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## Androgen receptor inhibition suppresses anti-tumor neutrophil response against bone metastatic prostate cancer via regulation of T $\beta$ RI expression

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### Abstract

Bone metastatic disease of prostate cancer (PCa) is incurable and progression in bone is largely dictated by tumor-stromal interactions in the bone microenvironment. We showed previously that bone neutrophils initially inhibit bone metastatic PCa growth yet metastatic PCa becomes resistant to neutrophil response. Further, neutrophils isolated from tumor-bone lost their ability to suppress tumor growth through unknown mechanisms. With this study, our goal was to define the impact of metastatic PCa on neutrophil function throughout tumor progression and to determine the

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potential of neutrophils as predictive biomarkers of metastatic disease. Using patient peripheral blood polymorphonuclear neutrophils (PMNs), we identified that PCa progression dictates PMN cell surface markers and gene expression, but not cytotoxicity against PCa. Importantly, we also identified a novel phenomenon in which second generation androgen deprivation therapy (ADT) suppresses PMN cytotoxicity via increased transforming growth factor beta receptor I (T $\beta$ RI). High dose testosterone and genetic or pharmacologic T $\beta$ RI inhibition rescued androgen receptor-mediated neutrophil suppression and restored neutrophil anti-tumor immune response. These studies highlight the ability to leverage standard-care ADT to generate neutrophil anti-tumor responses against bone metastatic PCa.

## 1. Introduction

Prostate cancer (PCa) is the second most common cause of cancer death among men in the United States, accounting for an estimated 34,000 cancer deaths in 2023, which are mostly associated with metastatic disease[1, 2]. The current standard of therapy for advanced prostate cancer is either prostatectomy or initial androgen deprivation therapy (ADT), with response to therapy being initially characterized by biochemical response i.e., changes in the levels of prostate-specific antigen (PSA)[3, 4]. Approximately 50% of PCa patients progress to advanced stage disease characterized by biochemical recurrence and often, progression to metastatic disease or metastatic castration-resistant prostate cancer (mCRPC)[5] which is incurable. Although mCRPC patients exhibit progressing disease, many will initially respond to additional androgen receptor (AR) directed therapy given in the form of more selective androgen inhibitors including, inhibitors of androgen synthesis and AR signaling, such as abiraterone acetate (which inhibits CYP17A1) and enzalutamide, respectively[6–8]. However, even with a ~4–6 month improvement in overall survival, mCRPC patients typically succumb to the disease within 3 years after diagnosis of metastases. These statistics demonstrate a significant need to identify additional therapies or personalized therapeutic approaches.

Bone is the most frequent tissue site for metastatic PCa. It has been classically shown that within the bone microenvironment PCa promotes bone remodeling, e.g., excessive bone degradation, which subsequently releases bone-sequestered growth factors such as transforming growth factor beta, TGF $\beta$ , and further promotes tumor growth in what has been characterized as a “vicious” cycle of continued bone remodeling and cancer growth in bone [9, 10]. Importantly, PCa interactions with bone stromal cells contribute significantly to the progression of bone metastatic PCa (BM-PCa). We recently identified that bone marrow neutrophils and neutrophil precursors, which account for ~50–60% of cells in bone marrow [11, 12], migrate to BM-PCa and are protective against PCa growth in bone. Further, we found that BM-PCa resists neutrophil anti-tumor response and suppresses neutrophil cytotoxicity as the tumor progresses. However, the role of neutrophils in the progression of BM-PCa remains unclear.

Neutrophils are the most abundant leukocyte population in blood, representing 50–70% of all immune cells. More than  $10^{11}$  neutrophils are produced daily in bone marrow and released into circulation, ready to elicit an innate immune response to pathogens and tissue

damage. Based on evidence from our lab suggesting that the PCa tumor-bone environment suppresses neutrophil function, we sought to determine whether neutrophils might be utilized as biomarkers of disease progression and therapeutic response, and for identifying the impact of prostate cancer disease stage on neutrophil immune response.

In this study, we utilized patient-derived PMNs for comparison to primary bone marrow neutrophils and ran a preclinical mouse trial which revealed several novel insights into neutrophils in PCa. Importantly, we observed a therapy-associated regulation of neutrophil cytotoxicity that was independent of disease stage and the tissue source of neutrophils. Therapeutic options are limited for BM-PCa patients and in this study, we identified: 1) a novel mechanism through which androgens regulate neutrophil function and 2) PCa-associated neutrophil properties that can be utilized for development of neutrophil-focused cancer immunotherapy.

## 2. Materials & Methods

### Patient samples.

*Prospective blood sample collection:* Healthy men and PCa patients were deemed eligible and consented for sample donation within the UNMC integrated Cancer Repository for Cancer Research (iCaRe2). The iCaRe2 is a multi-institutional resource created and maintained by the Fred & Pamela Buffett Cancer Center to collect and manage standardized, multi-dimensional, longitudinal data and biospecimens on consented adult cancer patients, high-risk individuals, and normal controls. Patients for this study were specifically recruited from the Genitourinary Cancer Registry (GU-CARE), and all samples collected prospectively under the guidance and approval of the UNMC Institutional Review Board (IRB) and under iCaRe2 during routine patient appointments. The study consisted of 4 groups (n= >17/group): 1) healthy (no cancer or pathological disease) (n=17), 2) localized PCa (n=22), 3) bone metastatic hormone-/castration-sensitive PCa (mCSPC) (n=19), and 4) bone metastatic castration-resistant PCa (mCRPC) (n=19). PCa patients in our study received treatments according to the National Comprehensive Cancer Network (NCCN) guidelines, including androgen deprivation therapy (ADT): Leuprolide (1<sup>st</sup> line ADT group), AR signaling inhibitors (ARSIs; abiraterone, enzalutamide, darolutamide, apalutamide), and chemotherapy (docetaxel). Some patients received bone-targeted radiation therapy, radium-223. Patient blood was collected and PMNs immediately isolated after collection and utilized for downstream analyses, including cell counts, viability, flow cytometry, RNA and protein collection, RNA sequencing, and co-culture assay. Although one patient presented with additional malignancies, the co-culture data in this manuscript includes only bone metastatic patients. *FFPE Rapid Autopsy samples:* FFPE prostate metastases were provided from patients who signed written informed consent under the aegis of the Prostate Cancer Donor Program at the University of Washington (IRB protocol # 2341).

### Mice.

Only male mice were used for these studies. For neutrophil isolations, C57BL/6 mice were utilized (Jackson Laboratory; # 000664). Mice (8–12 weeks) were used for all experiments using mouse primary neutrophils. C57BL/6 males (6 weeks of age) were

used for RM1 *in vivo* intratibial metastasis models. For human PCa cell intratibial metastasis models, immunocompetent SCID/Beige (C.B-17/IcrHsd-PrkdcscidLystbg-J) mice were utilized (6 weeks, Envigo).  $T\beta RI^{flox/flox}$  mice were purchased (Jackson Laboratory; #028701) and backcrossed 8 times to C57BL/6 to obtain a pure background. To generate neutrophil-selective  $T\beta RI$  knockout mice, Catchup mice[13] ( $Ly6G^{Cre/dtom}$ ) were crossed with  $T\beta RI^{flox/flox}$  mice; knockout  $T\beta RI^{-/-}$  was determined using PCR genotyping and confirmed using immunoblot protein analysis of mouse tissues. Mice were housed on a 12 hour light/dark cycle, with free access to food and water. All procedures performed were approved by IACUC (UNMC).

### Cell culture media and reagents.

LNCaP cells were cultured in RPMI complete media (RPMI (Hyclone), 10% FBS (Peak Serum), and 1% penicillin/streptomycin (Gibco)). C42B, PC3, and RM1 were cultured in DMEM complete media (DMEM (Hyclone), 10% FBS (Peak Serum), and 1% penicillin/streptomycin). To collect conditioned media (CM), cell lines were washed with Phosphate Buffered Saline (PBS) to remove serum, full medium was replaced with serum-free medium RPMI and cells were incubated for 24 hours. CM was collected and centrifugation was done to remove cellular debris and stored at 4 °C until usage. Total protein content of CM was measured using BCA assay (ThermoFisher) to ensure equal protein concentrations for treating neutrophils. Fresh media was collected every 2 weeks for experimental use.

### Neutrophil isolation.

From patients, polymorphonuclear leukocytes/neutrophils (PMNs) neutrophils were isolated from freshly collected blood, within 5 hours of collection. Neutrophils were isolated using a commercially available kit (Human-EasySep Neutrophil Isolation, StemCell; Mouse-MojoSort Neutrophil Isolation, Biolegend), that involves negative selection from other cells using magnetic beads. PMNs were isolated specifically using a negative isolation magnetic bead method to prevent premature activation. Purity was confirmed by flow cytometry for cell surface markers (Human-CD14<sup>-</sup>, CD15<sup>+</sup>, CD16<sup>+</sup>, CD66b<sup>+</sup>; Mouse-CD11b<sup>+</sup>Ly6G<sup>+</sup>) and morphology assessment using Giemsa stain, as described[14–16]. For mouse tumor-associated neutrophils (TANs) and primary neutrophil co-cultures, bone marrow neutrophils were isolated from mouse tibia and femurs of male C57BL/6 mice. Bones were cleared of tissue and muscle, and the epiphysis was removed and discarded. A hole was made in the bottom of a 0.65 mL tube, and one bone was placed individually per tube. This tube was placed into a 1.5-mL tube for bone marrow collection, where it was then centrifuged at high speed for for ~5 seconds. The bone marrow was re-suspended in 1 mL of neutrophil isolation buffer and filtered using a 70 micron filter. Bones from each mouse were pooled and counted, and the MojoSort Neutrophil Enrichment (Biolegend) protocol was followed, as per manufacturers' instructions. Neutrophil isolation buffer used for isolations consists of: 1X PBS, 2% FBS and 2 mM EDTA. All *in vitro* assays (viability analysis, flow cytometry, co-culture) were setup and/or performed immediately. Patient PMN protein and RNA were isolated immediately after PMN isolation from blood and stored at -80°C for downstream analyses.

### Real-time Glo MT cell viability assay.

PMNs were incubated in triplicate at 100,000 per well in PCa CM with 2x Real-Time Glo reagent (Promega). MT Cell Viability Substrate and NanoLuc<sup>®</sup> Enzyme were added in equal volumes to culture media to create the 2x Real-time Glo reagent. This media was added directly to the cells at time zero, and luminescence was read at the indicated time points using a luminometer. Mouse neutrophils were treated for 2 hours with media supplemented with enzalutamide (Selleckchem) at 3  $\mu$ M and 10  $\mu$ M, then luciferase was quantified.

### Flow cytometry.

From patient samples, isolated peripheral blood neutrophils were washed and reconstituted in FACS buffer (2% FBS in 1X PBS) at  $1 \times 10^6$  cells in 200  $\mu$ L. For tumor studies, bone marrow was flushed from tumor-bearing and saline-injected tibiae using a syringe and excess cells were further flushed out of the marrow with FACS buffer. For staining, cells were incubated on ice with fluorophore-conjugated antibodies added at a maximum of 1  $\mu$ L per  $10^6$  cells in appropriate antibodies listed in Supplemental Table 1. Cell viability dye, Live/Dead (Invitrogen), was also added at a concentration of 0.2  $\mu$ L per  $10^6$  cells. Stained cells were incubated with antibody on ice in the dark for 20 min, rinsed with 1X PBS and were fixed in 1% formaldehyde in 1XPBS for 30 min in the dark. Prior to analysis, cells were reconstituted in FACS buffer. For all analyses, single and live cells were gated and percentage of positive cells per marker was quantified (FACS Diva software, LSRII, BD Biosciences).

### RNA Library Preparation and Bulk RNA Sequencing.

For patient samples, peripheral blood neutrophils were isolated and reconstituted in Trizol reagent. RNA was purified from Trizol using DirectZol kit (Zymogen), according to manufacturer instructions. Libraries were prepared using RiboErase and RNA HyperPrep sample preparation kits (Roche Kapa Biosystems) from 100ng of RNA. RNA samples were fragmented at 94C for 8 minutes with 14 cycles of PCR post-adapter ligation according to manufacturer's recommendation. The finished dsDNA libraries were quantified by Qubit fluorometer and Agilent TapeStation 2200. Libraries were pooled in equimolar ratios and evaluated for cluster efficiency and pool balance with shallow sequencing on an Illumina MiSeq. Final sequencing was performed on an Illumina NovaSeq with paired-end 100bp reads at the Dana-Farber Cancer Institute Molecular Biology Core Facilities. *RNASeq Analysis*. Sequenced reads were aligned to the UCSC hg38 reference genome assembly and gene counts were quantified using STAR (v2.7.3a)[17]. Differential gene expression testing was performed by DESeq2 (v1.22.1)[18]. Initial RNAseq analysis was performed using the VIPER snakemake pipeline[19]. Data was further analyzed using Ingenuity Pathway Analysis software (IPA; Qiagen). The full gene list was uploaded into the NCBI Repository and can be accessed at GEO Accession: GSE197609.

### Real-time qPCR.

For RNA isolation, Trizol reagent was added to treated neutrophils and RNA was extracted using the standard Trizol isolation protocol. RNA (1  $\mu$ g) was used to synthesize cDNA using qSCRIPT Super mix (Quantabio) and PCR was performed using Perfecta SYBR Green

FastMix (Quantabio). PCR was run using Bio-Rad CFX Real-Time System. PCR conditions were as follows for all primer sequences (Integrated DNA Technologies; sequences listed in Supplemental Table 2): Step 1: 95° 30s; Step 2: 95° 5s, 57° 15s, 72° 10s, 95° 10s (×39 cycles); Step 3: Melt curve 65° to 95° at increments of 0.5° for 5s. For enzalutamide experiments: mouse primary bone marrow neutrophils were treated with 3 µM and 10 µM enzalutamide for 2 hours at 37 °C in complete RPMI media and RNA isolated using Trizol.

#### **PCa:Neutrophil Co-culture assay.**

For PCa co-cultures with neutrophils, PCa cells were plated at 30,000 cells/well in a 24-well plate, in triplicate per condition. Twenty-four hours later, neutrophils were isolated, re-suspended in complete medium, and plated in direct contact with cancer cells at a ratio of 10:1 (neutrophils/cancer) and 3 µM, 10 µM enzalutamide were added into the media or neutrophils were treated with enzalutamide for 30 minutes prior to addition to the culture, where noted, in complete RPMI. For TβRI co-culture assays, primary neutrophils were incubated in complete RPMI supplemented with RepSox (5 nM; Selleckchem) in the presence or absence of enzalutamide, for 30 minutes prior to addition to PCs cells. After incubation overnight, neutrophils were removed, and cancer cell viability was measured with Trypan Blue Exclusion assay, using a hemacytometer.

#### **Neutrophil Extracellular Trap (NET) Production assay.**

For analysis of PMN NET secretion, primary mouse neutrophils were incubated for 2 hours in prostate CM (LNCaP, C42B) with or without enzalutamide (3 µM) or complete RPMI supplemented with PMA (100 nM) as a positive control. Sytox Green dye (500 nM; Sigma) was added to each condition, and after 30 min, images were taken of each well by an EVOS FL Auto microscope (Invitrogen; AMAFD1000). The number of green fluorescent DNA traps/NETs was measured as a percentage of total cells to distinguish between dying cells that absorbed the Sytox Green dye. CM-treated neutrophils were compared to neutrophils incubated in serum-free media RPMI.

#### **Neutrophil migration assay.**

Primary neutrophils were isolated from mouse hind limbs using MojoSort and  $1 \times 10^5$  seeded in the top of transwell migration chambers, with a pore size of 5 microns (Costar; Ref # 3422). Neutrophils were allowed to migrate towards prostate cancer CM added to the bottom of the respective wells for one hour. To examine impact of androgen signaling, mouse neutrophils were pre-treated with 3 µM of enzalutamide or abiraterone (R&D Systems) for 30 minutes before addition the insert. Neutrophils were allowed to migrate towards specific conditions for 1 hour: serum-free media, serum containing 2% FBS, LNCaP CM, and C42B CM. Inserts were fixed in 100% methanol for 5 minutes, washed in 1X PBS to remove non-adherent cells and stained with hematoxylin for quantitation of the number of migrating neutrophils per insert. Insert membranes were mounted on a slide using Permount (Fisher Scientific) and imaged using an EVOS FL Auto microscope at 10x magnification.



### Immunoblot analysis.

Protein was isolated from patient PMNs using RIPA buffer (ThermoFisher, MA) and total protein quantified using BCA assay (Pierce, ThermoFisher, MA). Protein (50 µg) was run on a 12% SDS gel and then transferred to a PVDF membrane. The membrane was stained with 0.1% (w/v) Ponceau S in 5% (w/v) acetic acid for 5 minutes at room temperature. Background staining was removed with 3 washes of distilled water prior to capturing an image. Distilled water was used to destain the membrane, non-specific binding was blocked with 5% non-fat milk in 1X TBST for 1 hour at room temperature followed by overnight incubation with the primary antibody, 1:1000 in 5% non-fat milk, at 4°C. The following primary antibodies were used: MnSOD (#06-984, MilliporeSigma, Burlington MA), Catalase (#ab76024, Abcam, Boston MA), CuZnSOD/SOD1 (#ab51254, Abcam, Boston MA), GAPDH (#sc-32235, Santa Cruz Biotechnology, Dallas TX). The blot was washed in TBST prior to incubating with the secondary antibody, 1:4700 in 5% non-fat milk (anti-rabbit IgG #ADI-SAB-300-J, Enzo Lifesciences, Farmingdale NY or anti-mouse IgG #7076, Cell Signaling Technology, Danvers MA). The membranes were imaged using Azure Biosystems Radiance Plus substrate solution and an Azure c600 Imaging System.

### Immunohistochemistry.

Patient bone specimens and mouse tibia bone sections were dewaxed and hydrated through an alcohol gradient. Antigen retrieval for human and mouse specimens was performed using Tris-EDTA buffer (pH 9) in a pressure cooker on high temp for 6 minutes. Tissues were then blocked in 10% serum in 1X tris-buffered saline (TBS) for 1 hour prior to overnight incubation in primary antibodies (Myeloperoxidase (7.5 µg/ml), R&D MAB3174; TGFβ Receptor I (1:100), Millipore, ABF17-1). Following washes, species-specific secondary AlexaFluor antibodies were incubated 1:1000 on the tissues for 1 hour at room temperature. Fluorescent images were taken on a Zeiss Axio Imager Z2 at 20x.

### Two-photon microscopy.

An upright Olympus FVMPE-RS Multiphoton Laser Scanning Microscope equipped with a Spectra Physics dual line InSight X3 near infrared laser and 25x (1.05 NA) water-immersion objective was used to image inside intact tibial bone. Collagen autofluorescence (AF, 495–540 nm emission) and second harmonic generation (SHG, 410–455 nm emission) were collected using 880 nm excitation. Fluorescence from dTomato positive PMNs was collected using 1040 nm excitation and a 575–645 nm emission filter.

### Single cell RNA sequencing and data analysis.

Murine bone marrow samples were used to generate 5' gene expression libraries (10x Genomics) which were sequenced on a NovaSeq 6000 instrument. Demultiplexing was performed with Bcl2fastq. Alignment, tagging, and gene and transcript counting were performed with Cellranger. Quality control and analysis were performed using the R package Seurat. Dead or low complexity cells were filtered by removing cells with less than 200 features and cells with a percentage of mitochondrial reads greater than 6%. Doublets were removed by filtering cells with greater than 5000 features or greater than 30,000

transcripts. Filtered data was normalized with SCTransform, and dimensional reduction was performed by UMAP. Cluster ID was performed using the R package SingleR.

### In vivo mouse models.

**Androgen regulation study:** To examine the impact of androgen regulation on tumor burden and tumor-associated bone neutrophils (TBNs) function, we performed a preclinical/mouse study using standard-of-care therapy for BM-PCa, enzalutamide, in combination with exogenous testosterone (to mimic bipolar androgen therapy (BAT)). Male SCID Beige mice were chemically castrated using subcutaneous injection once with Degarelix (commercially available gonadotropin releasing hormone antagonist; 10mg/kg, Selleckchem) at a concentration which allowed for systemic depletion of androgen for the duration of the study [20, 21]. The following day, mice were injected intratibially with luciferase-expressing C42B prostate cancer cells. For injections, C42B were grown to confluence, trypsinized and washed with 1X PBS, and filtered using a 70-micron nylon filter. Cells were counted and reconstituted for intratibial injection of  $5 \times 10^5$  C42B per 40  $\mu$ L volume per mouse. Mice were anesthetized with isoflurane, and  $5 \times 10^5$  C42B injected into the right tibia. An equal volume of PBS was injected into the contralateral limb, as a control for the intratibial injection. At day 3 post-intratibial injection, tumor burden was imaged via bioluminescence.

For bioluminescence imaging, mice were given 10  $\mu$ L/g of D-luciferin (15 mg/mL) (Gold Bio) intraperitoneally and imaged using the IVIS Spectrum imager (PerkinElmer). Luciferase signal was quantified 15 min after injection, using the Living Image Software per manufacturer's instructions. Using bioluminescence intensity, mice were randomized (based on intensity above threshold minus background signal) into 6 treatment groups (n =4–5/group; based on available mice after confirmed tumor take) to receive either: 1) enzalutamide (10mg/kg) or 2) vehicle control (DMSO); 3) placebo subcutaneous pellet (Innovative Research), and 4) slow-release 5- $\alpha$ -dihydrotestosterone pellet (DHT;12.5mg 21-day release; Innovative Research); 5) enzalutamide plus placebo pellet and 6) enzalutamide plus DHT pellet. Pellets were subcutaneously transplanted 7 days after the start of enzalutamide treatment. IVIS imaging was used for longitudinal measurement of tumor burden. Mice were euthanized at 2 weeks to allow for isolation of neutrophils. Tumor-bearing and saline-injected tibia were flushed with sterile PBS and neutrophils isolated using MojoSort Neutrophil Isolation kit for analysis in co-culture and for protein analysis. Protein was isolated from TBNs using RIPA Lysis buffer, total protein content measured using BCA assay, and immune-modulating cytokines and chemokines examined by protein array (RayBiotech; Mouse Cytokine Array C3), according to protocol. As a control, tumor naïve neutrophils (from tibia injected with saline) were used for comparison. Flow cytometry was performed on neutrophils from tumor bearing and tumor naïve mice using the following markers: CD11b, CD45, Ly6C, Ly6G, F4/80 and CD11c (Biolegend).

**T $\beta$ RI knockout and inhibitor study:** To examine the impact of neutrophil-selective knockout of T $\beta$ RI on BM-PCa *in vivo*, the mouse RM1 prostate cancer model was utilized;  $3.5 \times 10^4$  luciferase-expressing RM1 were injected into tibia of 6–7 week old T $\beta$ RI knockout (T $\beta$ RI<sup>-/-</sup>) or floxed T $\beta$ RI mice (wildtype expression; T $\beta$ RI<sup>lox/lox</sup>). Additionally, luciferase-expressing RM1 was used to examine the outcome of pharmacologic T $\beta$ RI and AR



inhibition on PCa growth in bone. For this study,  $3.5 \times 10^5$  luciferase-expressing RM1 were injected into tibia; 3 days post-injection, mice were randomized via bioluminescence into 4 treatment groups (n=5–7/group based upon confirmation of tumor take): 1) vehicle control (DMSO), 2) enzalutamide (10mg/kg), 3) RepSox, T $\beta$ RI inhibitor (5mg/kg), and 4) combination enzalutamide and T $\beta$ RI inhibitor. After confirming the tumor take based on meeting luminescence threshold values (previously determined to reflect exponential tumor growth throughout the study), mice were randomized in drug groups based on histogram distribution compared to the median of all mice. For both studies, tumor burden was measured longitudinally throughout the study using bioluminescence and hind limbs collected at the end of the study for downstream analyses.

### Statistical power and analysis.

For patient samples, per power analyses for our studies by the UNMC Biostatistics Core, a minimum of 17 patients per group was needed to detect at least a 2-fold difference in gene expression. This calculation was based assumption of a sequencing depth of 20, and a coefficient of variation of 0.4, and alpha of 0.001 to adjust for multiple comparisons for the RNA-Seq study. This sample size also achieves 80% power to detect a large Cohen's d effect size of 1.0 at alpha=0.05 using a two-sided two-sample t-test. Additional subjects were recruited to account for any patients with low yields of PMN/working material. For statistical analyses: Graphpad Prism software was utilized for all other statistical analyses. Continuous patient data was compared between diagnosis and treatment groups using one way- or two-way analysis of variance (ANOVA) when appropriate. Preclinical data was analyzed using one-way ANOVA. Tukey's correction was used as a secondary test for comparing all groups to each other.

## 3. Results

### Impact of PCa progression on PMN number and viability.

Blood was prospectively isolated from men of various stages of PCa (localized/non-metastatic disease; bone metastatic hormone/castration-sensitive (mCSPC); bone metastatic castration-resistant (mCRPC). For comparison, blood was collected from healthy male donors. All patient groups and treatments are shown in Table 1. Usage of the neutrophil-to-lymphocyte ratio for determining prognosis has yielded varied results[22], thus we wanted to examine whether PMN numbers in blood are altered independently of other leukocyte populations. We found that absolute PMN numbers per volume of blood were similar between groups; however, there was a trend towards increased PMN numbers with progression to metastatic disease (comparing healthy to mCSPC) that was significantly reduced with progression from mCSPC to castration resistant disease (mCRPC) ( $p < 0.01$ ) (Figure 1A, left). This finding was independent of isolation-associated impact on viability as there were no significant differences noted in cell viability when comparing the three PCa groups. However, PMNs from patients with localized PCa showed increased numbers at 18 hours (173%;  $p < 0.05$ ) and 24 hours (141%) compared to healthy patient PMNs (Figure 1A, right), suggesting either prolonged viability or enhanced proliferation, as frequently seen with immature/undifferentiated PMNs.

### PCa progression induces a pro-inflammatory PMN gene signature.

To gain further insight into the impact of PCa progression on molecular changes in PMNs, we performed bulk RNA sequencing comparing disease stage (n=16 total (4/healthy; 4/localized; 3/mCSPC; 5/mCRPC); note: we initially intended to analyze 4 per group however, one mCSPC patient became resistant to androgen therapy after diagnosis, but prior to sample collection. There were >10,000 genes detected per treatment group out of approximately 25,000 genes analyzed (full gene list can be found at GEO Accession: GSE197609) (Figure 1B, left). Because PMNs are terminally differentiated cells, there was a low total number of genes altered per group. After controlling for the false discovery rate, we found that overall, there were more PMN genes turned “on” than “off” throughout PCa progression. When comparing disease stage, the largest number of PMN gene alterations occurred in mCRPC patients compared to healthy individuals; genes up-/down-regulated by more than 2-fold, ( $p<0.05$ : 135 up/22 down;  $p<0.01$ : 44 up/11 down) demonstrating that there is a change in the molecular profile of PMNs in PCa patients throughout progression. There were fewer genes altered between PCa groups and treatments (Figure 1B, right; Supp Figure 1A and 1B). When comparing PMNs from healthy men with CRPC patients, cancer-derived PMNs exhibited a pro-inflammatory molecular profile, including increased expression of *CD177*, *MMP8*, and *CYBB* (Figure 1C, left). There were some shared upregulated genes, but no shared downregulated genes, when comparing mCRPC vs. healthy and the 2 metastatic groups, mCRPC vs. mCSPC, including *CYBB* (Supp Figure 1C). There were some genes that were only altered with progression from metastatic hormone-sensitive PCa to metastatic castration-resistant disease, including complement 3 receptor 1 (*C3AR1*) and C-type Lectin Receptors 12A & 12B (*CLEC12A/12B*), which are all major inducers of inflammation suggesting a stage-specific shift to a highly pro-inflammatory PMN population (Figure 1C, right). These findings suggest that the biggest impact on the PMN transcriptome occurs at the early stage of PCa carcinogenesis and as PCa acquires resistance to hormone-targeted therapy.

Significantly altered genes were further analyzed by Ingenuity Pathway Analysis (IPA) software (Supp Figure 1D). The top 2 enriched canonical pathways in mCRPC PMNs compared to healthy, were: 1) glycolysis I (5 of 26 genes), and 2) 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase 4 (PFKFB4) signaling pathway (6 of 46 genes) both which are associated with altered redox signaling and glucose metabolism[23]; these findings are in support of our previous findings where we identified that BM-PCa alters redox metabolism genes in bone marrow neutrophils and increases neutrophils reactive oxygen species production (ROS)[24].

TGF $\beta$  was identified as a critical upstream mediator of several of the identified PMN gene changes including immune-regulating markers arginase 1 (*ARG1*), *WNT5B*, *IL4 receptor*, and *Cathepsin B* as well as ROS regulator, *CYBB* (Supp Figure 2). Based on evidence of PCa-induced *CYBB* expression in human bone marrow PMNs, increased PMN release of reactive oxygen species and activation of antioxidant signaling pathways [24], we examined antioxidant protein levels in patient-derived PMNs (n=8/group). We noticed there was reduced PMN protein content, seen by Ponceau Stain (Supp Figure 3A); this was to be expected in terminally differentiated cells. However, this reduction was also observed in

housekeeping genes, such as GAPDH (which also are metabolic enzymes) which prevented densitometry normalization. Thus, we quantified overall positivity of specific antioxidants, superoxide dismutases (SODs) and catalase per patient. There was an increase in SOD1 positive PMNs with PCa progression to metastasis and to therapy-resistant disease (Supp Figure 3B). Specifically, there was less SOD1-positivity in PMNs of patients with metastatic diseases (~2–3 of 8 patients compared to 6 of 8 patients from localized PCa and healthy men). This suggests a potential shift in the redox metabolism of PMNs from metastatic patients.

### **PMNs from prostate cancer patients show a combination of mature and immature myeloid cell markers.**

To gain insight into PMN differentiation status, we examined known cell surface markers classically found on all PMNs, along with markers associated with immunosuppressive PMNs and pro-inflammatory, activated PMNs[14, 25, 26]. Cell surface markers were examined on viable peripheral blood CD11b<sup>+</sup>CD15<sup>+</sup>CD16<sup>+</sup>CD14<sup>-</sup> PMNs. There were no differences in the percentage of CD15<sup>+</sup>(granulocyte marker), CD16<sup>+</sup> (marker for mature granulocytes; not shown), and CD14<sup>+</sup> (monocyte marker) cells per diagnosis (Figure 2A). There was a slight, though not significant, increase in LOX-1<sup>+</sup> PMNs with progression to metastatic disease (i.e., comparison of localized PCa to mCSPC) indicative of an immunosuppressive phenotype. Similarly, there was a reduction in CD10-positive PMNs in all PCa patients; it was most significantly reduced in patients with bone metastases (~21% reduction in mCSPC and mCRPC;  $p<0.01$ ) compared to patients with only localized PCa. PMN expression of CD10 has been associated with maturation[27], suggesting that PMN populations from PCa patients are less mature and immunosuppressive. Even still, ~50% of PMNs from all stages expressed CD10 and fewer than ~20% were LOX-1 positive. There was a slight reduction in CD66b, a marker of activated PMNs, on all PCa patient PMNs compared to healthy patients. However, another activation marker CD88 showed no difference when comparing healthy men with patients with localized PCa but was reduced with development of metastasis (in mCSPC). Interestingly, CD88<sup>+</sup> PMNs increased significantly increased in mCRPC, compared to mCRPC ( $p<0.01$ ). These data highlight disease-specific changes in PMN cell surface markers that may dictate PMN function throughout PCa progression.

Collectively, these findings reveal PCa disease stage-associated alterations in canonical markers of PMN activation suggesting enhanced PMN cytotoxicity with progression of PCa to metastatic disease.

### **Second generation hormone therapy suppresses neutrophil cytotoxicity.**

The changes in gene expression and cell surface markers suggests that cytotoxicity of PCa PMNs would be altered with cancer progression. To test this possibility, isolated patient PMNs (n= >17/group; Table 1) were cultured overnight with PCa cells (non-metastatic LNCaP, C42B (bone metastatic LNCaP cells), and PC3 (bone metastatic, AR-negative, PMN-resistant))[28–30]. Patient PMNs from all disease stages induced cell death of LNCaP (~25%) and C42B (~35–40%), though not significantly, due to some variability between patients (Figure 2B). In contrast, PC3 cells were resistant to PMNs from all disease stages

and there was significantly more C42B cell death, induced by healthy patient PMNs ( $p=0.0001$ ) and even mCRPC-PMNs ( $p<0.0001$ ), compared to PC3. We observed PMN cytotoxicity to PC3 (~40% cell death) and in some cases PMNs promoted proliferation of PC3 cells (Figure 2B).

We had previously shown PMN response to PCa to be somewhat dependent on PCa expression of STAT5, a mediator of AR signaling and target of neutrophils, such that neutrophils display targeted killing of STAT5 positive cells [31]. Our patient PMN co-culture data support the fact that there are cancer intrinsic and extrinsic factors that affect neutrophil-induced prostate cancer cell death. Patient PMNs were most cytotoxic towards the AR-positive PCa cells, however there was some variability in killing capacity such that some PMNs were both suppressive and promotive of each PCa cell line, suggesting that cytotoxicity is also dependent on PMN intrinsic factors.

The majority of all PCa patients in this study received 1<sup>st</sup> line ADT, i.e., Leuprolide, an antagonist of gonadotrophin-releasing hormone, while patients whose disease progressed (as determined by rising PSA levels) were administered 2<sup>nd</sup> generation non-steroidal anti-androgens (including abiraterone or enzalutamide) (Table 1). Previous studies have shown that, in addition to prostate epithelial cells, immune cells also express AR, which can regulate immune cell function and immune response [32, 33]. Based on the diversity of ARSIs prescribed in our patient cohorts and the known functions of AR signaling in neutrophil function, we next examined PMN cytotoxicity of mCRPC patients who had only received 1<sup>st</sup> line ADT (Leuprolide or orchiectomy) at the time of blood collection, in comparison to PCa patients who received 2<sup>nd</sup> generation ADT, including ARSIs. We focused on these patients because they represent the most aggressive form of PCa, exhibited the most variability in PMN cytotoxicity, and these patients were represented in both cohorts of 1<sup>st</sup> line ADT vs. 2<sup>nd</sup> gen ADT. We also included one patient who received chemotherapy (Docetaxal (Doc)). Other treatment groups were excluded from the co-culture analyses (treatment demographics shown in Table 1). Interestingly, we found that PMNs from patients treated with 1<sup>st</sup> line ADT, were more cytotoxic to C42B than PMNs from patients who received 2<sup>nd</sup> generation androgen therapy (~75% vs. ~40%, respectively;  $p<0.05$ ) which showed impaired cytotoxicity (Figure 2C). In total, PMNs from patients treated with 2<sup>nd</sup> generation androgen inhibition were significantly less cytotoxic than PMNs from patients treated with only 1<sup>st</sup> line ADT. Although AR is likely not the only mediator of PMN cytotoxicity, these data suggest that androgen regulation is a mediator of anti-tumor PMN response.

### **Bipolar androgen therapy manages BM-PCa progression and restores neutrophil immune response.**

Disease recurrence in PCa is typically associated with acquired resistance to androgen therapy. Metastatic PCa is treated with ARSIs upon disease recurrence; however, the tumor inevitably adapts to reduced AR signaling, by reducing AR expression[34]. A previous clinical study leveraged the dynamic tumor through applied high dose testosterone to “shock” the tumor by overloading it with more testosterone than feasible to utilize (bipolar androgen therapy (BAT)), followed by cycles of 1<sup>st</sup> line ADT (GnRH antagonists/agonists)

and testosterone to stabilize tumor progression [35, 36]. Along these lines, we next tested the potential of using androgen regulation to manipulate neutrophil immune response *in vivo* by establishing a modified BAT study, incorporating exogenous testosterone into a mouse bone metastasis model. Male mice were administered 1<sup>st</sup> line ADT/Degarelix to deplete systemic androgen[20, 21], followed by intratibial inoculation of C42B cells, and were then placed on 2<sup>nd</sup> generation ADT (i.e., enzalutamide). To examine the impact of exogenous testosterone on prostate tumor growth in bone and neutrophil function, mice were implanted with a DHT pellet, or placebo for comparison (Figure 3A schematic). In contrast to clinical BAT studies in which patients again receive 1<sup>st</sup> line ADT after androgen treatment, we continued treating mice with 2<sup>nd</sup> generation ADT (enzalutamide) in the presence of DHT.

Surprisingly, we found that DHT alone suppressed tumor burden, demonstrated by reduced bioluminescent intensity (Figure 3B, top graph and representative images). Although enzalutamide suppressed tumor burden more than DHT. Combined DHT and enzalutamide had the most significant impact on tumor burden compared to the control/placebo group ( $p<0.05$ ). Although enzalutamide suppressed tumor burden, the tumor growth rate appeared to be nearly identical when comparing sole treatment of DHT or enzalutamide. Combination DHT and enzalutamide significantly suppressed the tumor growth rate compared to DHT alone ( $p<0.01$ ) (Figure 3B, bottom graph).

Next, we isolated neutrophils from the tumor bones for analyses of function, in comparison to neutrophils from saline-injected bones. Neutrophils from non-tumor limbs treated with DHT were the most viable; 75% were still viable in serum-free media 48 hours after isolation compared to 50% from placebo-treated mice ( $p<0.0001$ ) and only ~25% remaining viable cells from all tumor mice ( $p<0.0001$ ) compared to non-tumor bearing mice (Figure 3C). Tumor-associated bone neutrophils (TBNs) and tumor-naïve bone neutrophils (from saline-injected tibia) were isolated from tibia and co-cultured *ex vivo* with C42B to examine the impact of treatment on TBN cytotoxicity. Neutrophils from both PBS and DHT-treated non-tumor bearing mice induced ~50% cell death ( $p<0.05$ ). Like PMNs from patients treated with 2<sup>nd</sup> Gen ADT, cytotoxicity of TBNs from enzalutamide-treated (enza-placebo group) mice was completely diminished compared to tumor placebo-treated mice ( $p<0.001$ ) and tumor DHT-treated mice ( $p<0.01$ ). However, the addition of DHT restored TBN cytotoxicity in the presence of enzalutamide ( $p<0.0001$ ) (Figure 3D).

Overall, there were very few changes in the percentages of isolated bone marrow myeloid cells though there were notably fewer myeloid cell numbers ((CD11b<sup>+</sup>, CD45<sup>+</sup>) and (CD11b<sup>+</sup>, Ly6C<sup>+</sup>/Ly6G<sup>+</sup>)) in enzalutamide-treated mice (Supp Figure 4A). We next examined cytokine expression in TBNs from treated groups (Figure 3E; Supp Figure 4B). Stem Cell Factor (SCF), a marker expressed by immature neutrophils, was significantly more expressed in TBNs from placebo-treated ( $p<0.01$ ) and Enza-treated mice ( $p<0.001$ ), compared to DHT treated; DHT-treated TBNs displayed little to no SCF suggesting that DHT may overcome tumor-mediated inhibition of neutrophil differentiation (Figure 3E). In comparison to placebo-treated TBNs, DHT-treated TBNs exhibited higher stromal derived factor 1(SDF) alpha, the ligand that binds CXCR4, a major retention factor for neutrophils in bone marrow[37]. Notably, CXCL1 and CXCL5, cytokines shown to be important for neutrophil migration and shown to promote progression of several tumor types[38],

was significantly reduced in DHT-treated TBNs compared to placebo-treated (Figure 3E). There were few changes in myeloid-regulating growth factors, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF). However, there was significantly more monocyte (M)-CSF expressed in TBNs from enza/DHT mice compared to DHT-treated mice (Supp Figure 4B). TBNs from mice treated with enza/DHT showed increased tumor necrosis factor alpha (TNF $\alpha$ ) and TNF receptor expression which supported the cytotoxic and reactive response against C42B identified in *ex vivo* analyses (Supp Figure 4B). Collectively, these findings suggest that high-dose testosterone therapy may alter the phenotype of tumor-bone neutrophils to support their maturation, cytotoxicity and retention in the bone microenvironment.

### **Androgen signaling regulates neutrophil cytotoxicity, viability, migration and NETosis.**

Our data suggests that androgen regulation of neutrophils can be leveraged to manage neutrophil anti-PCa immune response. To further test this possibility, C42B cells were co-cultured with bone marrow neutrophils isolated from C57BL/6 mice, in the presence of enzalutamide at 2 different doses (high dose-10  $\mu$ M, low dose-3  $\mu$ M). As previously seen, mouse neutrophils significantly killed approximately 45% of the C42B cells. However, high dose enzalutamide completely abrogated neutrophil killing ( $p < 0.05$ ) (Figure 4A). To delineate which cell population was most affected by enzalutamide, we pre-treated C42B cells or neutrophils with enzalutamide prior to combining the cell populations together in culture. C42B were resistant to enzalutamide alone but pre-treatment with low-dose enzalutamide enhanced their sensitivity to neutrophil killing (Figure 4B, left). Surprisingly, neutrophils pre-treated with low and high dose enzalutamide prior to culture with C42B were unable to induce cell death of C42B (Figure 4B, right). This phenomenon was also seen in neutrophil-LNCaP cell cultures (Figure 4C). These findings support our patient PMN data demonstrating that androgen inhibition of neutrophils reverses their cytotoxicity against PCa.

Although poorly understood, there is evidence that androgen signaling and specifically, AR-mediated signaling, is important for immune response[33]. To test the role of AR inhibition on neutrophil functions associated with activation, we examined neutrophil viability, migration and neutrophil extracellular trap (NET) production. Primary mouse bone marrow neutrophils were treated for 2 hours with C42B-conditioned media supplemented with enzalutamide (3  $\mu$ M and 10  $\mu$ M) and viability was measured. Compared to control (DMSO)-treated neutrophils, both doses of enzalutamide prolonged neutrophil viability over time and there was an increase in luminescence of low dose-enzalutamide treated neutrophils in the first 6 hours suggesting an increase in neutrophil numbers i.e., proliferation (Supp Figure 5A); these results were confirmed using Trypan Blue exclusion assay of high dose enzalutamide-treated neutrophils (Supp Figure 5B).

We next examined the impact of androgen inhibition on neutrophil migration in the context of prostate cancer [31]. Mouse bone marrow neutrophils were treated with enzalutamide or, for comparison, abiraterone, which inhibits CYP17A1[39], an enzyme important for androgen synthesis and allowed to migrate to C42B-CM in a modified Boyden Chamber assay. Significantly more neutrophils migrated to C42B CM significantly more (~5-fold;



$p < 0.01$ ) than to control serum-free media. Enzalutamide further enhanced neutrophil migration to C42B media (7-fold compared to CM;  $p < 0.05$ ) but did not suppress neutrophil migration; whereas abiraterone had little to no impact on neutrophil migration to C42B factors (Supp Figure 5C). This suggests that neutrophil migration toward BM-PCa is independent of AR signaling.

Our lab previously demonstrated that PCa induces neutrophil release of NETs[31]. To examine whether NETosis is regulated by androgen signaling, bone marrow neutrophils were treated with C42B-CM supplemented with either enzalutamide or the positive control, serum-free RPMI media supplemented with PMA to induce NET formation. Similar to migration data, low-dose enzalutamide further enhanced NETosis (4-fold;  $p < 0.01$ ) compared to untreated neutrophils in CM (Supp Figure 5D); high-dose enzalutamide showed no impact on NETs. These findings collectively suggest that neutrophil AR regulates neutrophil cytotoxicity and viability but is not the sole regulator of neutrophil migration and NETosis.

### **Second generation AR therapy regulates neutrophil gene expression *in vitro* and in patients.**

We next examined enzalutamide-mediated gene expression potentially contributing to neutrophil function. Based on the RNAseq results revealing TGF $\beta$  to be a mediator of PMN gene expression changes in mCRPC patients (Supp Figure 2), we examined expression of TGF $\beta$  receptors in patient PMNs. In patients treated with 2<sup>nd</sup> Gen ADT, we observed a significant increase in PMN gene expression of the serine-threonine kinase, Type 1 TGF $\beta$  Receptor (*TβRI*), and the Type 2 TGF $\beta$  receptor, which binds TGF $\beta$  ligands (*TβRII*) (~2-fold increase of both receptors;  $p < 0.05$ ), in comparison to patients treated with only 1<sup>st</sup> line ADT (Figure 5A).

For validation in our preclinical models, primary mouse bone marrow neutrophils were treated with 2 doses of enzalutamide (3  $\mu$ M and 10  $\mu$ M) and neutrophil gene expression measured. We detected abundant levels of AR and found that enzalutamide treatment significantly reduced neutrophil AR expression (3 $\mu$ M~9-fold,  $p < 0.001$ ; 10 $\mu$ M~2-fold,  $p < 0.05$ ) compared to vehicle control (DMSO)-treated neutrophils (Figure 5B). As seen with patient PMNs, AR inhibition increased TβRI and in a dose-dependent manner (10 $\mu$ M~3-fold,  $p < 0.01$ ); there was a slight, but significant, increase in TβRII, which binds TGF $\beta$  ligands in enzalutamide-treated neutrophils ( $p < 0.01$ ) (Figure 5B). In addition to TGF $\beta$  receptors, we measured expression levels of TNF $\alpha$ , a pro-inflammatory cytokine which has been shown to be inhibited by TGF $\beta$  and neutrophil AR [40–42]. There was also a 2-fold reduction in TNF $\alpha$  expression by both low- ( $p < 0.001$ ) and high-dose ( $p < 0.01$ ) enzalutamide treatment. These findings suggest that AR regulates TGF $\beta$  receptor expression in neutrophils which may be significant in neutrophil function.

### **Neutrophil-selective- and pharmacological TβRI inhibition rescues enzalutamide-mediated suppression of function.**

Based on known anti-inflammatory and immunosuppressive roles of TGF $\beta$ , we next tested the importance of neutrophil TβRI expression on neutrophil cytotoxicity against BM-PCa. Primary bone marrow neutrophils were pre-treated with enzalutamide alone or in

combination with RepSox, a small molecule kinase inhibitor of T $\beta$ RI, and then subsequently cultured them with C42B and PC3 for comparison. Notably, inhibition of neutrophil T $\beta$ RI reversed enzalutamide suppression of neutrophil cytotoxicity and significantly enhanced neutrophil-induced C42B cell death resulting in 75% C42B cell death with RepSox/enzalutamide combination ( $p < 0.0001$ ), compared to ~50% cell death by vehicle-treated neutrophils; ( $p < 0.01$ ) (Figure 5C). Further, RepSox-treated neutrophils induced significant death of PC3 cells ( $p < 0.001$ ) which are resistant to neutrophil responses. This phenomenon was also verified using mouse RM1 prostate cancer cells demonstrating that our findings are conserved between species (Figure 5D). Collectively, these data suggest that inhibition of neutrophil AR suppresses neutrophil killing response via T $\beta$ RI.

TGF $\beta$  is a pleiotropic cytokine utilized by multiple cells within the bone marrow, which complicates therapeutic intervention. To gain insight into T $\beta$ RI expression in mouse bone marrow and the potential for T $\beta$ RI-focused therapy, we analyzed unpublished single cell RNA sequencing data from male C57BL/6 bone marrow with a specific focus on T $\beta$ RI expression in Ly6G<sup>+</sup> cells and overall T $\beta$ RI expression in bone marrow cells (Supp Figure 6A). There was T $\beta$ RI expression in bone marrow neutrophils and all neutrophil subpopulations within the bone marrow (Supp Figure 6A). However, T $\beta$ RI was not expressed in all Ly6G<sup>+</sup> cells i.e., granulocytes, suggesting that T $\beta$ RI expression may be more expressed in the presence of androgen inhibition and expression is low under normal conditions i.e., in normal bone marrow.

To gain additional insight into T $\beta$ RI expression in PCa metastases, we performed fluorescent immunohistochemistry on matched liver and bone metastasis from FFPE samples collected in the University of Washington Rapid Autopsy Prostate Cancer program (n=3; all who received 2<sup>nd</sup> Gen ADT), to identify T $\beta$ RI<sup>+</sup> neutrophils, identified by myeloperoxidase (MPO). We found co-localization of T $\beta$ RI and MPO (in 3 of 3 patients) whereas there was little to no co-localization of T $\beta$ RI and MPO in the matched liver metastases (co-localization was seen in 1 of 3 patients) suggesting that T $\beta$ RI-expressing neutrophils may be more conserved in prostate cancer bone metastases, compared to liver metastasis (Supp Figure 6B).

Based on these data, we generated a neutrophil-selective T $\beta$ RI knockout mouse model using the Catchup mouse model, which expresses Cre-recombinase and dTomato under the Ly6G locus (Figure 6A, left). Ly6G is a cell surface marker specifically expressed by mouse granulocytes, which allows for a more specific targeting to neutrophils instead of all myeloid cell populations[43]. Protein analysis of multiple mouse tissues revealed a neutrophil-specific loss of T $\beta$ RI protein expression, with no change in other tissues (Figure 6A, right). There were no differences in neutrophil cell number with genetic deletion of T $\beta$ RI, however there were significantly fewer myeloid cells (CD45<sup>+</sup>CD11b<sup>+</sup>) in the bone marrow (Figure 6B). Although there appeared to be significantly fewer Ly6G<sup>+</sup> cells in *T $\beta$ RI*<sup>-/-</sup> knockout mice ( $p < 0.0001$ ) (Figure 6C, left), this was not surprising considering that the Ly6G knock-in disrupts Ly6G expression without impacting cellular function[13]. Surprisingly, there was a significant increase in CD19<sup>+</sup> B cells ( $p < 0.001$ ), and an increase in (though not significant) CD8<sup>++</sup> cells in *T $\beta$ RI*<sup>-/-</sup> bone marrow (Figure 6C, right) suggesting an indirect impact of neutrophil TGF $\beta$  signaling on other immune cell populations.

Next, we examined killing capacity of T $\beta$ RI knockout neutrophils against BM-PCa cells (C42B, PC3, and 22Rv1). Neutrophils induced death of C42B, independently of T $\beta$ RI expression as there was no difference in C42B death induced by wildtype T $\beta$ RI neutrophils (T $\beta$ RI<sup>fl/fl</sup>) and T $\beta$ RI knockout (T $\beta$ RI<sup>-/-</sup>) neutrophils (Figure 6D, left). Similar to our findings using pharmacologic blockade of T $\beta$ RI, T $\beta$ RI<sup>-/-</sup> neutrophils induced significant cell death of PC3, neutrophil-resistant (Figure 6D, middle;  $p < 0.001$ ), compared to T $\beta$ RI<sup>fl/fl</sup> neutrophils. Neutrophils also induced 22Rv1 death, but there was little additional impact in the absence of T $\beta$ RI (Figure 6D, right).

To examine the impact of neutrophil-selective T $\beta$ RI in the context of androgen-mediated activity, we treated bone marrow neutrophils from T $\beta$ RI knockout and T $\beta$ RI floxed mice with low-dose enzalutamide (3  $\mu$ M) (or vehicle control) prior culture with C42B and PC3 cells. As seen in our previous experiments, there was ~50% C42B cell death induced by untreated T $\beta$ RI<sup>fl/fl</sup> neutrophils which were completely suppressed by enzalutamide treatment (0% C42B cell death by enza-treated T $\beta$ RI<sup>fl/fl</sup> neutrophils;  $p < 0.01$ ) demonstrating that enzalutamide treatment inhibits T $\beta$ RI<sup>fl/fl</sup>-induced C42B cell death. In contrast, enzalutamide-treated T $\beta$ RI<sup>-/-</sup> neutrophils sufficiently killed ~50% of C42B ( $p < 0.05$ ), (Figure 6E). Interestingly, untreated T $\beta$ RI<sup>-/-</sup> neutrophils killed ~40% of PC3 cells ( $p < 0.01$ ) and, further, enzalutamide-treated T $\beta$ RI<sup>-/-</sup> killed ~75% of PC3 (Figure 6E). Additionally, mouse RM1 prostate cancer cells were cultured with T $\beta$ RI knockout neutrophils in the presence of enzalutamide. Similar to human PCa, enzalutamide suppressed neutrophil killing of RM1 and this phenomenon was significantly reversed with T $\beta$ RI knockout neutrophils ( $p < 0.05$ ) compared to wildtype neutrophils (Figure 6F). These data suggest that T $\beta$ RI inhibition activates neutrophils against androgen insensitive metastatic PCa and that combined loss of T $\beta$ RI and AR may enhance neutrophil response.

Last, we sought to determine whether these data translated to the prostate tumor-bone microenvironment. Luciferase-expressing RM1 cells were injected into tibia of T $\beta$ RI<sup>fl/fl</sup> and T $\beta$ RI<sup>-/-</sup> mice and tumor growth measured throughout the study. Based on bioluminescent changes, genetic deletion of T $\beta$ RI in neutrophils alone significantly suppressed RM1 growth in bone (Figure 7A). Despite these findings, it would be difficult to target T $\beta$ RI inhibition solely to neutrophils in the bone microenvironment. Thus, we tested a more clinically feasible therapeutic approach and examined the impact of systemic T $\beta$ RI or AR inhibition single or in combination in BM-PCa. We found that RM1 *in vivo* did not respond to enzalutamide and grew more aggressively compared to vehicle control. We observed that T $\beta$ RI inhibition via RepSox appeared to suppress prostate tumor growth in bone which was reduced ~2-fold by Day 10/the end of the study ( $p < 0.001$ ) but there was an added effect on tumor burden in the presence of enzalutamide (~2.5 fold;  $p < 0.001$ ) (Figure 7B).

Collectively, we have identified PMN markers, genetic changes and phenotypes that emerge with progression of prostate cancer, including development of metastasis and acquired resistance to 1<sup>st</sup> line ADT. Further, our findings strongly support the conclusion that androgen deprivation, specifically AR inhibition, increases neutrophil T $\beta$ RI and suppresses their cytotoxicity against PCa (Figure 8). Pharmacologic and genetic inhibition of neutrophil T $\beta$ RI completely rescued this phenomenon. Thus, T $\beta$ RI inhibition can be used to overcome neutrophil suppression and to simultaneously target both AR-responsive and AR-insensitive

bone metastatic prostate cancer. Importantly, our study reveals insights from neutrophils that can be used to optimize therapeutic strategies and highlights a novel regulatory mechanism of androgen regulation in the regulation of neutrophil immune response.

#### 4. Discussion

Androgen deprivation remains the primary therapeutic approach for treating metastatic PCa. However, patients frequently develop resistance to ADT e.g., develop CRPC and present with bone metastases. Newer cancer therapies, namely immunotherapy approaches, have had limited success for the treatment of advanced PCa. However, our lab previously identified that bone marrow neutrophils are activated to induce metastatic PCa death, highlighting a phenotype that may be utilized as a novel immunotherapy strategy for PCa patients. In this study, we investigated the impact of prostate cancer disease progression on neutrophils to determine their potential as biomarkers for disease progression. Consequently, we identified several conclusions suggesting that: 1) classical PMN characterization is not parallel to PMN cytotoxicity against PCa, and 2) PMN anti-tumor response is highly regulated by androgen-receptor inhibition and resultant upregulation of TGF $\beta$  receptor 1.

Neutrophils/PMNs have been classically ignored in the conversation of immunotherapy due to their propensity to be short-lived terminally differentiated cells, most well characterized in infectious diseases, with emerging findings of cancer-related properties that seem to differ depending on tissue context. To date, there has been very little known of how neutrophils contribute to metastatic PCa and importantly, how neutrophils are systemically altered throughout the progression of PCa to advanced and metastatic disease. Here, we identified PCa stage-dependent differences in PMN populations that may be useful in downstream studies to develop PMN-focused therapeutic interventions. A major benefit of interrogating peripheral blood-derived PMNs as markers for disease progression is their abundance in circulation and non-invasive collection from patients. Using this to our advantage, our findings reveal that PMNs are indeed altered throughout progression and specifically, as patients transition from mCSPC to mCRPC, become more inflammatory though not necessarily more/less cytotoxic. We acknowledge that blood-derived PMNs can substantially differ in molecular pattern from bone-marrow derived PMNs, as previously discussed[44–46], which likely will be the cell populations interacting with PCa in the bone compartment. Thus, future studies are needed to compare bone marrow neutrophils throughout progression. This type of study would require substantial efforts to procure data in a non-invasive/non-painful method for patients but would provide critical data towards the characterization of PMNs in the tumor-bone environment.

Along those lines, our previous studies showed that neutrophils in bone lose their cytotoxicity as the PCa tumor progresses in bone[31]. Analysis of patient PMNs revealed a significant increase in the viability of all PCa-derived PMNs, specifically of those with localized disease compared to healthy men, suggesting the emergence of a less mature population since mature PMNs are terminally differentiated and lack mitotic ability[47]. Further, maturation markers, such as CD10, were significantly reduced with progression of disease along with CD66b. Both CD10 and CD66b have been associated with PMN maturity and are highly expressed on fully differentiated PMNs, compared to immature

precursors[27, 48]. There was also a significant increase in PMNs expressing CD88 (C5aR) in mCRPC compared to mCSPC. Complement C5a is a potent chemoattract for leukocyte migration, promotes granule enzyme release and oxidative burst and thus, often is associated with neutrophil activation[49]. This suggests more activated PMNs in mCRPC compared to less aggressive prostate cancer, a finding that was highly surprising. This finding was supported by RNA sequencing data which revealed a gene signature associated with neutrophil activation and degranulation; this conclusion was based upon increased expression of granule enzymes, complement receptors, and genes associated with migration and oxidative burst. Additionally, there was an increase in LOX-1 (marker for immunosuppressive cells[50]) positive cells in both cohorts of metastatic patients compared to healthy men and localized PCa. However, in total fewer than 20% of collected PMNs expressed LOX-1 suggesting that LOX-1 expression alone may not be an appropriate marker for determining PMN function in BM-PCa. This is in consideration of previous data demonstrating bone-derived neutrophils to be both cytotoxic to PCa and immunosuppressive though further investigation would be required to determine the correlation between LOX-1 status and cytotoxicity of peripheral blood PMNs. Importantly, the changes in cell surface markers could be utilized as supportive indicators of disease stage as an accompaniment to PSA, which is sometime less reliable for more advanced PCa.

Our results showed that patient PMN cytotoxicity was not dependent on PCa disease stage, as we had initially expected. In fact, even PCa patients at the most aggressive PCa stage harbor anti-tumor PMNs. Importantly, we discovered that PMN cytotoxicity can be suppressed by 2<sup>nd</sup> generation ADT, specifically enzalutamide. Progression of PCa disease to CRPC is associated with dysregulated androgen signaling, including, but not limited to: 1) increased AR expression, 2) therapy-induced AR-activating mutations, or splice variants (e.g., AR-V7), 3) bypass of AR signaling pathways, such as STAT5 or glucocorticoid receptor (GR) signaling), and 4) AR independent signaling that occurs upon loss of AR expression (i.e., Myc activation)[34]. Based on these mechanisms of acquired resistance to 1<sup>st</sup> line ADT, ARSIs have significantly improved survival for patients with castration-resistant disease. Even still, eventually those patients become resistant to 2<sup>nd</sup> generation ADT and ~90% develop bone metastatic disease. The findings from this study suggest that, although ARSIs may inhibit PCa growth, it may also be contributing to the development of tumor resistance to drug and anti-tumor neutrophil immune responses. Further, patients with advanced disease also are frequently treated with radiation therapy and/or chemotherapy, which both are myeloablative[51]. Thus, it is possible that the standard-of-care treatment regimen for PCa patients may, in tandem, be altering the tumor-immune microenvironment and creating a more tumor-tolerant environment. Although some of our patients received other non-androgen focused therapies, a longer more comprehensive study would be needed to examine the importance of individual therapies on neutrophil immune response in metastatic PCa.

Second generation androgen inhibition and ARSI prove to be temporarily beneficial for treating disease recurrence in CRPC however, immune cells also rely on AR for function and our data verified that neutrophils also express and utilize AR for functions that need to be interrogated further. AR is present on all PMNs and PMN precursor cells within the bone. Previous studies using conditional AR knockout mice revealed that granulopoiesis

i.e., the formation of granulocytes in the bone marrow, is regulated by androgen signaling [33, 52]. This evidence was supported by our findings from the modified BAT mouse and emphasizes the importance of androgens in neutrophil function. We modified this BAT protocol to reflect the treatment regimen of the mCRPC patients within our study and interrogated the impact of giving those patients exogenous testosterone upon biochemical recurrence after 2<sup>nd</sup> Generation ADT. A major limitation in the clinical translation of this work is that our hypothesis is based upon peripheral blood neutrophils which may not be representative of the bone marrow neutrophils[45]. Additionally, the C42B cancer cell line is mCRPC and has not had to go through the spontaneous development of androgen resistance prior to use in our BAT study. Nonetheless, we found that systemic DHT treatment could rescue enzalutamide-mediated suppression of neutrophil cytotoxicity and altered neutrophil markers associated with differentiation and function (Figure 3). Specifically, AR-inhibited TBNs expressed more SCF/cKit ligand, which has been shown to be associated with immature neutrophils[53], suggesting that one method that AR impacts neutrophil function is through the suppression of neutrophil maturation. Further, AR inhibition increased neutrophil migration and proliferation, the latter being characteristic of immature (non-differentiated) neutrophils. Notably, DHT seemed to significantly suppress growth of CRPC tumors *in vivo* (in support of clinical BAT study data[35]) and this evidence, combined with TBN functional data from our mouse study suggests that androgens might be utilized to target CRPC both directly, and indirectly, through manipulation of the neutrophils in the immune microenvironment.

Based on known roles for TGF $\beta$  in the-bone microenvironment [54–56] and its identification as a master regulator of PMN gene changes in PCa patients (Supp Figure 2), we examined the role of TGF $\beta$  receptor in AR-mediated neutrophil cytotoxicity. *In vitro* preclinical data revealed that AR mediated suppression of neutrophils is predominantly caused by an increase in neutrophil T $\beta$ RI expression (Figure 8). Several studies have identified a role for T $\beta$ RI in cancer immunity, namely as an immunosuppressant and a known regulator of inflammation[57–59], however, our findings emphasize a connection between neutrophil AR and T $\beta$ RI that should be investigated further for defining its role in neutrophil anti-tumor response. Canonical TGF $\beta$  signaling is propagated through ligand binding of the T $\beta$ RII receptor which then forms a heterodimer complex with the kinase receptor, T $\beta$ RI. T $\beta$ RI activation results in phosphorylation of Smad2/3 docked at the receptor, dimerization with cytoplasmic Smad4 and translocation into the nucleus for transcriptional regulation [60]. It is unclear how androgens regulate T $\beta$ RI expression. We and others have shown TGF $\beta$  receptor expression to be regulated by TGF $\beta$  signaling [55, 56]. A previous study showed that AR regulates TGF $\beta$ -mediated signaling through direct association with Smad3, thereby suppressing Smad-mediated transcription. These data suggest that ARSIs disrupt the AR-Smad binding which indirectly increases T $\beta$ RI expression. Recently, Paller et al. showed that T $\beta$ RI inhibition using the small molecule kinase inhibitor, Galunisertib, combined with a dominant negative T $\beta$ RI suppressed primary prostate tumor growth and enhanced response to enzalutamide in the neuroendocrine transgenic adenoma mouse prostate (TRAMP) model[61]. In a separate study, scRNAseq of prostate primary tumors from patients with advanced disease showed a tumor increase in T $\beta$ RI expression and correlation with enzalutamide resistance demonstrating a connection between AR inhibition



and TGF $\beta$  signaling in both the epithelial cancer cells as well as stromal cells[62]. In our study, we observed that AR-negative PC3 cells are resistant to neutrophil killing; however, neutrophil-specific loss of T $\beta$ RI expression was able to sensitize PC3 to neutrophil killing and enzalutamide treatment suggesting that both neutrophil T $\beta$ RI suppression combined with AR inhibition, significantly enhances neutrophil cytotoxicity to even the resistant BM-PCa. Further studies will be required to determine the mechanisms of T $\beta$ RI and AR-mediated neutrophil function in the tumor-bone microenvironment.

Targeting TGF $\beta$  remains complicated because of the pleiotropism of TGF $\beta$  and its role in regulating an abundance of cellular processes. scRNAseq of mouse bone marrow data revealed expression of T $\beta$ RI predominantly in granulocytic cells (Supp Figure 6). Further, T $\beta$ RI appears to be expressed more abundantly in neutrophils in patient bone metastases, compared to matched liver metastases (Supp Figure 6), suggesting that systemic T $\beta$ RI inhibition may be a relevant target for enhancing neutrophil anti-tumor response in bone mCRPC with limited off-target effects though this would need to be investigated further. Further, the rapid turnover of neutrophils within the bone marrow would likely require consistent T $\beta$ RI inhibition, this was supported by the suppression on tumor burden in neutrophil-selective T $\beta$ RI knockout mice (Figure 6A). However, sustained combined AR and T $\beta$ RI inhibition may circumvent neutrophil turnover, as we observed in the suppression on tumor growth with pharmacologic inhibitors (Figure 6B), although extensive studies are needed to understand how neutrophil turnover would impact therapeutic intervention in the long-term.

Collectively, our data demonstrate that peripheral blood PMNs can give insight into PCa disease progression, particularly in the context of bone-metastatic disease. We identified reveal a novel finding that neutrophil anti-tumor immune response is suppressed by AR inhibition via T $\beta$ RI, independently of the PMN tissue source (Figure 8). With the benefit of current standard-of-care ADT on PCa progression, this information can be used as a two-pronged approach, leveraging T $\beta$ RI as a viable target for enhancing neutrophil anti-tumor response in the presence of ADT. Importantly, we emphasize that peripheral blood PMN functional assay may be a feasible method for optimizing therapeutic intervention of mCRPC.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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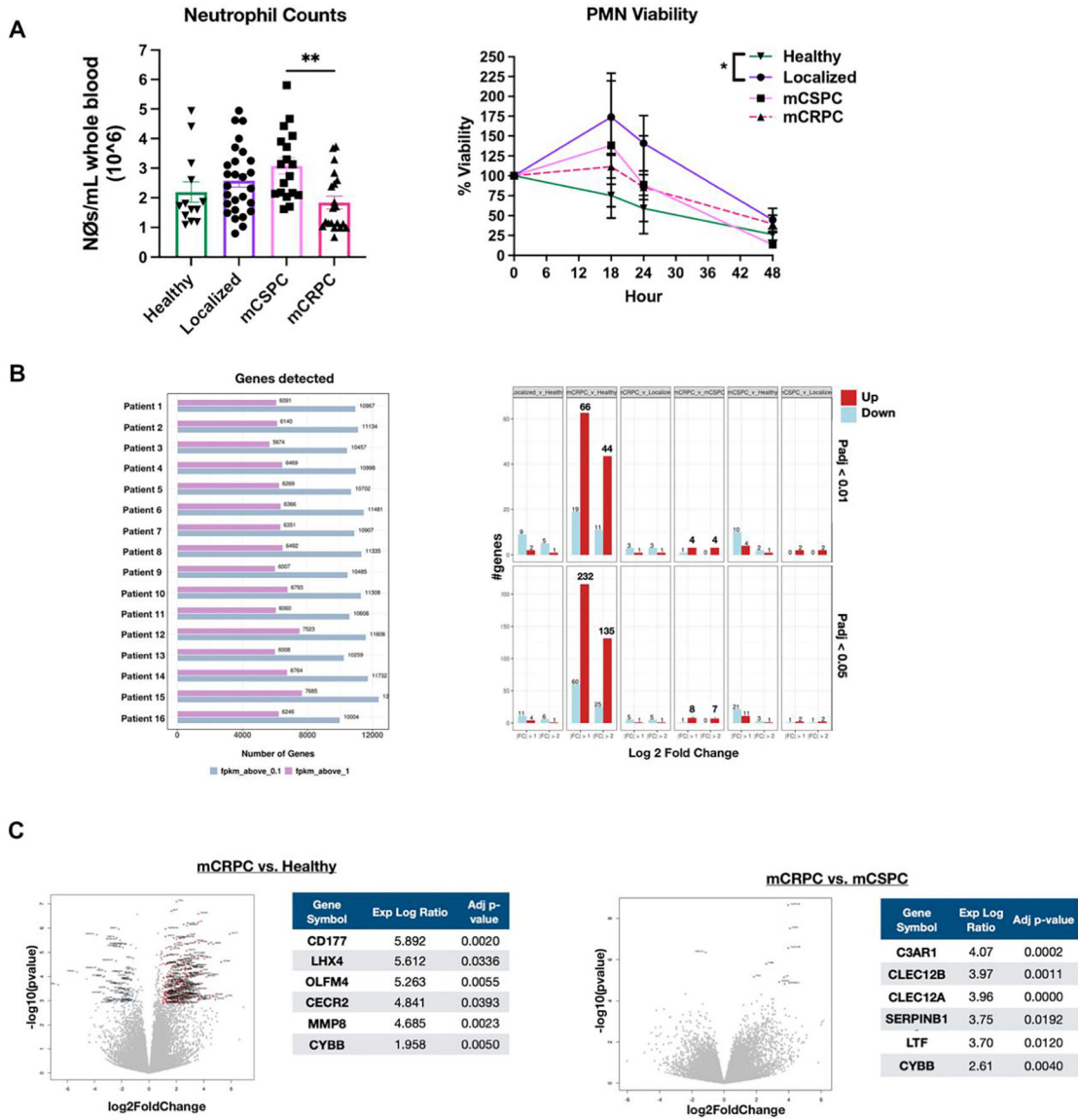
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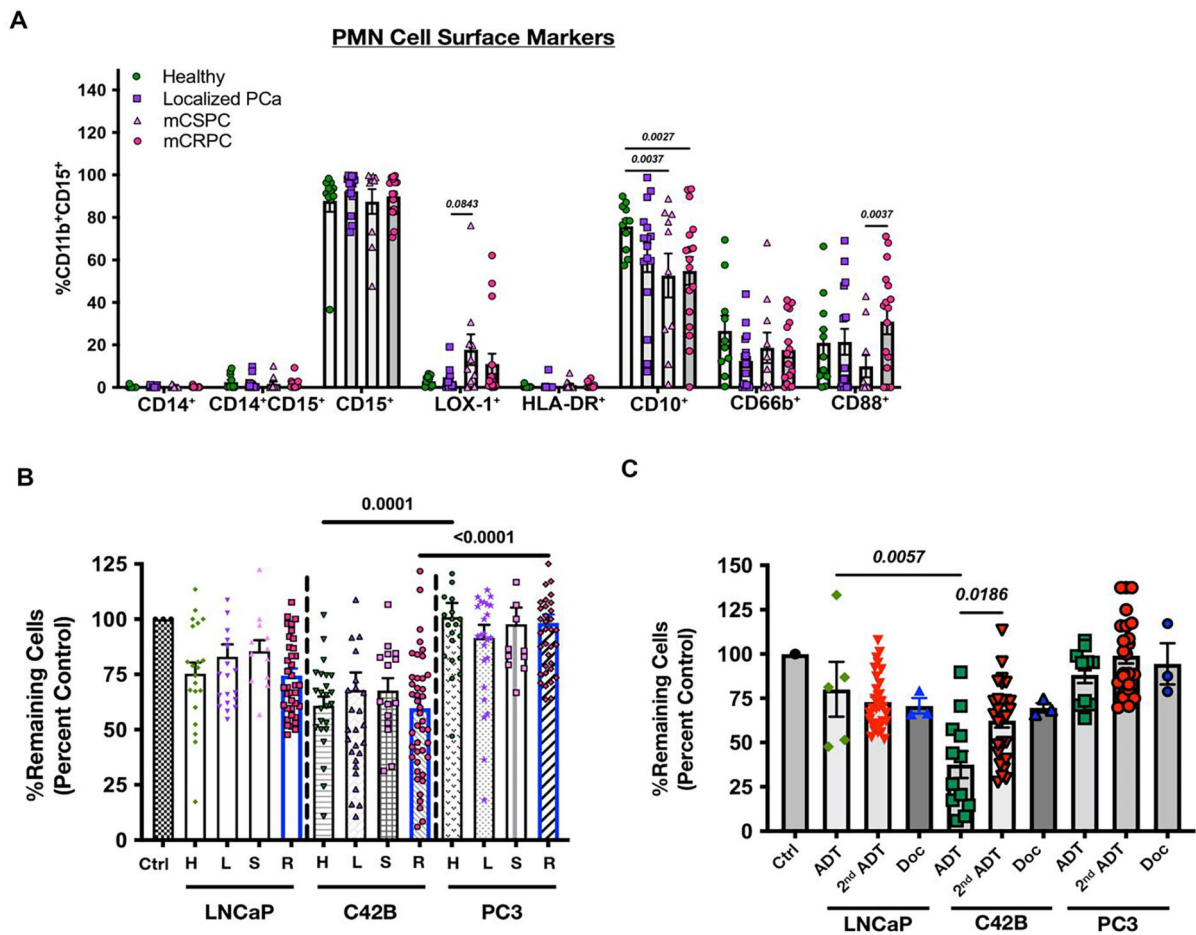
### Highlights

- Prostate cancer most frequently metastasizes to bone compared to other tissues and reduces patient survival to ~2–3 years after diagnosis of bone metastatic disease
- Current standard-of-care androgen deprivation therapy provides minimal improvement for metastatic disease
- Neutrophils suppress prostate cancer growth in bone
- Standard-of-care androgen therapy abrogate neutrophil immune response
- Androgen regulation can be optimized to enhance neutrophil cytotoxicity

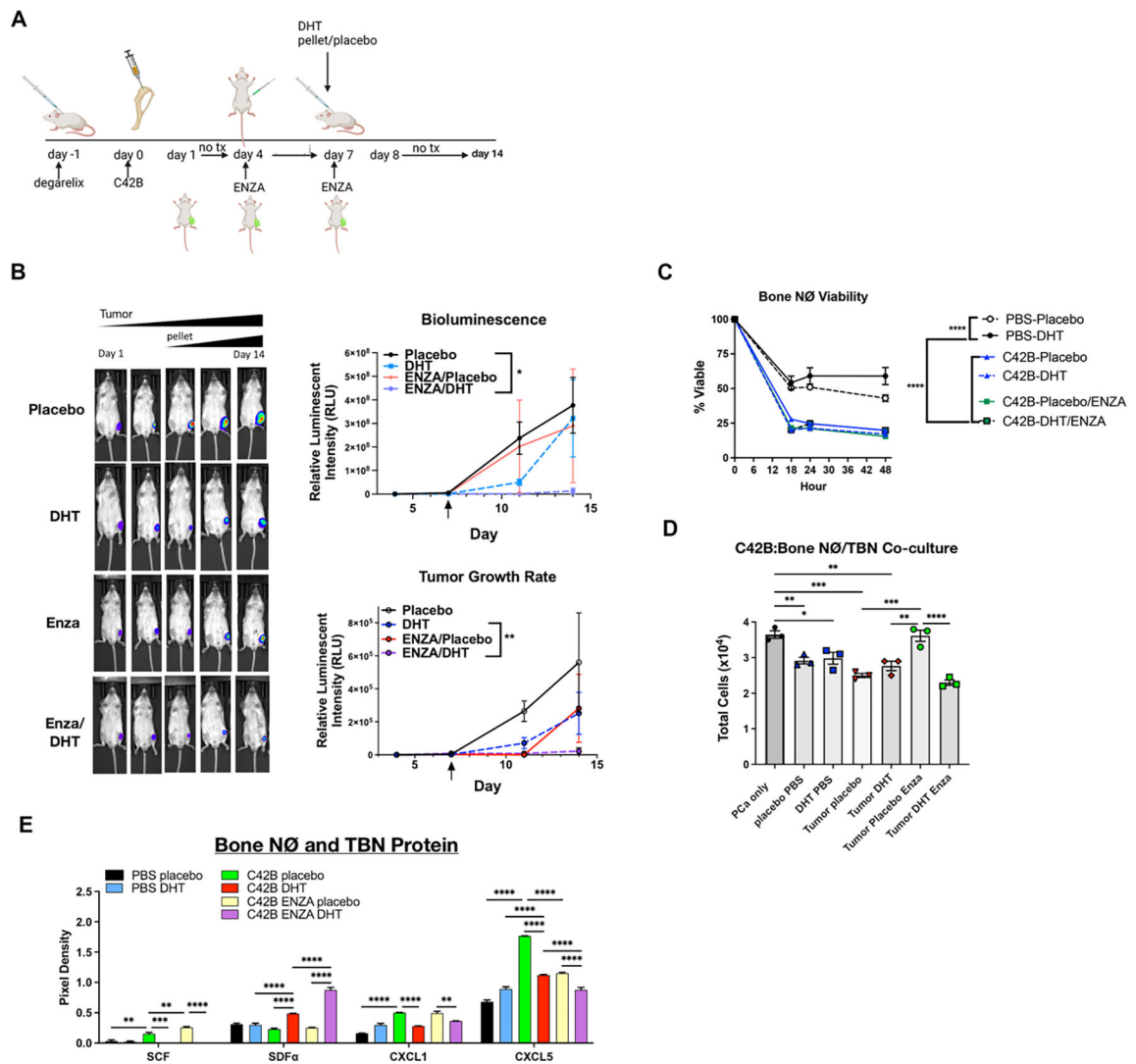




**Figure 1. Characterization of peripheral blood PMNs reveals molecular and functional parameters of PMN cytotoxicity against BM-PCa.** (A) Left, PMN absolute counts per mL of blood per disease stage. One-way ANOVA was performed.  $**p < 0.01$ ; Right, PMN viability per specified hours post isolation. Statistics using two-way ANOVA identified statistical significance at 18 hours between healthy and localized patients;  $**p < 0.05$  (B) RNA sequencing analysis of PCa-derived PMNs. Bulk RNA sequencing of blood PMNs was performed on an Illumina NovaSeq with paired-end 100bp reads (n per group=4/healthy; 4/localized; 5/mCSPC; 3/mCRPC). Left, plot shows number of genes dictated per patient per fpkm. Right, number of genes significantly up-regulated (red) or down-regulated (blue) per disease stage group. Changes are separated by significance; top- ( $p < 0.10$ ) bottom- ( $p < 0.05$ );(C) Tables show 5 most up-regulated genes when comparing most aggressive stage with non-cancer patients (mCRPC vs. healthy, left) and metastatic cohorts that differ in responsiveness to androgen therapy (mCRPC vs. mCSPC, right).



**Figure 2. Prostate cancer patient PMN surface markers and cytotoxicity.** (A) FACS analysis was performed using specified cell surface markers. Plot shows percentage of marker-positive cells. Two-way ANOVA statistical analysis was performed; graph shows p-values; (B) Co-culture of patient PMNs per PCa stage with PCa cell lines (LNCaP, C42B, and PC3); 10:1 PCa to neutrophil ratio plated in triplicate. PCa cell numbers were normalized to 100% and graph shows percent remaining PCa cells per well (technical replicates) after overnight culture with PMNs. H= healthy; L= localized; S= Castration-sensitive; R= Castration-resistant. (C) Co-culture of patient PMNs treatment with PCa cell lines (LNCaP, C42B, and PC3); graph shows percent remaining PCa cells per well (technical replicates). Ctrl= Control; ADT= 1st line androgen deprivation therapy; 2nd Gen ADT= 2nd generation androgen deprivation; Doc= docetaxel (chemotherapy).



**Figure 3.** In vivo and ex vivo androgen-mediated regulation of PMN cytotoxicity. (A) Schematic of pre-clinical study; generated by [Biorender.com](#). (B) Quantitative bioluminescence imaging, representative mouse images. Top graph shows quantitation of relative luminescent intensity (RLU) in photons/cm<sup>2</sup>/second over time. Bottom graph shows RLU normalized to bioluminescence at Day 1 post tumor inoculation. Arrows point to beginning of ADT treatment. (C) Real Time Glo MT cell viability assay of tumor-bone neutrophils (TBNs) isolated from tumor-bone at the end of study, day 14. Two-way ANOVA statistical analysis performed; \*\*\*\*p<0.0001 at 18 hours postisolation. (D) C42B ex vivo co-culture with TBNs from each mouse group: placebo PBS (saline-injected mice with placebo pellet), DHT PBS (saline-injected mice with DHT pellet), tumor placebo (C42B-injected mice with placebo pellet), tumor DHT (C42B-injected mice with DHT pellet), tumor placebo Enza (C42B-injected, enzalutamide-treated, placebo pellet), and tumor DHT Enza ((C42B-injected, enzalutamide-treated, DHT pellet). For co-culture, C42B were plated in triplicate, cultured with TBNs overnight, and remaining C42B counted using Trypan Blue exclusion

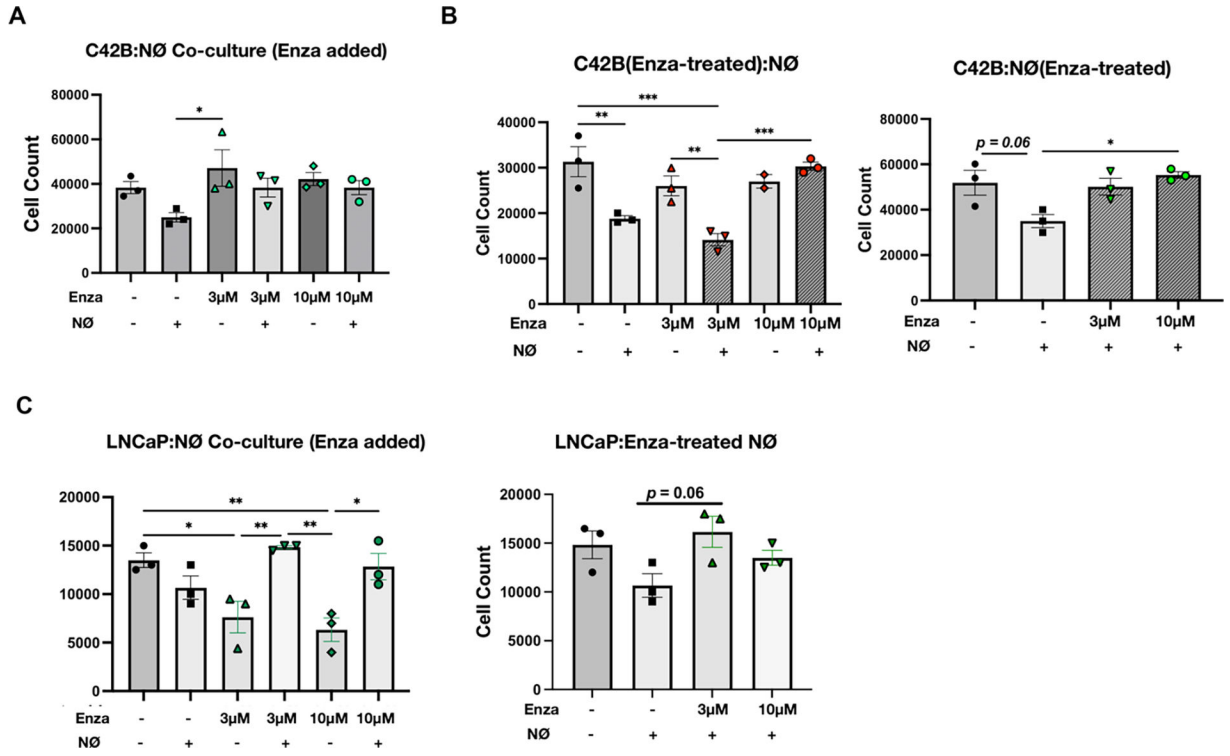
assay. Graph shows C42B numbers after culture with TBNs. (E) Cytokine array of TBNs. Two-ANOVA statistical analysis was performed. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.000$ .

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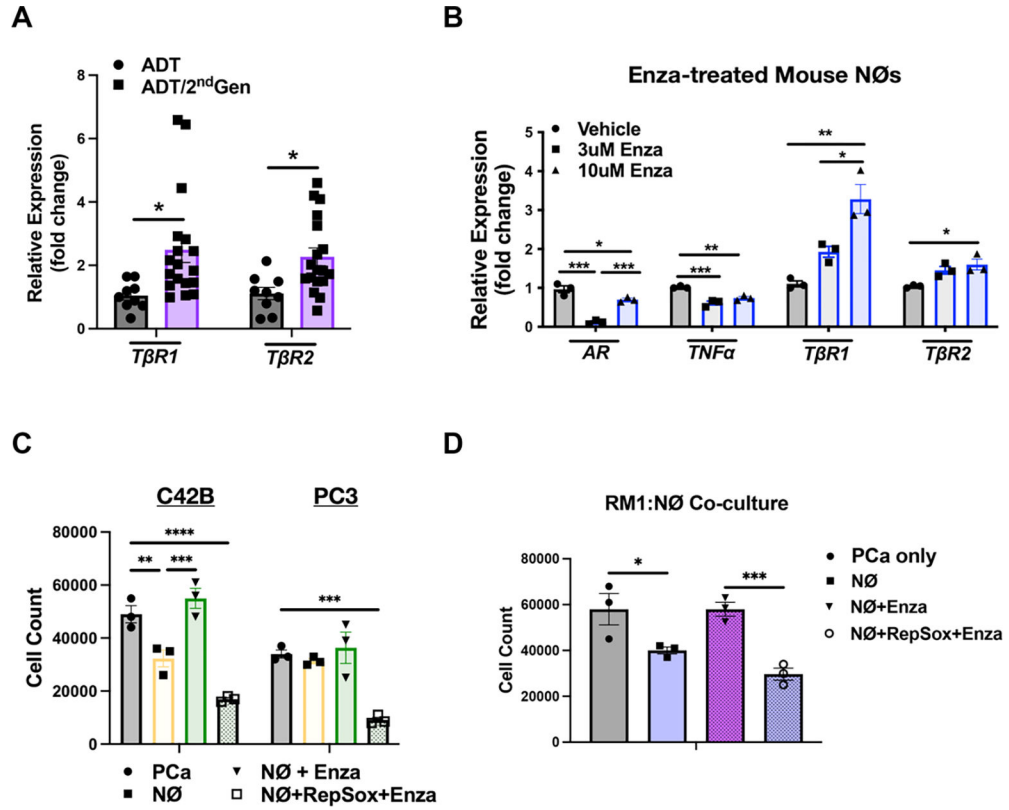
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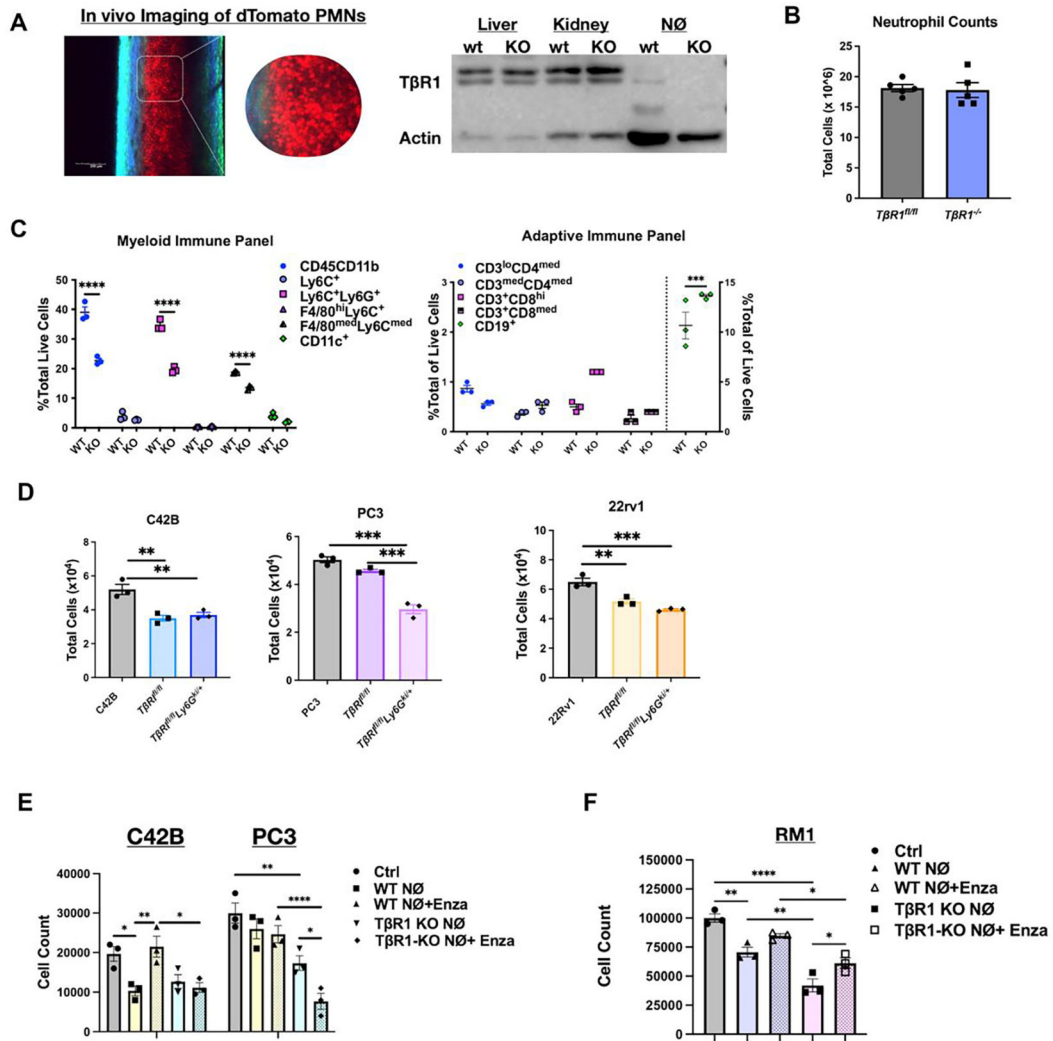


**Figure 4.** enzalutamide suppresses neutrophil cytotoxicity. (A) C42B direct co-culture with primary mouse bone marrow neutrophils. enzalutamide (Enza) was added to the culture when neutrophils were seeded. (B) Left, C42B were treated with enzalutamide (6 hours) and neutrophils added for overnight culture; right, primary mouse bone marrow neutrophils were treated with enzalutamide for 30 minutes and then added to C42B for direct co-culture overnight. Graphs show C42B cells after overnight culture via Trypan Blue exclusion assay. (C) Left, LNCaP direct co-culture with mouse neutrophils and enzalutamide added directly to culture. Right, neutrophils were treated with enzalutamide for 30 minutes before addition to culture. Graphs show C42B cells after overnight culture via Trypan Blue exclusion assay. One way ANOVA statistical analysis was performed; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.



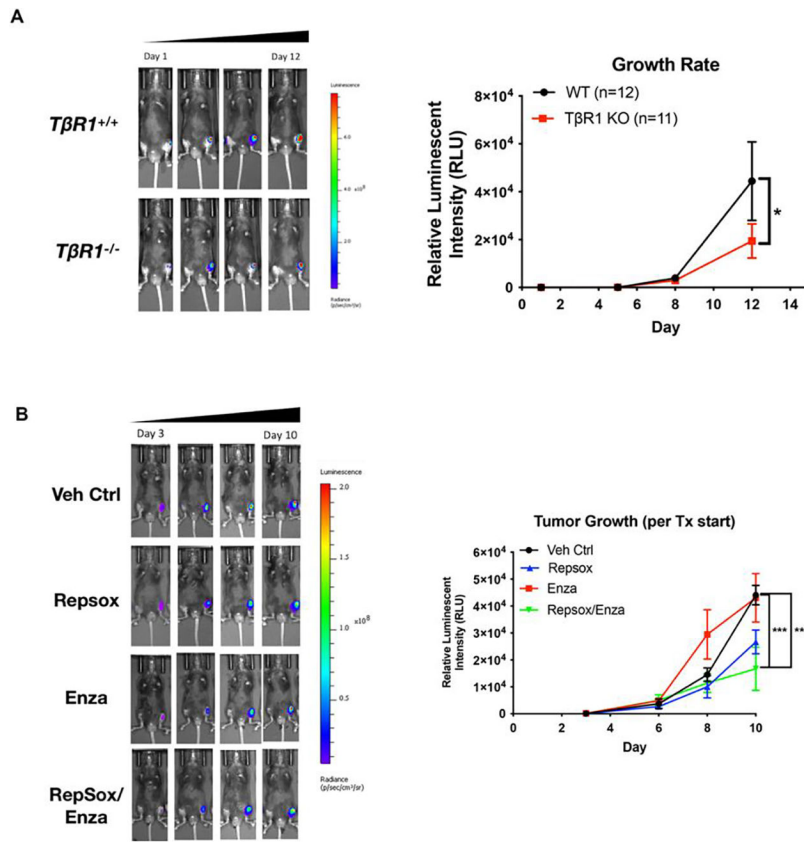
**Figure 5. ARSIs suppresses neutrophil anti-tumor response via regulation of TβR1 expression.** (A) RT-qPCR of human patient PMNs treated with either 1st line androgen deprivation therapy (ADT) or 2nd generation ADT (includes all ARSIs plus 1st line ADT). (B) RealTime quantitative PCR (RT-qPCR) of mouse primary bone marrow neutrophils treated with enzalutamide. Genes profiled: androgen receptor (AR), tumor necrosis factor alpha (TNFα), transforming growth factor beta receptor 1 (TβR1). (C) C42B and PC3 co-culture assay with mouse bone marrow neutrophils pre-treated for 30 minutes with either RepSox (small molecule kinase inhibitor of TβR1; 5nM), enzalutamide (3 μM) or a combination prior to addition to overnight culture. Graph shows PCa cell counts after overnight culture. (D) Mouse RM1 PCa culture with mouse neutrophils pre-treated with RepSox and/or enzalutamide for 30 minutes prior to addition to culture. Graph shows RM1 cell counts after overnight culture. One-way or two-way ANOVA statistical analysis were performed where appropriate. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.





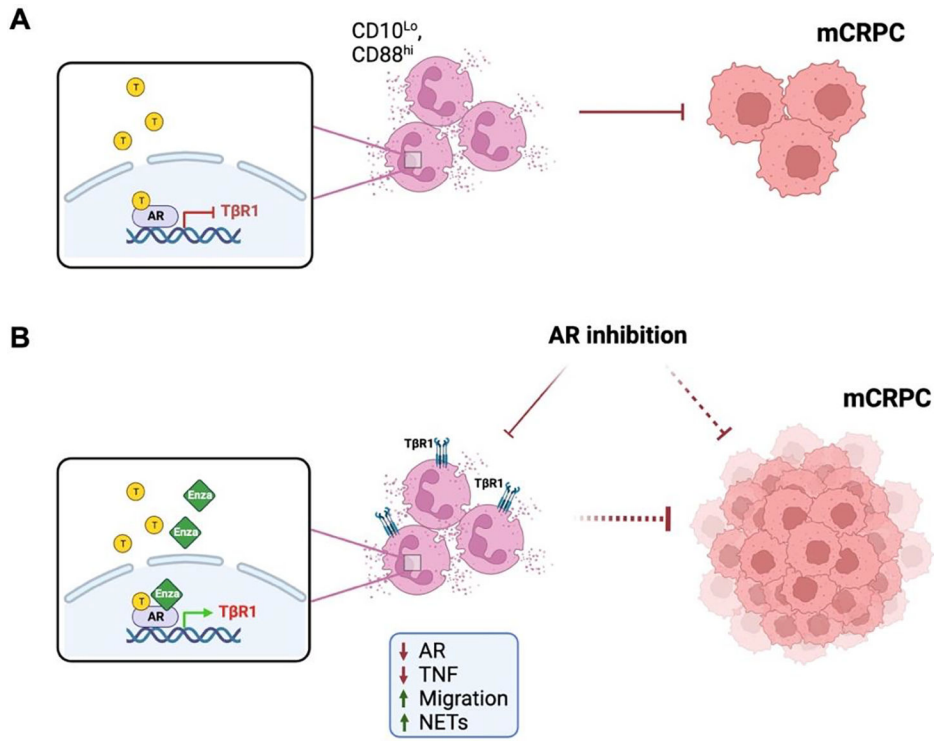
**Figure 6. Neutrophil-selective genetic deletion of TβRI rescues enzalutamide neutrophil suppression.**

(A) Left, two-photon microscopy of bone marrow of Catchup mouse model. Right, western blot of TβRI in tissues from Catchup/TβRI flox/flox crossed mice. (B) Neutrophil counts post-isolation from mouse bone marrow. (C) Flow cytometry of myeloid cell markers (left) and adaptive immune markers (right) from mouse bone marrow from floxed TβRI (*TβRI<sup>fl/fl</sup>*) and TβRI-null (*TβRI<sup>-/-</sup>*) mice. (D) Co-culture of wildtype TβRI and TβRI-null mouse bone marrow neutrophils with BM-PCa cell lines C42B, PC3 and 22Rv1. (E) Co-culture of mouse bone marrow neutrophils in the presence of enzalutamide (Enza). C42B and PC3 co-culture assay with *TβRI<sup>fl/fl</sup>* and *TβRI<sup>-/-</sup>* mouse bone marrow neutrophils pre-treated with Enza (3 μM) prior to addition to overnight culture. Graph shows PCa cell counts after overnight culture. Two-way ANOVA statistical analysis was performed. \*p<0.05, \*\* p<0.01, \*\*\*\* p<0.0001. (E) RM1 co-culture assay with *TβRI<sup>fl/fl</sup>* and *TβRI<sup>-/-</sup>* neutrophils pre-treated with Enza (3 μM), where shown, prior to addition to overnight culture. Graph shows PCa cell counts after overnight culture. One-way ANOVA statistical analysis was performed.



**Figure 7. Impact of neutrophil-targeted deletion of TβRI and combined TβRI/enzalutamide inhibition on BM-PCa growth.**

(A) RM1 intratibial bone metastasis model in TβRI KO mice. Luciferase-expressing RM1 cells were injected into tibia of 6–7 week male floxed *TβRI* (n=12) or neutrophil-selective *TβRI*-null mice (n=11) and tumor burden measured longitudinally for the entire study using bioluminescence imaging. Representative image (left) and bioluminescence quantitation (right, photons/cm<sup>2</sup>/sec). Two-way ANOVA statistical analysis was performed. \*p<0.05 at Day 12 of the study. (B) RM1 intratibial metastasis in preclinical therapeutic trial. Luciferase-expressing RM1 was injected intratibially in 6-week C57BL/6 mice (n=5–7) and randomized via bioluminescence into 4 treatment groups: 1) vehicle control (DMSO), 2) RepSox (5mg/kg), 3) enzalutamide (Enza; 10mg/kg), 4) RepSox combined with Enza. Representative images (left) and quantitation (right). Two-way ANOVA statistical analysis was performed; graph shows significance at end of study (Veh. Ctrl vs. combo treatment and Enza vs. combo treatment) at Day 10. \*\*\*p\* < 0.001



**Figure 8. AR inhibition regulates neutrophil cytotoxicity via TβR1.**  
 (A) Neutrophil function is driven, in part, by androgen signaling through AR; neutrophil AR signaling inhibits TβR1 expression and allows for neutrophil suppress of PCa growth. “T” = testosterone; “AR” = androgen receptor. mCRPC= Metastatic (bone) castration-resistant prostate cancer. (B) AR inhibition moderately suppresses metastatic PCa and promotes neutrophil TβR1 expression which inhibits neutrophil cytotoxicity. It also suppresses AR and TNF expression while increasing neutrophil migration and NETs. “Enza” = enzalutamide. Dashed lines represent inhibition of function. Created by [Biorender.com](https://www.biorender.com).

**Table 1.**

Patient Diagnosis and Treatment Demographics.

	n	1 <sup>st</sup> Line ADT	2 <sup>nd</sup> Gen ADT	Chemotherapy (Docetaxel)	ADT+Radium 223	Survivorship/no longer on therapy
Healthy	17	<i>n/a</i>				
Localized	22 <sup>*†</sup>	4	8			9
Metastatic castration-sensitive prostate cancer (mCSPC)	19	5	12	2		
Metastatic castration-resistant prostate cancer (mCRPC)	19	4 <sup>**</sup>	9	3	2	

\* Includes one patient on ADT and immune checkpoint blockade (ICB)

<sup>†</sup> Includes one patient on ICB with other malignancies; this data was included in neutrophil counts, viability, and cell surface markers but was excluded from co-culture and sequencing analyses

\*\* Excludes one patient on ADT with evidence of other malignancies, in addition to prostate cancer

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