APPROVAL SHEET

Title of Thesis: Role of TRPV1 channels in mediating chronic pelvic pain and the role of inflammation in prostate cancer

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Abstract

Title of Document: Role of TRPV1 channels in mediating chronic pelvic pain and the role of inflammation in prostate cancer

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Chronic Prostatitis/ Chronic Pelvic Pain Syndrome (CP/CPPS) forms around 90% of the 2 million prostatitis cases. CP/CPPS is accompanied by excruciating pelvic pain for which the etiology is unknown. Current analgesics include tricyclic antidepressants and anti-convulsants that have a plethora of side effects. Identification of the etiology of chronic pelvic pain is instrumental for the development of better therapies. Transient Receptor Potential (TRP) channels have been shown previously to play an important role in mediating pain responses. TRPV1 channels are vanniloid receptors that are known to play a role in inflammatory pain. In this study we show the role of TRPV1 channels in mediating chronic pelvic pain in our mouse model of IL-1β mediated prostate inflammation. ABT-102 is a selective TRPV1 antagonist that has been shown to alleviate pain in murine models of bone cancer and post-operative pain. In addition, we show that blocking these TRPV1 channels with ABT-102 successfully and reversibly blocks pain sensation in our mouse model. Chronic prostatitis has also been linked to an increased risk of prostate cancer. Phosphatase tensin homolog (PTEN) is a tumor suppressor that is the most commonly mutated gene in prostate cancer. We have developed a mouse model by crossing our IMPI model with the Cre/PTEN$^{fl/fl}$ model to generate mice with IMPI/Cre/PTEN. We show that upon induction of prostate inflammation combined with loss of one allele of PTEN using doxycycline we see infiltration by immune cells but no cellular atypia at 15 weeks. We also show that long term chronic inflammation in the IMPI model leads to the development of prostatic intraepithelial neoplasia (PIN) with a high penetrance. Using these results we further show the link between chronic inflammation and incidence of neoplastic lesions in the prostate.

ROLE OF TRPV1 CHANNELS IN MEDIATING CHRONIC PELVIC PAIN AND THE ROLE OF INFLAMMATION IN PROSTATE CANCER

by
Anupam Prakash

Thesis submitted to the faculty of the Graduate School at the University of Maryland, Baltimore County in partial fulfillment
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List of Abbreviations:

CP/CPPS	**Chronic Prostatitis/Chronic Pelvic Pain Syndrome**
IL	**Interleukin**
LPS	**Lipopolysaccharide**
TNF	**Tumor Necrosis Factor**
HIV	**Human Immunodeficiency Virus**
TRP	**Transient Receptor Potential**
NGF	**Nerve Growth Factor**
PTEN	**Phosphatase and Tensin Homolog**
PI3K	**Phosphoinositide 3-Kinase**
mTOR	**Mammalian Target of Rapamycin**
AR	**Androgen Receptor**
PIN	**Prostatic Intraepithelial Neoplasia**
Cre	**Cre Recombinase**

Introduction:

Human prostate anatomy and function:

The prostate is a tubular-alveolar gland located at the base of the bladder and surrounds the urethra (Thomson and Marker, 2006; Shen and Abate-Shen, 2010). The prostate connects to the seminal vesicles through the vas deferens, which carries the seminal fluid. Its main function is to produce an alkaline secretion that contributes to 10-30% of seminal fluid volume to provide a suitable environment for spermatazoa inside the female reproductive tract (Javed et al., 2013; Lin and Wang, 2010).

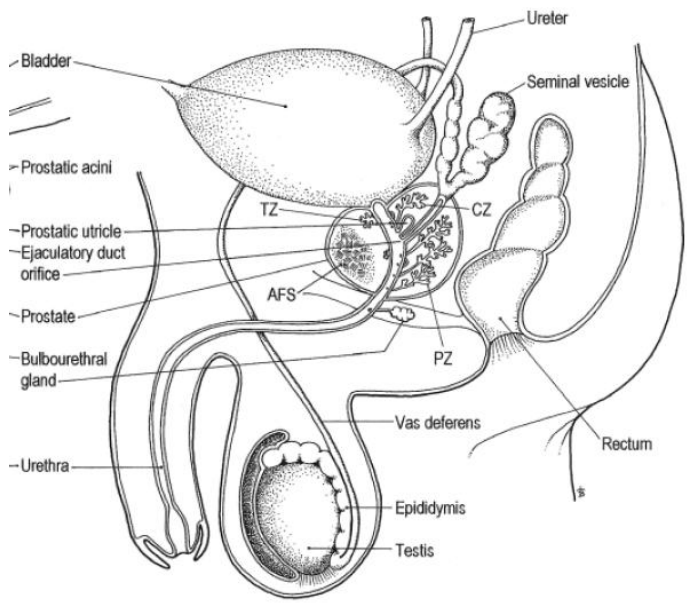

Figure 1. Male accessory sex organs

From Timms et al 2008

The human prostate is divided into three zones; peripheral, transition and central. The peripheral zone is the largest zone, located behind the urethra wrapping around to the front. The central zone comprises most of the base of the prostate and surrounds the ejaculatory ducts while the transition zone surrounds the proximal urethra.

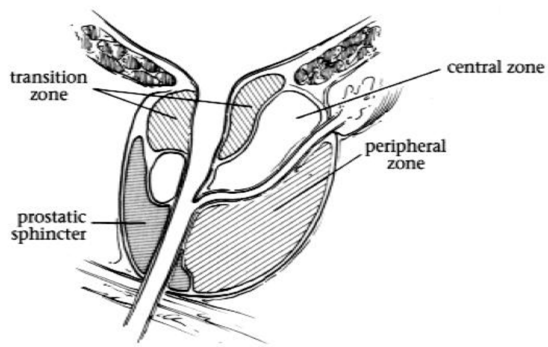

Figure 2. Zones of the human prostate. CZ, central zone; E, ejaculatory duct; PZ, peripheral zone; TZ, transition zone; UP, urethra proper

From Abate-Shen and Shen et al 2010 and Timms et al 2008

The prostate has a ductal morphology with an inner epithelial layer surrounded by a layer of smooth muscle (Thomson and Marker, 2006). These ducts are composed of three epithelial cell types, luminal, basal and neuroendocrine, each with a different gene expression profile (Figure 3). Luminal cells line the inside of the ducts and are characterized by a columnar morphology. These cells secrete the proteins necessary for the seminal plasma, are high in androgen receptor (AR), cytokeratins 8 and 18 (CK8 and CK18) and are believed to be the origin of prostate adenocarcinoma (Shen and Abate-Shen, 2010; Wang et al., 2009). Beneath the luminal cells are the basal cells that lie just inside the basement membrane. Basal cells are low in AR, but express p63 and high molecular weight cytokeratins (Shen and Abate-Shen, 2010). Finally, neuroendocrine cells are irregularly dispersed among the basal cells, with currently unknown function. They are indistinguishable from the basal cells unless immunohistochemmically stained to reveal Chromogranin A, a neuroendocrine marker. Outside of the epithelial layers is the fibromusclar stroma surrounding the entire gland, which serves as an inducer of epithelial growth.

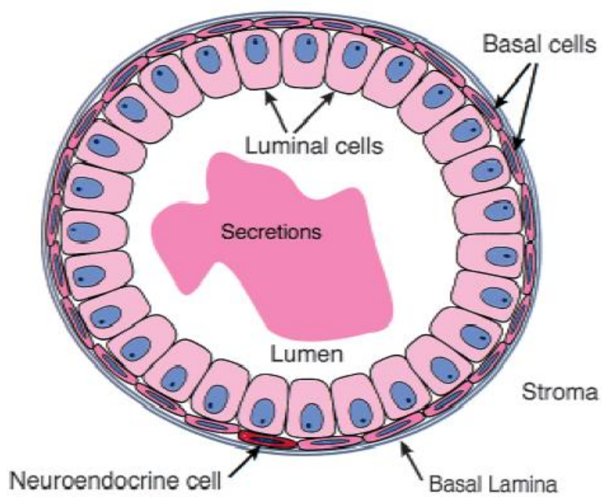

Figure 3. Cells of the prostate duct

Adapted from Abate-Shen and Shen 2000

Mouse Prostate Anatomy

In contrast to the unitary, compact organization of the human prostate, the mouse has a multi-lobed and highly branched prostatic morphology (Thomson and Marker, 2006; Abate and Abate-Shen, 2010; Thomson, 2008). It is separated into four distinct lobes that have left and right mirror symmetry; ventral, anterior, dorsal and lateral, with dorsal and lateral often combined into dorsolateral (Figure 4).

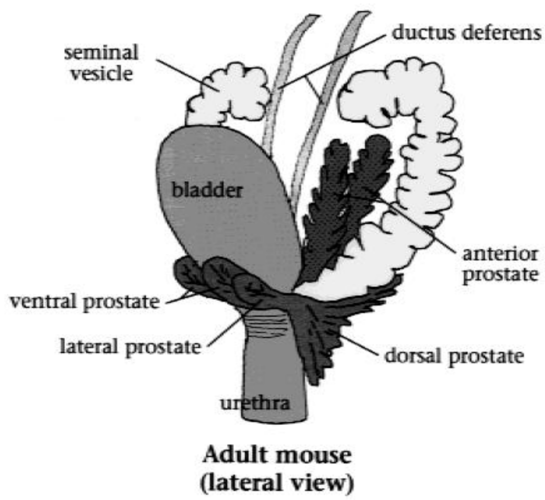

Figure 4. Mouse prostate lobes

Adapted from Abate-Shen and Shen 2000

Most studies that have been done to investigate prostate organogenesis have been completed in mouse or other rodent models. In the mouse, initial epithelial budding occurs around 17 days post conception, while ductal elongation and major branching span the first two weeks after birth (Figure 5). About 80% of branching is complete by postnatal day 10 with completion by two to three months of age (Lin and Wang 2010). Initiation of bud formation is androgen dependent, but the further development is only affected by androgens in terms of size of branches and overall prostate volume (Lin and Wang, 2010; Timms, 2008; Thomson, 2008).

Chapter 1

Role of TRPV1 channels in mediating chronic pelvic pain

Introduction:

Chronic Prostatitis

Chronic prostatitis is a heterogeneous condition with broad diagnostic criteria such as urologic pain or discomfort in the pelvic region, and/or the sexual dysfunction in the absence of identifiable pathology such as cancer, curable infection or anatomic abnormalities. The prostate is a gland responsible for the production of alkaline fluid that is part of the male ejaculate. There are four types of prostatitis such as Chronic Prostatitis/ Chronic Pelvic Pain Syndrome (CP/CPPS), Chronic Bacterial Prostatitis, Acute Bacterial Prostatitis and Asymptomatic Inflammatory Prostatitis. CP/CPPS is the most common type of prostatitis and forms ninety percent of all CP cases (Collins et al., 1998). Symptoms of CP/CPPS include painful urination, muscle tenderness, sexual dysfunction and pain originating from the urogenital organs (Rees et al., 2015). The etiology of CPPS still remains a mystery and studies trying to correlate lifestyle factors such as obesity, smoking and hypertension revealed no association with the risk of developing CPPS (Zhang et al., 2015). The predominant symptom of CPPS is pain and therefore therapies that can alleviate pain may prove to be therapeutically effective (Nickel et al., 2007). Current treatment options for pain include tricyclic antidepressants and anticonvulsants that have side effects such as dry mouth, dizziness and somnolence (Nickel et al., 2007). Thus, there is a pressing need to develop alternative treatment options that are effective at treating pain with minimum side effects.

In order to study pain associated with CPPS, animal models that recapitulate the CPPS phenotype are required. Our lab has developed a mouse model that over-expresses the pro-inflammatory cytokine IL-1β that has been implicated in CPPS. Cytokines are small, soluble, immunomodulatory molecules that can regulate the immune response. Over-expression of certain cytokines can over-activate the immune system and is implicated in various autoimmune disorders (Cope, 1998; Dinarello, 2011; O'Shea et al., 2002). IL-1β is a multi-functional protein and belongs to the IL-1 family of cytokines (Barksby et al., 2007). The IL-1 receptor is present on various cell types such as monocytes or macrophages, epithelial cells, endothelial cells, chondrocytes and fibrablasts and has synergistic roles with other cytokines and signaling molecules (Jura et al., 2008; Vincenti and Brinckerhoff, 2001, Weber et al., 2010). IL-1β expression can be induced by a range of factors: lipopolysaccharide (LPS), other cytokines (TNF-α,IL-2, IL-3), cell matrix proteins, clotting factors, lipids, inflammatory by-products (c-reactive protein, neuro-active substances (substance P, melatonin)), and stress factors (Dinarello, 1996). Elevated levels of IL-1β have been observed in rheumatoid arthritis, asthma, HIV infections, Alzheimer's disease, solid tumors, leukemia, transplant rejections, and pancreatitis (Apte et al., 2006; Elaraj et al., 2006; Forlenza et al., 2009; Lewis et al., 2006).

Existing mouse models of prostate inflammation:

The pathogenesis and etiology of prostate inflammation is quite obscure and there is a need for good animal models that recapitulate human disease. There are multiple existing mouse models of prostate inflammation, each having its advantages and limitations.

1) **<u>Spontaneous Prostate Inflammation Model:</u>** An existing model of spontaneous prostate inflammation uses the Non-obese diabetic (NOD) strain of mice that develop spontaneous autoimmune prostatitis at 20 weeks of age (Penna et al., 2007). In this

model, mice with type-1 insulin dependent autoimmune diabetes exhibit leukocytic infiltration into the prostate by 20-30 weeks. It was further shown that a T cell response primed against prostate antigens was found in the spleens of these mice and that IFN-g might contribute to the development of prostatitis. As seen in Figure 5, inflammatory cell infiltration can be clearly observed in the prostates of NOD mice at different time points.

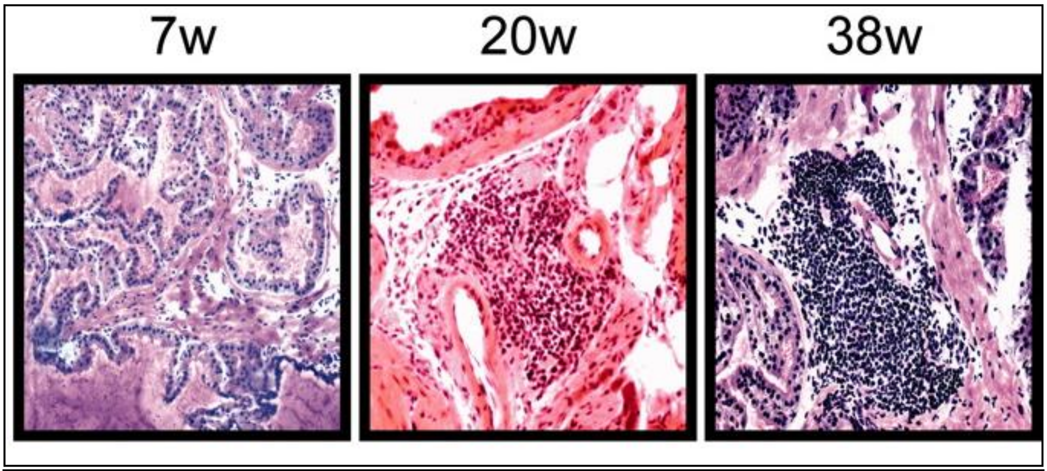

Figure 5. H&E staining of prostates from NOD mice at different time points.

(From Penna et al, 2007)

2) **Infectious Prostatitis Model:** Chronic bacterial prostatitis is often a consequence of acute bacterial prostate inflammation. Even though there exists a well defined management protocol using antibiotics, its effect is limited due to the poor ability of antibiotics to reach therapeutic levels within the prostate (Nickel, 2000, Nickel, 2003). It was shown that there is a strain dependent response to bacterial inoculation. It was demonstrated that BALB/c, C57BL/6J and (BALB/c X C3H/HeJ) mice at 13 weeks of age

show no apparent prostate infection 5 days post inoculation with *E.coli*. In contrast, it was shown that C3H/HeJ and C3H/HeOuJ mice exhibited high incidence of acute prostate infection at the same time point (Elkahwaji et al., 2005). These inconsistencies further emphasize the need for better mouse models for prostate inflammation. However, more recently it was shown that C3H/HeOuJ mice develop acute prostate inflammation 5 days post inoculation with *E.coli* using histological analysis. This mouse model is quite advantageous due to the availability of mice strains and that these mice develop acute infectious prostate inflammation that might eventually lead to the development of chronic prostate inflammation. This model could be used to study the influence of chronic prostate inflammation on the development of prostate cancer.

3) **Hormone induced prostatitis:** The prostate gland is an androgen-dependent organ in males. Therefore, androgens play a key role in the regulation of prostatic growth, function and disease. Supra-physiologic doses of exogenous testosterone do not stimulate additional prostate growth; however, it should be noted that normally occurring neonatal hormone surges may permanently imprint the prostate and determine its future growth in adulthood (Naslund et al., 1986). It has been shown in rats that neonatal administration of 17 β-estradiol followed by testosterone administration several months later induced severe prostatitis in the ventral and lateral lobes of the prostate (Naslund et al., 1988). It was reported that 67% of testosterone treated LH knockout (LuRKO) male mice showed marked inflammation in the prostate.

This was characterized histologically by the abundant presence of infiltrating lymphocytes in the stroma and between tissue layers in the prostate gland (Pakarainen et al., 2004). This testosterone replacement was done in pre-pubescent 21 day old mice to restore accessory sex organ function and anatomical abnormalities in hypogonadal LuRKO mice (Zhang et al., 2001, Lei et al., 2001, Zhang et al., 2004). Additionally, it was found that in some of these mice, testosterone replacement lead to inflammation in the coagulating glands, *vasa deferentia* and epididymis (Pakarainen et al., 2004). Long term administration of testosterone in certain strains of rats have been shown to induce invasive prostate adenocarcinoma (Pollard et al., 1987, Drago, 1984). It has been previously shown that testosterone blocks 17 β-estradiol-induced prostate inflammation but prostatitis has not been shown to occur in mice treated with testosterone alone. Therefore, the mechanisms by which testosterone induces prostate inflammation in LuRKO mice is largely unknown. This mouse model of testosterone induced prostate inflammation in LuRKO mice will help us gain an insight into the mechanism of testosterone-induced prostate inflammation and may hold some promise for future studies.

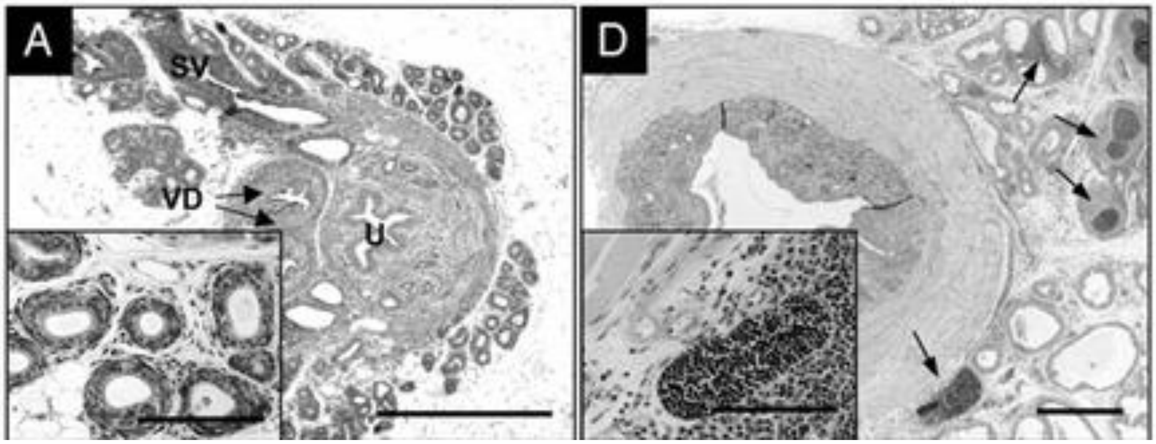

Figure 6. Representative light microscopy images of prostates from testosterone treated LuRKO mice.

(From Pakarainen et al, 2004)

4) **Immune-induced prostatitis model:** One of the first mouse models of immune-induced prostatitis was created in the 1990s (Keetch et al., 1994). They reported that 100% of C57BL/6 male mice immunized once with prostate tissue extract developed autoimmune prostatitis by day 30. Only ventral lobes of the prostate from syngeneic mice were used for preparation of the extract. Mice were immunized with different doses, up to a maximum of 0.75 mg protein extract and supplemented with pertussis toxin. A prominent interstitial infiltration of multi-nucleated cells was noted, predominantly surrounding blood vessels and prostatic acini. The inflammation was localized to the dorso-lateral prostate and the degree of inflammation was dose-dependent. The authors were able to induce prostatitis in syngeneic naive recipients by adoptive cell transfer. The autoimmune nature of the induced prostatitis was supported by the identification of circulating serum antibodies to unknown prostatic homogenate proteins with molecular weight 25 kDa. This study demonstrated that the C57BL/6

mouse strain was more susceptible to the induction of prostatitis than other strains such as SJA, AJ and BALB/c (Keetch et al., 1994).

NOD mice are a well-characterized model of type I diabetes. They are also prone to develop inflammatory reactions in multiple exocrine and endocrine glands including the salivary and lachrymal glands, (van Blokland et al., 2002) and the thyroid (Damotte et al., 1997). The induction of urogenital organ inflammation in NOD mice after xenoantigen challenge was also studied and it was demonstrated that 10 days and 21 days after vaccination with 1.0 mg pooled myelin associated glycoprotein (MAG) homogenate from Wistar rats with complete freund's adjuvant (CFA), all mice showed inflammation in the dorso-lateral prostate, regardless of whether they had been exposed once or twice to MAG in CFA (Rivero et al., 2002). Additionally, foci of moderately severe inflammation were also found in the ventral prostate, seminal vesicles and coagulating glands, although less frequently. Further experiments have shown that 1.0 mg Wistar rat prostate homogenate or 30 µg of Wistar rat PSBP injected twice resulted in the induction of prostatitis in 80–100% of young male NOD mice but in only 30% of C57BL/6 mice (Rivero et al., 2002). Although precise sites of prostate inflammation were not specified in this study, histologic changes in the prostate were characterized by perivascular and stromal MNC infiltration accompanied by edema. The edema was more severe in mice immunized with prostate homogenate than in those immunized by prostatic steroid binding protein (PSBP). These experiments with NOD mice also demonstrated that immunized mice specifically responded to prostate xenoantigen challenge by increased T-cell proliferation, increased release of INF-γ by T cells, and by increased production of circulating IgM and IgG auto-antibodies. More recently, it was reported that NOD mice are characterized by the spontaneous development of autoimmune prostatitis which becomes apparent at about 20 weeks of age and is stably maintained thereafter (Penna et al., 2007). In contrast to BALB/c and C57BL/6 mice, significant upregulation of PSBP-specific autoreactive T cells are detectable in NOD males before the spontaneous development of autoimmune prostatitis, indicating lack of tolerance to the self-antigen. There are indications that disease development was markedly delayed in IFN-γ deficient mice and prostate lymphocyte infiltration could be transferred into NOD.SCID recipients by CD4+ cells from 24-week-old NOD males. Experimental autoimmune prostatitis was detected in NOD mice 30 days after priming of 8-week-old NOD males with mouse prostate homogenate, purified PSBP, and synthetic PSBP peptides. Moreover, both PSBP and prostate homogenate induced comparable levels of intraprostatic CD4+ cell infiltration of NOD mice (Penna et al., 2007). The advantages of this mouse model of immunological prostatitis are that relevant mouse strains are readily available, and that there is a wide variety of immunological techniques available to study of immunological aspects underlying prostatic inflammation. On the other hand, the major limitation of this model is that the prostate tissue auto-antigen involved in the induction of autoimmune prostatitis is not well characterized. Furthermore, NOD mice frequently develop diabetes and other organ-specific autoimmune diseases that may interfere to specify study of prostate inflammation.

In the mid-1980s, another immune-mediated prostatitis mouse model was characterized (Taguchi et al., 1985, Taguchi et al., 1987). They reported that 70% of C3H/HeMs × 129/J F1 male mice that were thymectomized on day 3 (Tx-3) developed prostatitis which was found predominantly in the anterior prostate (coagulating gland) and to a much lesser degree in the ventral prostate (Taguchi et al., 1985, Taguchi et al., 1987). The autoimmune prostatitis was characterized by loss of secretory products in the lumen and massive lymphocyte infiltration in the stroma. Marked inflammation in the prostate was accompanied by circulating auto-antibodies to prostate epithelial cells in 42 of 60 cases (70%) of the mice aged 50–150 days. The authors observed that performing a thymectomy on day 0 or 7 of life did not reproduce this phenotype and castration on day 0 (Orx-0) of life eradicated it. At the same time, seven of 13 Orx-0+Tx-3 mice (54%) developed autoimmune prostatitis when they were given a testosterone pellet at 90 days of age. These data indicate that Tx-3 mice develop autoimmune prostatitis by auto-sensitization of the immune system to an unknown prostate antigen normally expressed in the differentiated prostate gland. In the mid 1990s, Taguchi et al. reported that depletion of the tissue-specific CD4+ T-suppressor cell population in Tx-3 mice results in autoimmune prostatitis after the onset of puberty. They demonstrated that the development of the prostate lesions in Tx-3 (C57BL/6N × A/J) F1 male mice could be prevented by a single injection of CD4+ spleen cells from normal syngeneic mice, but not from normal females or Orx-0 mice. However, the splenocytes from Orx-0 mice could prevent prostatitis in Tx-3 recipients if the Orx-0 donors had received a dihydrotestosterone (DHT) pellet in adulthood, resulting in prostate maturation. The data of this study indicate that immune tolerance to prostate antigen(s) is maintained by a tissue-specific T regulatory cell subset, which is activated by specific auto-antigens in the mature prostate. The autoimmune nature of prostatitis in the studied mice was also supportive by induction of prostate inflammation in nude recipients after adoptive cell transfer of autoreactive CD4+ cells from syngeneic Tx-3 mice with prostatitis. Moreover, the findings that even CD4+ cells from Orx-0 mice that were thymectomized as adults and treated thereafter with DHT were effective in preventing prostatitis may suggest that activation of this T suppressor population takes place in the peripheral lymphoid organs (Taguchi et al., 1994). It was later shown that this depletion of CD4+CD25+ T regulatory cells by 3 day thymectomy in this model is an underlying cause of prostatitis (Sakaguchi et al., 1995, Sakaguchi et al., 2004). More recently it was reported that eight of 13 (62%) of Tx-3 lupus-prone (NZB × SWR) F1 male mice developed prostatitis and antibody to PSAs was found in 11 of 15 cases (73%). These authors also reported that prostatitis was detected in 73% of Tx-3 males of another lupus-prone mouse strain (NZM2328).108 It has been also shown that the transfer of CD25+ T cells to Tx-3 NZM2328 mice completely suppressed prostatitis (Bagavant et al., 2005). In summary, these data strongly suggest the role of T regulatory cells in this model of prostate inflammation. One of the major drawbacks with this mouse model is that the mice develop other non-specific conditions such as thyroiditis, oophoritis, gastritis, orhitis and insulitis.

The cytotoxic T lymphocyte antigen-4 (CTLA-4) is a second T cell counter-receptor for surface B7 molecule that plays a critical role in attenuating T-cell responses. CTLA-4 engagement may inhibit the initiation of T cell responses by raising the threshold of signals needed for full activation, or may also play a role in terminating ongoing T-cell responses. Anti-CTLA-4 antibodies that block CTLA-4/B7 interactions enhance in vivo T-cell responses to peptides, superantigens and parasites, and can exacerbate experimental autoimmune encephalomyelitis or induce the rejection of newly implanted and well established tumors in several transplantable murine tumor systems. Using this principle, CTLA-4 blockade was used to create primary prostate cancer in a transgenic mouse model. It was shown that significant accumulation of inflammatory cells in prostate interductal spaces when TRAMP mice were treated with anti-CTLA-4 and a granulocyte–macrophage colony-stimulating factor (GM-CSF) expressing tumor-derived vaccine account for a significant reduction of tumor incidence. The authors demonstrated that antitumor response is directed against antigens expressed by both prostate adenocarcinoma and normal prostate, because immunization of nontransgenic mice with GM-CSF-expressing tumor cell vaccines under conditions of CTLA-4 blockade can also result in marked dorso-lateral prostatitis evident 28 days post-treatment. However, they found no evidence of significant inflammation or tissue damage in the dorso-lateral or ventral lobes of the prostates of nontransgenic mice immunized only with GM-CSF tumor vaccine (Hurwitz et al., 2000).

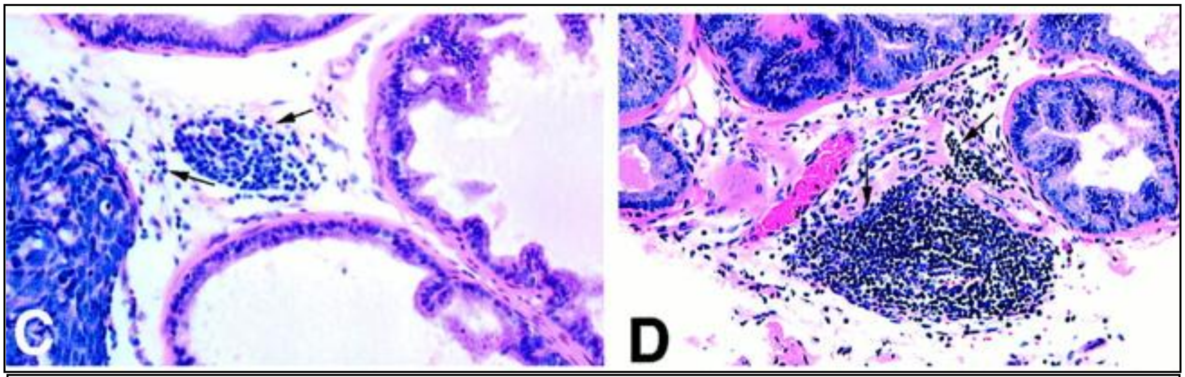

Figure 7. H&E staining of prostates from anti-CTLA4 treated TRAMP mice

(From Hurwitz et al, 2000)

Another popular mouse model for prostate inflammation was the prostate ovalbumin expressing transgenic (POET-1) model. This model was developed as a way to address the problem of the inability to monitor specific T cell populations in prostate inflammation. POET-1 mice express a membrane-bound ovalbumin fusion protein (mOVA) at high levels in the ventral and dorso-lateral prostate lobes following adolescence (Zhang et al., 2000, Adriani et al., 2001). The effectiveness of activated T cells in infiltrating and damaging POET prostate was evaluated using adoptive transfer of either CD8+ or CD4+ T cells that recognize of mOVA in prostate tissue (Kurts et al., 1997). It was shown that initiation of inflammation by ovalbumin specific CD8+ T cells using adoptive transfer resulted in the development of acute prostatitis in the anterior, ventral and dorso-lateral lobes of the prostate. This was characterized by recruitment of the adoptively transferred CD8+ T cells, autologous CD4+ and CD8+ T cells, regulatory T cells and myeloid derived suppressor cells. This was further confirmed by the elevated levels of pro-inflammatory cytokines and chemokines. It was also seen that inflammation led to marked proliferation of epithelial cells that was sustained for up to

80 days. This mouse model can be used to study acute and chronic non-bacterial prostatitis. Further, these mice could be crossed with other mouse models of prostate cancer to study the influence of chronic prostatitis on prostate cancer development (Haverkamp et al., 2011).

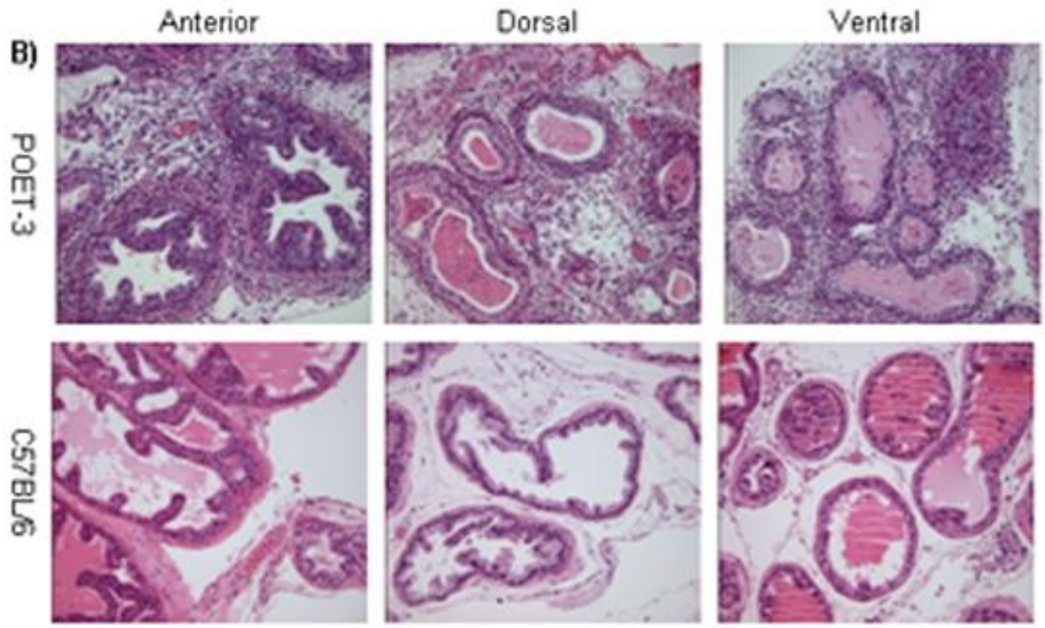

Figure 8. H&E staining of prostates from POET-3 mice and C57BL/6 control mice

(From Haverkamp et al, 2011)

These observations indicate that specific antigen priming or interference with immune signals is necessary to induce immune mediated prostatitis. However, using these mouse models it is difficult to further our understanding of prostate inflammation and the underlying cause of chronic pelvic pain.

Tetracycline Dependent Gene Activation System

Our mouse model uses the TetO-IL-1β|Hoxb13-rtTA system to induce expression of IL-1β specifically in the prostate gland upon induction by doxycycline. The Tet-On system makes use of the reverse tetracycline transactivator (rtTA) protein, which is created by fusing one protein, TetR (tetracycline repressor), found in E.coli with the activation domain of another protein, VP16, found in the Herpes Simplex Virus (Allen et al., 2000). In our mouse model, rtTA is placed under the control of Hoxb13, a prostate specific promoter. The resulting rtTA protein is able to bind to DNA at specific TetO operator sequences. The TetO operator is placed upstream of the human IL-1b gene. Upon induction with doxycycline, a tetracycline analog, rtTA can fuse with a doxycycline molecule and bind to TetO sequences, thus driving IL-1b expression in a prostate specific manner. A schematic of this system can be seen in Figure 5.

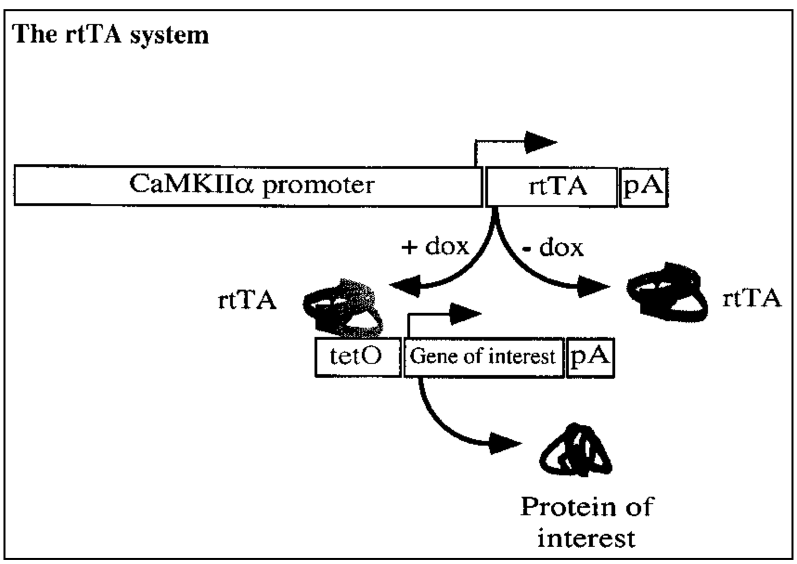

Figure 9. The rtTA system

Adapted from Mansuy et al., 2014

We have shown that these mice exhibit pelvic pain upon doxycycline induction.

Transient Receptor Potential channels

The Transient Receptor Potential (TRP) superfamily of cation channels have been shown to play an important role in sensation including vision, taste, olfaction, hearing, touch and thermo-sensation (Venkatachalam et al., 2007). The Vanilloid Receptor Type 1 (TRPV1) is a member of the transient receptor potential channel (TRP) superfamily and can be activated by exogenous vanilloids (such as capsaicin), heat (>43 degrees), low pH and various lipids such as anandamides that are present during inflammatory conditions. TRPV1 activity can also be enhanced by the presence of inflammatory mediators such as bradykinins, NGF and prostaglandins (Szallasi et al., 2007, Honore et al., 2009). Consequently, TRPV1 channels have been shown to be mediators of inflammatory pain and mice lacking these channels have been shown to have a decreased response to heat and mechanical induced inflammatory pain and hypersensitivity (Walder et al., 2012, Everaerts et al., 2011, Koivisto et al., 2014, Brain, 2011).

The TRPA1 channel has been identified as a target for noxious and inflammatory irritants such as mustard oil, implicating a role in pain and neurogenic inflammation (Tai et al., 2008, McMahon et al., 2006). Previous experiments have shown the effects of TRPV1 and TRPA1 inhibitors in alleviating inflammatory pain in other animal models (Borghi et al., 2013, Chen et al., 2015). The small molecule drug, ABT-102, developed by Abbott is a TRPV1 antagonist that has successfully completed Phase-1 clinical trials (Othman et al., 2012). It has been shown that ABT-102 has been successful in blocking nociception in rodent models of inflammatory, post-operative, osteoarthritic, and bone cancer pain (Honore et al., 2009).

Our aim is to study the role of TRPV1 and TRPA1 channels in mediating pelvic pain in our model of prostate inflammation. We further aim to evaluate the potential of ABT-102 in alleviating pelvic pain.

Materials and Methods:

Animal housing

Animal care was provided in accordance with NIH's Guide for the care and use of laboratory animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed in an animal facility with controlled temperature at 22±2°C, relative humidity at 55±15% and maintained on a 12h light/ 12 h dark cycle. Water and food were made available to the mice ad libitum. Aspen shavings were used as bedding within the cages (7.25"W x 11.5"D x 5"H). Mice were fed diet purchased from Harlan Laboratories (Ref: 2020).

Transgenic mice

IMPI transgenic mice were used for the experiments that were generated previously in our laboratory.

Breeding of mice

We bred the homozygous Hoxb13-rtTA+/+|TetO-IL-1ß+/+ mice onto both TRPA1 and TRPV1 null strains. The IMPI mice on an FVB background were crossed with B6.129 TrpV1null mice to generate triple heterozygous IMPI+/-|TRPV1+/- offspring. Multiple breeding pairs of triple heterozygous offspring were intercrossed to generate a large cohort of IMPI+/-|TRPV1+/- animals to finally generate the IMPI+/+|TRPV1null genotype. An identical strategy was employed to generate IMPI+/+|TRPA1null mice. These IMPI+/+|TRPV1null and IMPI+/+|TRPA1null mice will be used to further experiments. In order to negate any effects due to background of the mice, we bred our IMPI mice to the TRPA1-/- mice and selected the mice with intact channels upon inbreeding of the F1 generation. A schematic for the breeding can be seen in Figure 3.

Tactile Allodynia testing in mice

Measurement of tactile allodynia was done using von Frey filaments as previously described in rodent behavioral testing (Radhakrishnan and Nallu, 2009; Rudick et al., 2008). Filaments were tested in ascending order using five individual fibers with forces of 0.02 g, 0.04 g, 0.16 g, 0.4 g and 1.0 g (Stoelting) and applied perpendicularly to the pelvic region and the hind paw. During testing, mice were individually placed on a wire mesh and covered using tinted cups. Mice were allowed to acclimate for 45 minutes before the test began. Each filament was applied for 1-2s, 10 times, with 3 seconds intervals between each stimulus. The area tested was restricted to that of the pelvic region and the hindpaw. Such region specificity was designed to test for prostate specific pain sensitivity in males originating from Doxycycline induced prostate inflammation. In addition to the pelvic region, the plantar region of the hind paw was used as a control region to demonstrate reproducible response to the von Frey filaments in the presence and absence of Doxycycline. Reactions such as rapid abdominal withdrawal, scratching the stimulated area, jumping movements or micturation were recorded as positive reactions to filament pressure. The response frequency was recorded as the number of positive responses to the stimuli of the filament (number of positive responses to stimuli out of the 10 trials). For the hind paw, a positive response was defined as either a sharp withdrawal of the paw or licking of the paw tested. Standardized testing was applied to diminish variability, including fixed time of day and blinding of the experimenter.

Administration of ABT-102

ABT-102 ((R)-1-(5-tert-butyl-2,3-dihydro-1H-inden-1-yl)-3-(1H-indazol-4-yl)urea was synthesized at Abbott Laboratories. ABT-102 was dissolved in 100% polyethylene glycol (PEG400) for oral administration at a dosage of 10.45mg/kg body weight. ABT-102 was administered twice-daily for 2.5 days before tactile allodynia testing.

Histological Analysis

After euthanasia, the prostates of the mice were separated into different lobes and fixed in 10% Formalin. After 48 hours, formalin was replaced with 1X PBS. These tissue samples were sent to Oncology Tissue Services at Johns Hopkins University for H&E staining.

Results:

IL-1β mediated inflammation causes pelvic pain:

The first step was to demonstrate that our mouse model develops chronic pelvic pain as a result of prostatitis shown by inflammation in the prostate. Mice at 6 weeks of age were put on doxycycline at a dosage of 2mg/ml that was administered via their drinking water. One week following induction by doxycycline, the mice were tested for tactile allodynia using von Frey filaments (Radhakrishnan and Nallu, 2009; Rudick et al., 2011; Rudick et al., 2008; Traub et al., 2008). Pre-calibrated filaments were used in ascending order using five individual fibers with forces of 0.04 g, 0.16 g, 0.4 g, 1.0 g and 4.0 g and applied perpendicularly to the pelvic region and the hind paw. During testing, mice were individually placed on a wire mesh and covered using tinted cups to restrict movement and prevent escape. Before testing, the mice were allowed to acclimatize to the new environment for 45 minutes. Only one tester was allowed inside the room to prevent any behavioral changes in the mice. Each filament was applied ten times and the response frequency was measured for each stimulus. The area tested included the pelvic region and the hind paw. This region specificity was designed to test for prostate specific pain sensitivity in males originating from Doxycycline induced prostate inflammation. In addition to the pelvic region, the plantar region of the hind paw was used as a control region. Rapid abdominal withdrawal, scratching or licking the stimulated area, jumping movements or micturation was recorded as positive reactions to filament pressure. A cohort of mice homozygous for all the transgenes were chosen for the experiment. Before induction, the von Frey assay was done on the entire cohort to obtain a baseline reading of the pain. Tactile allodynia was checked in the pelvic region and the hindpaw was used as a control. Von Frey filaments of increasing thickness were used that each exerted a pre-calibrated amount of force. Following the baseline reading, the mice were put on doxycycline water while the control group was given regular water. After one week of treatment, the von Frey assay was performed on the cohort. We observed a significant increase in response frequency in the pelvic region of the group that received Doxycycline compared to the control group as seen in Figure 10A. The response frequency of the hindpaw did not significantly change as shown in Figure 10B.

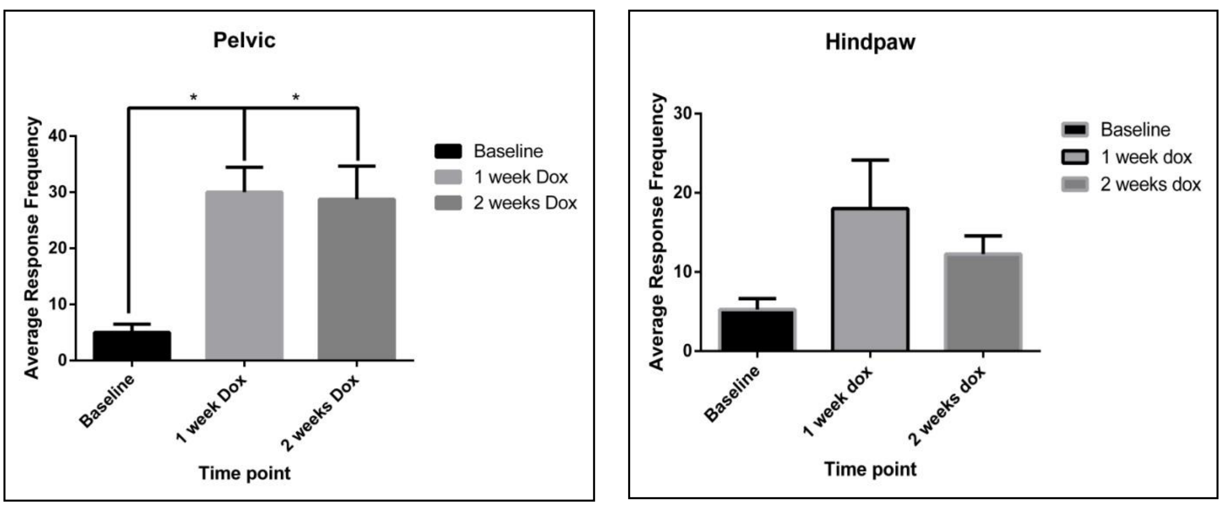

Figure 10(A): Average response frequency per mouse measured for the pelvic area. (B): Average response frequency per mouse measured for hindpaw. * represents p<0.05 as measured by paired Student's t-test. N=4 Error bars represent Mean±SEM

Besides showing increased pelvic pain, the mice that received doxycycline showed general irritability, were jumpy and sensitive compared to the control group. At the end of the experiment, the mice were euthanized and the prostates were fixed and stained. As can be seen in Figure 11, mice that were put on doxycycline for the course of the experiment showed inflammation compared to the control mice. This shows of corelation of pelvic pain and prostatic inflammation that appears at 1 week and 2 weeks post-induction with doxycycline.

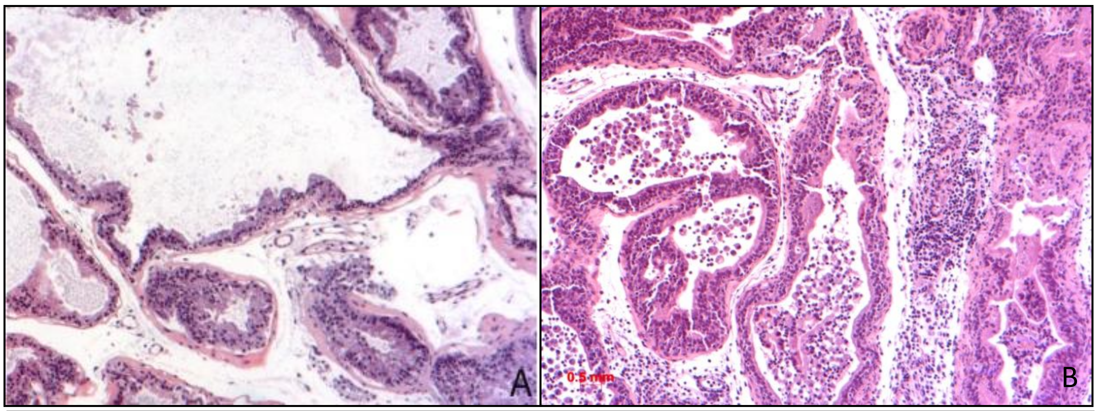

Figure 11(A): H&E stained sections of the ventral prostate in control mice. (B) H&E stained sections of the ventral prostate in mice induced for 2 weeks (arrows show infiltrating inflammatory cells).

The role of TRP channels in mediating chronic pelvic pain:

In order to identify the role of TRP channels, we measured tactile allodynia in mice using Von Frey filaments. We had three groups of animals in our cohort: IMPI mice that have intact TRP channels (IMPI+/+|TRPA1+/+|TRPV1+/+), IMPI Mice lacking TRPA1 channels (IMPI+/+|TRPA1-/-|TRPV1+/+) and IMPI mice lacking TRPV1 channels (IMPI+/+|TRPA1+/+|TRPV1-/-). These groups of mice were tested for tactile allodynia using the aforementioned von Frey filaments before the start of the experiment to obtain a baseline reading and were administered doxycycline for 1 week and tested again to obtain another reading. This was repeated for another week. We found a decreased response in mice lacking either of the TRP channels along with a lower baseline response compared to the control animals as seen in Figure 12. Stained sections of the prostates were prepared at the end of the experiment to confirm inflammation. We observed no significant difference in the level of inflammation as shown in Figure 13.

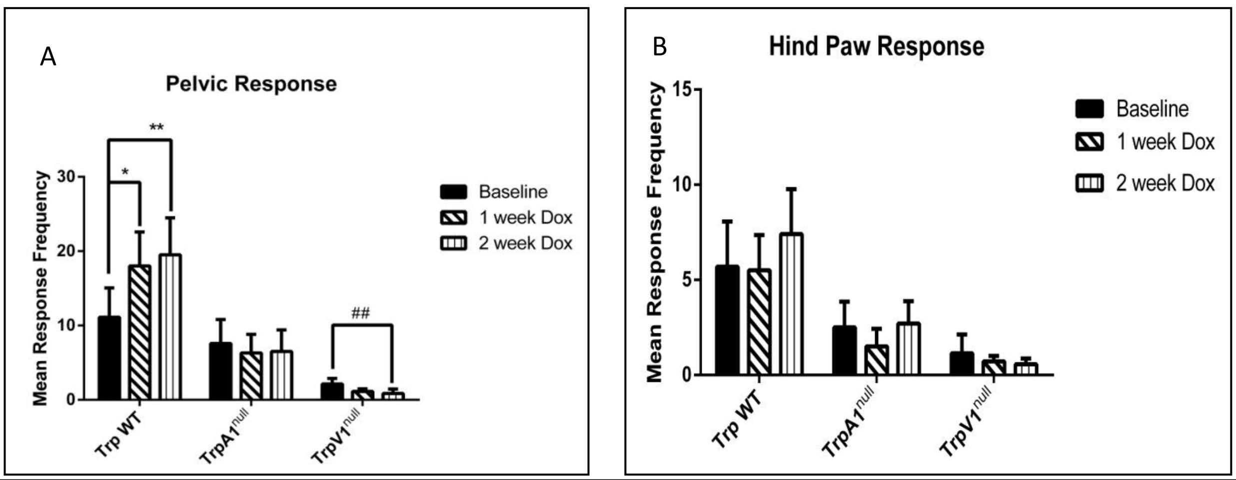

Figure 12(A): Average response frequency per mouse measured for the pelvic area. (B): Average response frequency per mouse measured for hindpaw. * represents $p<0.05$ compared to Baseline, ** represents $p<0.01$ compared to Baseline, ## represents $p<0.01$ compared to Baseline. All statistics were done using Student's t-test. Error bars represent Mean±SEM. N=10 for WT and TRPA1 KO; N=7

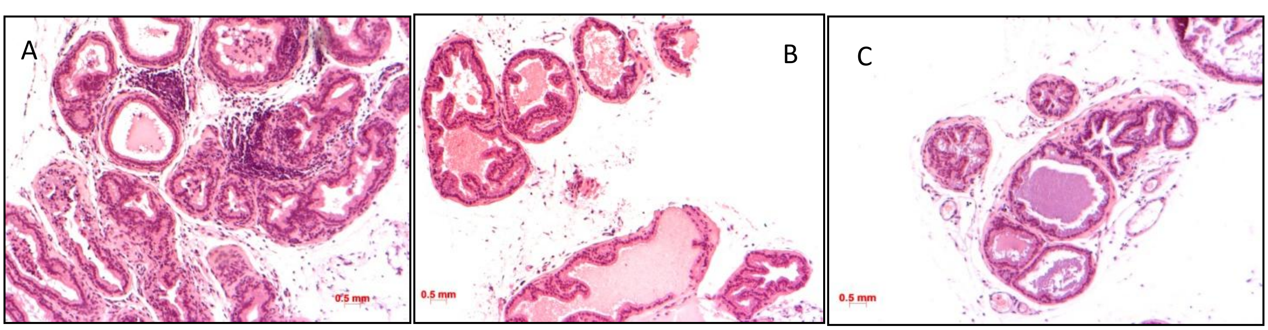

Figure 13(a): H&E stained sections of the Ventral Prostate from IMPI^{+}/TRPA1$^{+/+}$/TRPV1$^{+/+}$ mice. (B): H&E stained sections of the Ventral Prostate from IMPI^{+}/TRPA1$^{-/-}$/TRPV1$^{+/+}$ mice. (C): H&E stained sections of the Ventral Prostate from IMPI^{+}/TRPA1$^{+/+}$/TRPV1$^{-/-}$ mice.

The effects of blocking TRPV1 channels on pelvic pain:

In order to study the effect of blocking TRPV1 channels in our model of IL-1β mediated pelvic pain, we used the experimental drug ABT-102. ABT-102 is a selective TRPV1 antagonist and has been shown to specifically block TRPV1 channels in vivo by preventing capsacin-induced TRPV1 activation (Honore et al., 2009). ABT-102 was formulated as explained previously. Before the start of the experiment, mice were tested for tactile allodynia using the von Frey filaments to obtain a baseline response frequency. These mice were administered doxycycline for one week and response frequency was measured at the end of the week. ABT-102 was administered orally via gavage for a period of 2.5 days for a total of five doses. The control group received an analogous volume of solvent only (PEG400). Shortly after the fifth dose, the mice were tested for tactile allodynia using the von Frey filaments as previously discussed. We found a significant decrease in the response frequency of the drug group. However, no such decrease was seen in the control mice. In order to show that the effects of ABT-102 were reversible, these mice were then administered of doxycycline for another week without ABT-102 or PEG400. At the end of the week, tactile allodynia testing showed a significant increase in the response frequency in the drug group and a maintenance of response frequency in the control group showing that the effects of ABT-102 were reversible. In order to show that ABT-102 could successfully decrease tactile allodynia following a hiatus, a second course of ABT-102 was administered on the same schedule as before. We found a significant decrease in response frequency following repeated dosing of ABT-102 in the drug group in contrast to the persistent levels in the control group. In order to further confirm that this decreased response frequency was due to blocking of TRPV1 channels and not due to any differences in the levels of inflammation, these mice were euthanized at the end of the study and their prostates were fixed for H&E staining. No significant difference in levels of inflammation were seen between the drug group and the control group, further strengthening our claim that ABT-102 successfully blocked TRPV1 channels, leading to a decrease in response frequency. We found no adverse effects that could be attributed to ABT-102. However, we did find softening of stools in the mice from both groups. This could be attributed to the polyethylene glycol and not the drug.

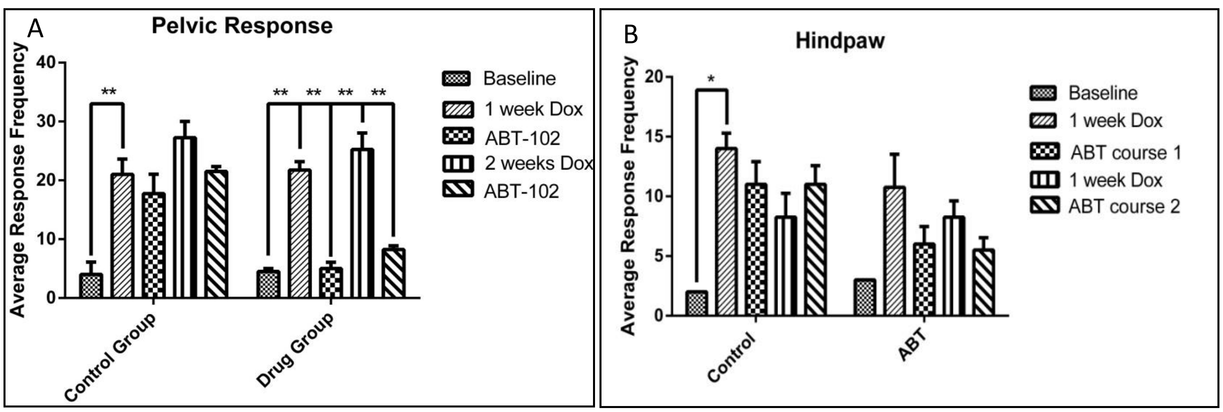

Figure 15(A): Average response frequency per mouse measured for the pelvic area. (B): Average response frequency per mouse measured for hindpaw. * represents p<0.05 compared to Baseline, ** represents p<0.01. All statistics were done using Student's t-test. Error bars represent Mean±SEM.

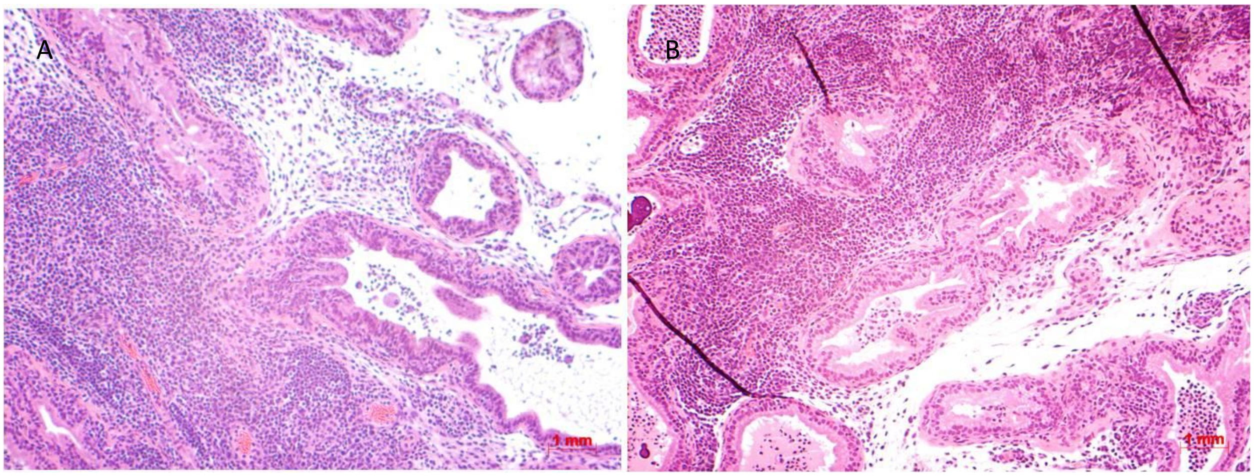

Figure 16(A): H&E stained ventral prostate sections of control mice. (B) H&E stained ventral prostate sections of control mice showing the presence of inflammatory cells.

Conclusion and Future Directions:

Using our IMPI mouse model, we have shown that IL-1β induced chronic prostatitis leads to chronic pelvic pain that was seen using von Frey filaments. By crossing these mice with TRPV1 KO mice we have shown the role of TRPV1 channels in mediating chronic pelvic pain. We further show that blocking these TRPV1 channels using an experimental TRPV1 antagonist, ABT-102 leads to an alleviation in pelvic pain as seen by tactile allodynia measurements using von Frey filaments. We have also observed that there is no significant difference in inflammation between the experimental groups and control groups as demonstrated by histological analysis. This shows that the alleviation of pain is not likely due to reduced inflammation but due to the blocking of these TRPV1 channels furthering reinforcing our finding that TRPV1 channels play an important role in mediating chronic pelvic pain in our mouse model of IL-1β mediated inflammation.

As we shown in our experiments, ABT-102 has the potential to alleviate chronic pelvic pain due to chronic prostatitis. In the future, this drug could be moved to clinical trials as a therapeutic for chronic pelvic pain in patients suffering from CP/CPPS.

Chapter 2

Role of inflammation in prostate cancer

Introduction:

Molecular biology of PTEN

PTEN was the first phosphatase gene identified to be a tumor suppressor (Li and Sun, 1997). PTEN plays a pivotal role in the regulation of the PI3K/AKT/mTOR pathway that is important for cell survival. It is known to counteract PI3K function, leading to inactivation of AKT and mammalian target of rapamycin (mTOR).

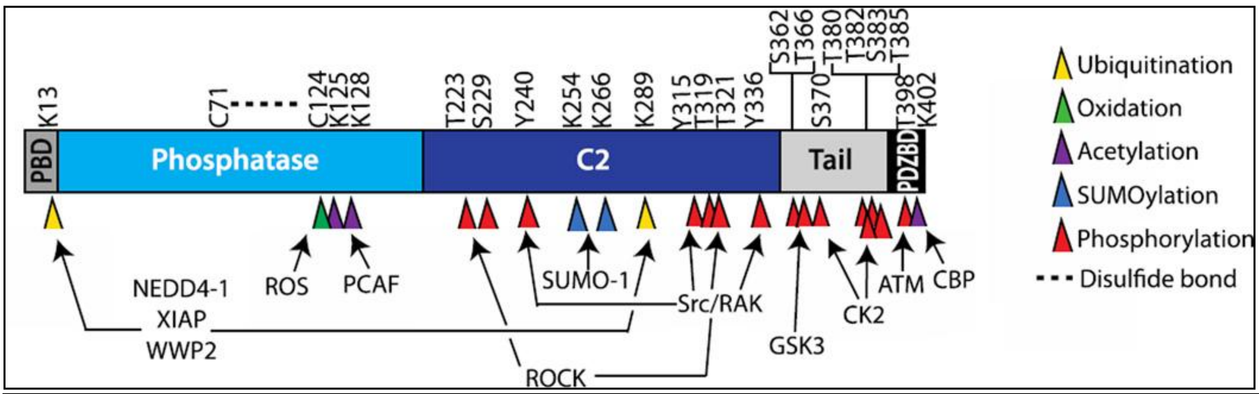

Figure 17: A schematic of the domains of the PTEN protein
From Hopkins et al., 2015

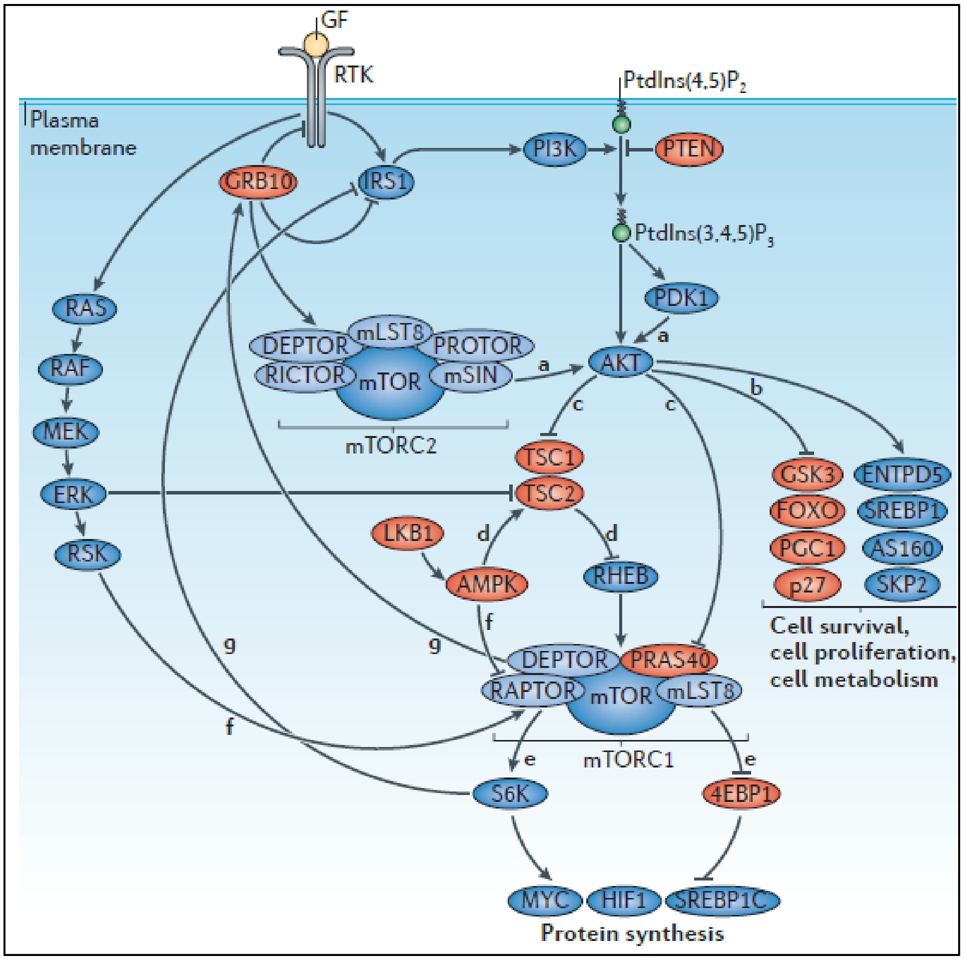

Figure 18: A schematic showing the PI3K/AKT/mTOR pathway
From Song et al., 2012

Since its discovery, most PTEN research has been targeted to study its role as a tumor suppressor. PTEN is also essential for normal embryonic development since mice with null mutation for PTEN die at 6.5 to 9.5 days post coitum (Di Cristofano et al., 1998). PTEN has been shown to be mutated in prostate cancer and its loss of function coupled with low levels of AR has been shown to result in worse prognosis (Choucair et al., 2012, Phin et al., 2013).

Mouse models with loss of PTEN:

It has been found that loss of one allele of PTEN causes a 50% reduction in PTEN levels and leads to pre-cancerous PIN lesions but not cancer, demonstrating that loss of one allele is sufficient to initiate tumorigenesis but not tumor progression (Trotman et al., 2003, Alimonti et al., 2010). However, reduction in PTEN levels by 70-80% causes it to progress to invasive adenocarcinoma of the prostate indicating that a more profound decrease in required for cancer progression to occur in the prostate (Alimonti et al., 2010).

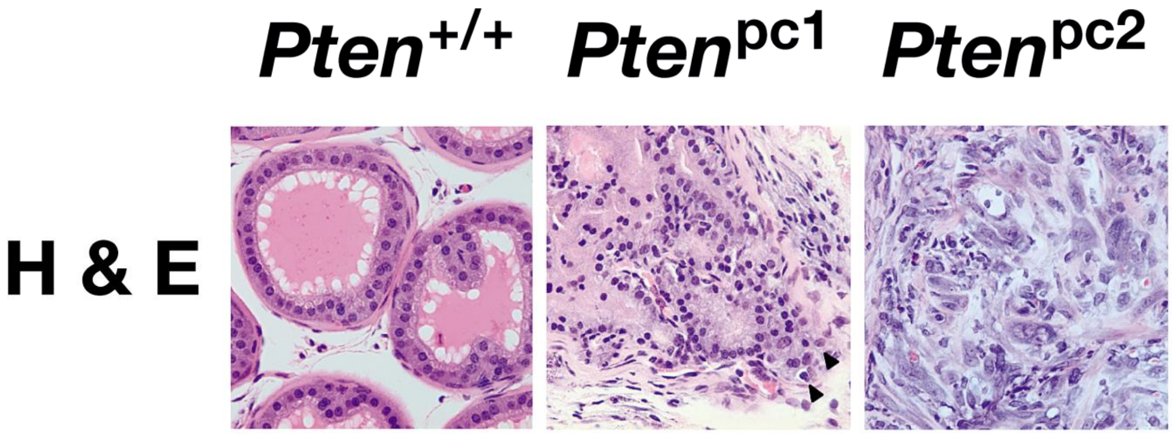

Figure 19: H&E staining of prostate from WT and PTEN knockout mice showing PIN lesions and invasive adenocarcinoma
From Trotman et al., 2003

Chronic inflammation has been shown to be a precursor for prostate cancer in patients and an increased risk of prostate cancer has been associated with incidences of chronic prostatitis (Huang et al., 2016, Sfanos and De Marzo, 2012, De Marzo et al., 2007). IL-1β has been shown to activate a signaling pathway that ends with the activation of the transcription factor NF-kB (Weber et al., 2010). Constitutive expression of NF-kB has been correlated to prostate cancer progression (Shukla et al., 2004). Constitutive expression of IKK2, a molecule that plays a role in the activation of NF-kB in mouse models promotes prostate tumorigenesis and in combination with heterozygous loss of PTEN causes an increase in tumor size (Birbach et al., 2011). These studies show the role inflammatory cytokines in progressing prostate tumorigenesis in cooperation with loss of PTEN leading to invasive adenocarcinoma.

Our aim is to investigate the effect of prostate-specific PTEN loss in the context of chronic inflammation in our mouse model that over-expresses IL-1β.

Materials and Methods:

Animal housing

Animal care was provided in accordance with NIH's Guide for the care and use of laboratory animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed in an animal facility with controlled temperature at 22±2°C, relative humidity at 55±15% and maintained on a 12h light/ 12 h dark cycle. Water and food were made available to the mice ad libitum. Aspen shavings were used as bedding within the cages (7.25"W x 11.5"D x 5"H). Mice were fed diet purchased from Harlan Laboratories (Ref: 2020).

Transgenic mice

IMPI and CRE/PTEN transgenic mice were used for the experiments that were generated previously in our laboratory.

Breeding of mice

We bred the homozygous IMPI mice with Cre/Pten$^{fl/fl}$ mice to generate IMPI$^{+/-}$|HoxB13-Cre$^{+/-}$/Pten$^{fl/+}$ mice.

Histological Analysis

After euthanasia, the prostates of the mice were separated into different lobes and fixed in 10% Formalin. After 48 hours, formalin was replaced with 1X PBS. These tissue samples were sent to Oncology Tissue Services at Johns Hopkins University for H&E staining.

Results

Long term effects of chronic prostate inflammation

In order to study the long term effects of chronic prostate inflammation, IMPI mice were put on doxycycline. At the end of 20 months, these mice were euthanized and their prostates were fixed for H&E staining. We found over 87% (7/8) of the mice had Prostatic Intraepithelial Neoplasia (PIN) lesions in the ventral and anterior lobes of the prostate. We also found multifocal PIN and carcinoma in situ in a subset of these mice. These results point to a direct correlation between chronic prostate inflammation and the incidence of pre-cancerous lesions.

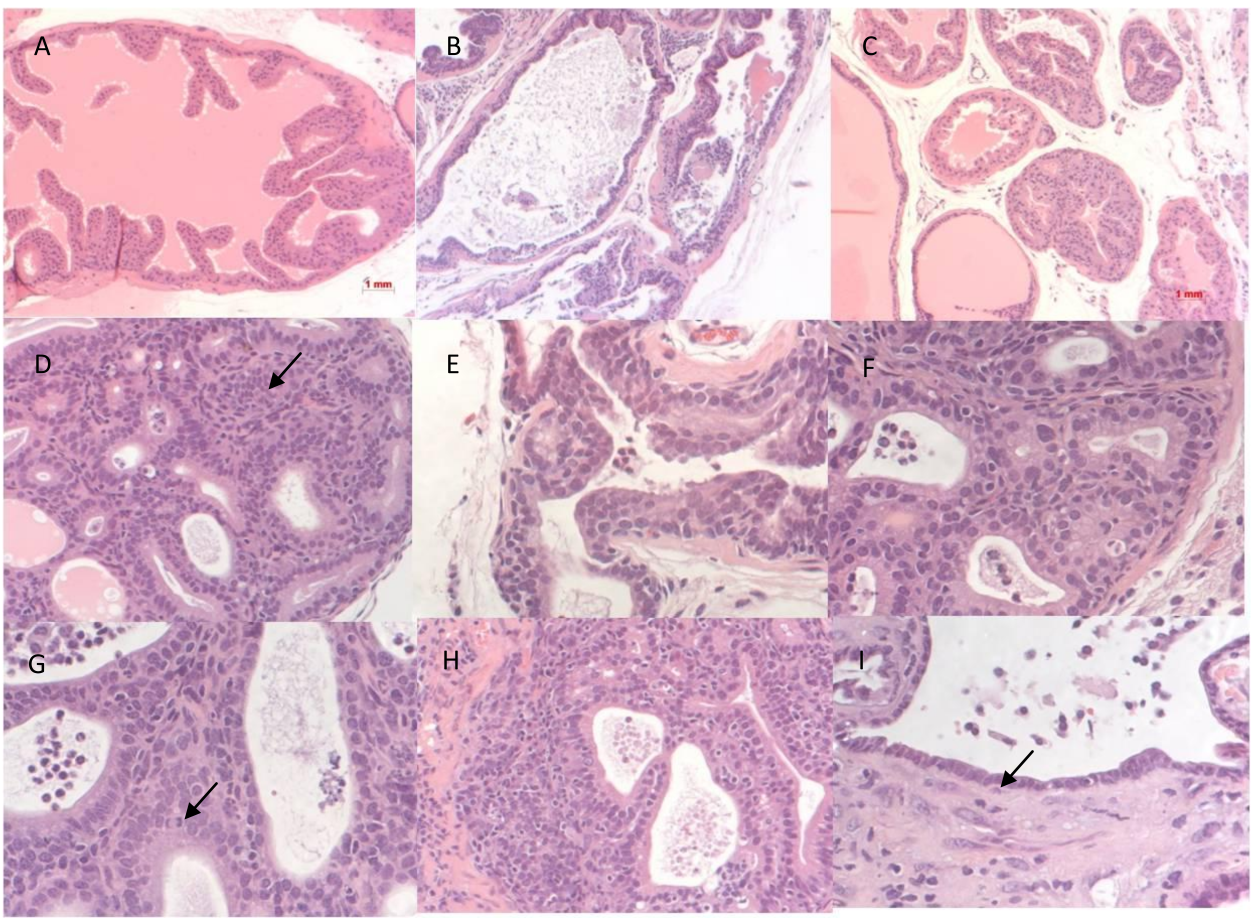

Figure 20: H&E staining of prostate from IMPI mice on doxycycline for 20 months. (A) Normal AP (B) Normal VP (C) Normal DLP (D) Invasive cells in AP (see arrow head) (E) CRIB-PIN in VP (F) PIN in DLP (G) Mitosis in AP (Arrow head shows cell undergoing mitosis) (H) Invasive AP (I) PIA in VP (Arrow head shows atrophic epithelium)

Effect of prostate specific over-expression of IL-1β in the context of PTEN loss

In order to study the effect of overexpression of IL-1β in the contest of prostate specific PTEN loss we used IMPI$^{+/-}$|HoxB13-Cre$^{+/-}$/Pten$^{fl/+}$ mice. These mice were put on doxycycline to turn on expression of IL-1β. At the end of 15 weeks, they were euthanized and their prostates were fixed for histological analysis. We found a significant inflammation in the mice that were put on doxycycline as characterized by the infiltration of immune cells compared to syngenic controls. We however did not find any atypical cellular morphology suggesting that a 15 week time point

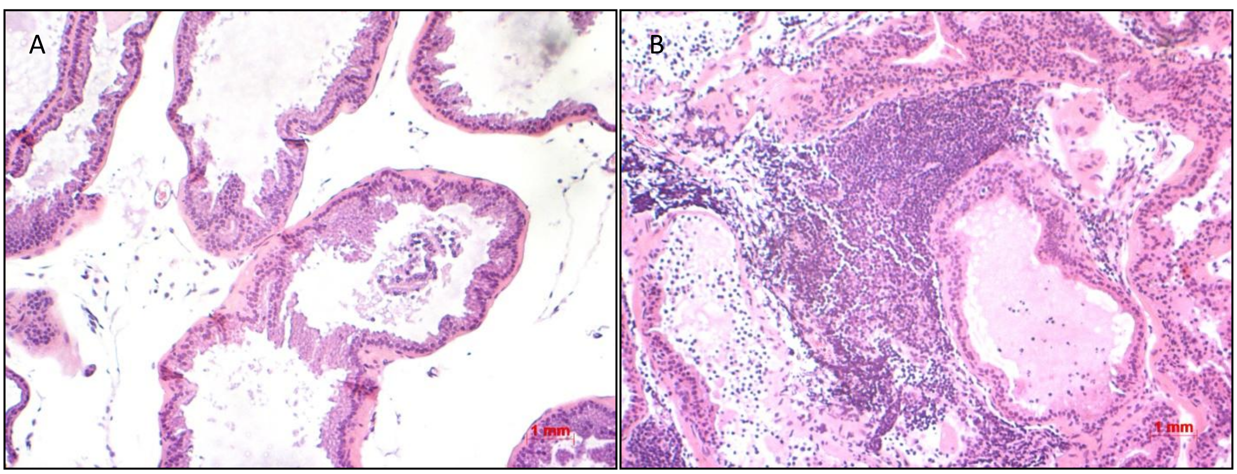

Figure 21: H&E staining of prostate from control mice (A) and dox-treated mice (B). Presence of inflammatory cells can be seen in (B) compared to the control mice

Conclusions and Future Directions:

The high penetrance of PIN in the IMPI mice at 20 weeks further reinforces the link between chronic prostate inflammation and the incidence of prostate neoplasia. In the future, we would like to study these further by using immunohistochemistry for markers such as Ki-67 and smooth muscle actin. These findings further demonstrate the usefulness of our IMPI mice in modeling chronic prostate inflammation.

Using the IMPI/Cre/PTEN model, we were unable to detect any malignant phenotype in the mice. However, this new transgenic mouse model could potentially lead to a better understanding of how inflammation plays an important role in tumor progression in the prostate by further histological analysis at advanced time points. These IMPI+| Cre+/PTEN+/- mice could be inbred to generate IMPI+| Cre+/PTEN-/- mice that can be further studied to elucidate disease progression. Immunohistochemistry for markers such as PTEN, Myc, Ki-67 could be done to study the effect on cell proliferation. If this mouse model is successful in demonstrating how chronic prostate inflammation correlates with a high incidence of cancer, various anti-cancer therapies that are currently being tested in our lab for other mouse models could be studied on these mice. Furthermore, transcriptome analysis of the prostates from these mice could lead us to a better understanding of gene expression and could give us insight into various biomarkers of prostate cancer pertaining to our model.

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