD1, while also inducing IL10 expression [43]. IL10 can then act to reverse EAP-induced increases in IL17 via either direct expansion or increased activity of T-regs. This reset in immune responses of the EAP prostate may enable a dampening of the referred allodynia through either interactions with activated mast cells or direct interactions with neurons themselves. The data above support this hypothesis but more research is needed to elucidate the exact signaling cascades and specific cell types involved and to determine whether multiple treatments could further ameliorate allodynia responses.

This paper demonstrates the capacity of a specific commensal bacteria and a specific cell wall constituent to reduce referred allodynia in a murine model of CPPS. We demonstrate not only the specificity of bacterial isolates from the human prostate in modulating behavioral and immune responses but also highlight the role of the host immune response in these effects. In terms of the etiology of CPPS, this increases our understanding of how both environmental factors and underlying host defects may contribute to disease development and progression. We have isolated a potential therapeutic that could manipulate the host immune response to counteract referred pain by resetting a disturbed microflora.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Intra-urethral instillation of human derived NPI does not induce pelvic tactile allodynia

(a) Pelvic tactile allodynia response frequencies, as assessed by Von Frey filament testing, in naive and NPI-instilled NOD mice. Pain response is depicted as increased percentage response frequency above baseline when tested every 7 days for 28 days. $n=3/4$ mice per group. No significant differences were observed based on two-way ANOVA with Tukey's multiple comparison test. (b) QRT-PCR for T-cell markers, Th1: IFN γ and Tbet, Th2: GATA3 and IL4 and Th17: IL17 and ROR γ T from RNA isolated from the prostate tissues of naive and NPI-instilled NOD mice, normalized against the L19 housekeeping gene followed by normalization against fold-change expression levels of CD4. $n=2/3$ mice per group. Data displayed is the averaged expression level of three mice per group prior to normalization and fold change calculation. All statistics were performed in Excel and GraphPadTM Prism.

Figure 2. NPI instillation reduces EAP induced pelvic tactile allodynia in C57BL/6 mice
 (a) Pelvic tactile allodynia response frequencies, in naïve, EAP and EAP with NPI instillation at day 21, in C57BL/6 mice, every 7 days for 28 days. Responses are depicted as increased percentage response frequency above baseline. (b) Pelvic tactile allodynia response change following instillation with NPI, using day 21 as baseline. * Naïve vs EAP, # Naïve vs EAP & NPI, ^ EAP vs EAP & NPI. *** $p < 0.001$, **** $p < 0.0001$ based on two-way ANOVA with Tukey's multiple comparison. $n=4/5$ mice per group, $N=2$, representative experiment shown, error bars are SD. (c) Quantification of IL17A expression by flow cytometry based on gating for CD4+ve lymphocytes in single cell suspensions of prostate tissues and (d) iliac lymph nodes, from naïve, EAP and EAP & NPI C57BL/6 mice harvested at day 28 of EAP (7-days post-instillation). **** $p < 0.0001$ based on one-way ANOVA with Tukey's multiple comparison. $n=4/5$ mice per group. All statistics performed in Excel and GraphPad™ Prism.

a.

Tactile Allodynia

b.

Figure 3. Modulation of pelvic tactile allodynia by NPI is strain specific and not due to differential colonization of the mouse prostate

(a). Response change frequencies from baseline (Day 0) after 21 days of EAP development. Values for C57BL/6 mice with EAP, EAP & NPI or EAP & 7244 are shown, before and 1, 3 & 7 days after bacterial instillation/infection. * $p < 0.05$ based on a two-way ANOVA with Tukey's multiple comparison. $n=4/5$ mice per group, error bars are SD. (b) Colony count data from serial dilutions of colony formation assays from whole prostate and bladder homogenates are shown for mice at days 1, 3 and 7 post-instillation/infection with NPI or 7244 strain. No significant differences based on two-ANOVA statistical analyses. $n=4/5$ mice per group, error bars are SD. All statistics performed in Excel and GraphPad™ Prism.

Figure 4. Intraurethral treatment of EAP mice with LTA from the NPI strain instillation ameliorates pelvic tactile allodynia in a strain specific manner

(a) Pelvic tactile allodynia response frequencies, in Naïve, EAP and EAP with LTA treatment at day 21, in C57BL/6 mice, every 7 days for 28 days. Responses are depicted as increased percentage response frequency above baseline. (b) Pelvic tactile allodynia response change following treatment with LTA, using day 21 as baseline. * Naïve vs EAP, # Naïve vs EAP & LTA, ^ EAP vs EAP & LTA. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ based on two-way ANOVA with Tukey's multiple comparison. $n=4/5$ mice per group, $N=2$, representative experiment shown, error bars are SD. (c) Quantification of IL17A expression by flow cytometry based on gating for CD4+ve lymphocytes in single cell suspensions of

prostate tissues and (d) iliac lymph nodes, from naive, EAP and EAP & LTA C57BL/6 mice harvested at day 28 or EAP, (7-days post-instillation). * $p < 0.05$, ** $p < 0.01$ based on one-way ANOVA with Tukey's multiple comparison. $n = 4/5$ mice per group, error bars are SD. (e) Response change frequencies from baseline (day 0) after 21 days of EAP development. Values for C57BL/6 mice with EAP, EAP & PBS (vehicle control), EAP & SELTA (*S. epidermidis*), EAP & SELTA II (second batch), EAP & SALTA (*S. aureus*) and EAP & BSLTA (*B. subtilis*) are shown, before and 7 days after LTA treatment. * $p < 0.05$, *** $p < 0.0001$ based on two-way ANOVA with Sidak's multiple comparison test. $n = 4/5$ mice per group, error bars are SD. All statistics were performed in Excel and GraphPad™ Prism.

Figure 5. LTA induces PDL1/2 expression on the surface of APCs of the prostate

(a) Quantification of PDL1 and (b) PDL2 expression by flow cytometry gated on CD11b+ve cells in single cell suspensions of prostate tissue from naïve, EAP and EAP with LTA, C57BL/6 mice, at day 28 of EAP (7-days post-LTA treatment). ** $p < 0.01$, *** $p < 0.001$ based on one-way ANOVA with Tukey's multiple comparison. (c) Representative images of immunofluorescent staining for PDL1 and (d) PDL2 with DAPI on paraffin sections of murine prostate from naïve, EAP and EAP with LTA, C57BL/6 mice at day 28 of EAP (7-days post-LTA treatment). (e) Quantification of expression of PD1 by flow cytometry on CD4+ve lymphocytes from single cell suspensions of naïve, EAP and EAP with LTA,

C57BL/6 mice at day 28 of EAP (7-days post-LTA treatment) * $p < 0.05$ based on one-way ANOVA with Tukey's multiple comparison. $n=4/5$ mice per group. $N=2$, representative experiment shown, error bars are SD. All statistics performed in Excel and GraphPad™ Prism.

Figure 6. Effects of NPI and LTA on pelvic tactile allodynia is dependent on IL10, PD1 and CD25 expressing cells

(a) Pelvic tactile allodynia response frequencies, in naïve, EAP, NPI only and EAP with NPI instillation, in IL10-KO mice, every 7 days for 28 days. Responses are depicted as increased percentage response frequency above baseline. (b) Pelvic tactile allodynia response change in IL10-KO mice following instillation with NPI using day 21 as a baseline. Statistics were performed using a two-way ANOVA and Tukey's multiple comparison test showing no significant difference at day 7 post-NPI instillation with day 21 of EAP as baseline. (c) Response change frequencies from baseline (day 0) after 21 days of EAP development and with antibody treatment for CD25 and PD1 where stated. Values for C57BL/6 mice groups with EAP, EAP & LTA, EAP & IgG (isotype control), EAP & anti-PD1, EAP & anti-PD1 & LTA, EAP & anti-CD25 and EAP & anti-CD25 & LTA are shown, before and 1, 3, 4 and 7 days after LTA treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ based on

two-way ANOVA with Tukey's multiple comparison test. n=4/5 mice per group, error bars are SD. All statistics were performed in Excel and GraphPad™ Prism.

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