Differential expression of the TP α and TP β isoforms of the human T Prostanoid receptor during chronic inflammation of the prostate: Role for FOXP1 in the transcriptional regulation of TP β during monocyte-macrophage differentiation.

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Research highlights:

- TP α and TP β isoforms differentially upregulated in prostate inflammatory infiltrates
- TP β found in 94% of infiltrates, including in B- & T-lymphocytes and in macrophages
- TP α found in 72% of infiltrates, but expression is mainly confined to macrophages
- TP α and TP β upregulated after monocyte-to macrophage differentiation of THP-1 cells
- Tumour suppressor FOXP1 transcriptionally regulates TP β expression in macrophages

Abstract

Inflammation is linked to prostate cancer (PCa) and to other diseases of the prostate. The prostanoid thromboxane (TX)A₂ is a pro-inflammatory mediator implicated in several prostatic diseases, including PCa. TXA₂ signals through the TP α and TP β isoforms of the T Prostanoid receptor (TP) which exhibit several functional differences and transcriptionally regulated by distinct promoters Prm1 and Prm3, respectively, within the *TBXA2R* gene. This study examined the expression of TP α and TP β in inflammatory infiltrates within human prostate tissue. Strikingly, TP β expression was detected in 94% of infiltrates, including in B- and T-lymphocytes and macrophages. In contrast, TP α was more variably expressed and, where present, expression was mainly confined to macrophages. To gain molecular insight into these findings, expression of TP α and TP β was evaluated as a function of monocyte-to-macrophage differentiation in THP-1 cells. Expression of both TP α and TP β was upregulated following phorbol-12-myristate-13-acetate (PMA)-induced differentiation of monocytic THP-1 to their macrophage lineage. Furthermore, FOXP1, an essential transcriptional regulator down-regulated during monocyte-to-macrophage differentiation, was identified as a key *trans*-acting factor regulating TP β expression through Prm3 in THP-1 cells. Knockdown of FOXP1 increased TP β , but not TP α , expression in THP-1 cells, while genetic reporter and chromatin immunoprecipitation (ChIP) analyses established that FOXP1 exerts its repressive effect on TP β through binding to four *cis*-elements within Prm3. Collectively, FOXP1 functions as a transcriptional repressor of TP β in monocytes. This repression is lifted in differentiated macrophages, allowing for upregulation of TP β expression and possibly accounting for the prominent expression of TP β in prostate tissue-resident macrophages.

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Key words

Thromboxane receptor; prostate; inflammation; cancer; macrophage; lymphocytes; T cells; B cells; Forkhead box protein P1 (FOXP1); tumour suppressor gene.

Abbreviations

AA, arachidonic acid; ADT, androgen deprivation therapy; AR, androgen receptor; BPH, benign prostate hyperplasia; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase/prostaglandin G₂/H₂ synthase; C-tail, carboxyl-terminal tail; DAB, 3,3'-diaminobenzidine; DAPI, 4'-6-diamidino-2-phenylindole; ERK, extracellular signal regulated protein kinase; FBS, foetal bovine serum; FHBS, forkhead binding sites; FOXP1, forkhead box protein P1; HIC, hypermethylated in cancer; HRP, horseradish peroxidase; IgG, immunoglobulin G; M-CSF, macrophage colony stimulating factor; MCP, monocyte chemoattractant protein; NKX3.1, homeobox protein NKX3.1; NSAIDs, non-steroidal *anti*-inflammatory drugs; PCa, prostate cancer; PG, prostaglandin; pGL3B, pGL3Basic; PIA, proliferative inflammatory atrophy; PIN, prostatic intraepithelial neoplasia; PMA, phorbol 12-myristate 13-acetate; PRK, protein kinase C-related kinase; pRL-TK, pRL-Thymidine Kinase; Prm, promoter; PSA, prostate specific antigen; qRT-PCR, real-time quantitative reverse transcriptase PCR; RLU, relative luciferase unit; SDM, site-directed mutagenesis; siRNA, *small interfering* RNA; TAMs, tumour-associated macrophages; TSG, tumour suppressor gene; TURP, transurethral resection of the prostate; TXA₂, thromboxane; TP, T Prostanoid receptor; WT, Wilms' tumour.

1. Introduction.

Prostate cancer (PCa) is a leading cause of cancer-related death in men [1, 2]. While many cases of localised PCa can be successfully treated with surgery and/or radiation therapy, androgen deprivation therapy (ADT) remains the principal treatment option available for locally advanced or metastatic disease [3]. Unfortunately, despite an initial response, many patients eventually fail in this and progress to the terminal castrate-resistant PCa stage, which remains a clinically challenging disease with no cure yet available [4]. Inflammation has long been linked to the development of PCa [5, 6] and indeed is often associated with other conditions of the benign prostate [7]. Histologically, inflammatory infiltrates are frequently observed in prostate specimens, often co-occurring within areas of proliferating atrophic glandular epithelium [5, 8]. These proliferative inflammatory atrophy (PIA) lesions occur most frequently within the peripheral zone of the prostate, the primary site where PCa originates, and have been proposed to represent precursors of prostatic intraepithelial neoplasia (PIN), a precursor stage of PCa [5]. Hence, defining the molecular pathways contributing to prostatic inflammation is a key goal of current research and may help to identify alternative therapies for PCa [6, 9].

The inflammatory microenvironment consists of a diverse leukocyte population including neutrophils, macrophages, B- and T-lymphocytes/cells, all of which can support the development of malignancy by producing a host of pro-inflammatory mediators, such as cytokines and prostanoids, as well as various proteases and angiogenic factors [10]. Prostanoids, including the prostaglandins (PGs) and thromboxane (TX) A₂, are synthesised from arachidonic acid (AA) by the sequential actions of cyclooxygenase (COX)-1/COX-2 in concert with the specific PG/TX synthases and play a major role in the promotion and resolution of inflammation by modulating the actions of immune cells and the surrounding microenvironment [11]. Accumulating evidence therefore suggests that the complex local actions of these lipid mediators and their receptors may represent a potential link between inflammation and cancer [12, 13]. Evidence for this is underpinned by recent longitudinal studies showing the prophylactic benefits of Aspirin and other non-steroidal *anti*-inflammatory drugs (NSAIDs) in reducing the risk of several cancers, particularly gastrointestinal and prostate cancers [14]. Moreover, the expression of COX-2 is increased during both inflammation and malignancy, also leading to enhanced prostanoid biosynthesis within the tumour microenvironment [15, 16].

Among the prostanoids, TXA₂ acts as a potent pro-inflammatory mediator while also regulating platelet activation status and vascular tone [17]. Numerous studies also strongly support a role for TXA₂ in cancer [18] and indeed in other conditions of the prostate including in benign prostate hyperplasia (BPH; [19]). In humans, TXA₂ signals through the α and β isoforms of its T Prostanoid (TP) receptor, which differ exclusively in their intracellular carboxyl (C)-tail domains and arise by alternative splicing of a 1° RNA transcript [20, 21]. TP α and TP β display distinct patterns of cell/tissue expression due to their transcriptional regulation by different promoters (Prm) within the same TP gene (*TBXA2R*) whereby Prm1 exclusively regulates TP α and Prm3 regulates TP β expression [22-25]. Moreover, the TPs exhibit several key differences in their modes of signalling and regulation which can also impact on their relative contribution to disease processes, including cancer progression in different cellular or clinical settings [26-30].

Notably, in the context of PCa, in a recent histological evaluation, we established that expression of the individual TP α and TP β receptor isoforms are strongly but differentially increased in prostate tumour tissue relative to the benign prostate [31]. Furthermore, within the PCa patient cohort ($N = 529$), elevated levels of TP β , and to a lesser extent of TP α , significantly correlated with adverse features of disease including with increasing Gleason score and with early biochemical recurrence, signified by rising prostate specific antigen (PSA) levels [31]. Such findings suggested that interrogation of TP β , or a combination of TP α and TP β , expression levels may have significant clinical potential as a diagnostic biomarker/predictor of PCa disease recurrence [31]. In addition, in that same study [31], through the use of laser-dissected tumour specimens from the same patient cohort, we also

discovered that the individual Prm1 and Prm3 promoter regions which regulate TP α and TP β , respectively, are subject to differential CpG methylation as a function of PCa staging accounting, at least in part, for the differential expression of the TPs in the tumour-derived tissue [31]. Moreover, in terms of their transcriptional regulation, the Wilms' tumour (WT) 1 and hypermethylated in cancer (HIC) were identified as key transcription factors that regulate TP α expression through Prm1 in the prostate adenocarcinoma PC-3 and LNCaP cell lines [32]. On the other hand, FOXP1 and NKX3.1, two tumour suppressor gene (TSG) products strongly implicated in PCa development, were identified as the key transcription factors regulating TP β expression through Prm3 in the PCa setting [33].

Mechanistically, the impact of TXA₂ on tumour development and metastasis can be partly explained by the ability of TP α /TP β to regulate key mitogenic/ERK- and RhoA-mediated signalling cascades [27, 34]. Interestingly, during our recent histology study in PCa [31], it was notable that TP α /TP β expression was not only found within the benign and tumour prostate epithelium but was also expressed in the fibromuscular stroma as well as in regions of inflammatory cell infiltrate (*Mulvaney & Kinsella, unpublished data*). While deemed beyond the scope of the previous study [31], such unpublished observations raised the possibility of a role for TP α and/or TP β in the regulation of local inflammation and immunity within the prostate microenvironment. Indeed, as stated, TXA₂ is a potent pro-inflammatory and immune-modulatory agent, being a major product not only of platelets but also of monocytes/activated macrophages [17]. For instance, TXA₂ negatively regulates the interaction of T-cells with dendritic cells, a process essential for adaptive/acquired immunity [35, 36], and also plays a critical role in early B-cell development [37]. TXA₂ also induces monocyte chemoattractant protein (MCP)-1 expression in lung cancer cells, leading to the recruitment of tumour-associated macrophages (TAMs) [38, 39]. Hence, in principle the impact of TXA₂/TP on tumorigenesis may also be potentially explained, at least in part, by its ability to regulate local inflammation and immunity within the tumour microenvironment [40].

While a role for TXA₂ signalling in the modulation of immune cell function has been shown, the precise role of the individual TP α and TP β receptor isoforms in mediating the immunoregulatory actions of TXA₂ in various immune cell types remains unknown. Moreover, while previous studies have demonstrated that COX-2 expression is up-regulated focally in prostate tumour areas with chronic inflammation [41, 42], to our knowledge no study to date has reported on the expression of key downstream components of the tumour-promoting COX cascade with regards to local inflammation of the prostate. Therefore, given our recent histological findings of a clinically significant role for TP β > TP α expression in PCa [31], coupled with the recognised role of TXA₂ as a pro-inflammatory and immune-modulatory mediator, including potentially within the tumour microenvironment, the initial aim of the current study was to histologically evaluate the expression pattern of the individual TP α and TP β receptors in regions of chronic inflammatory cell infiltration in benign and tumour specimens of human prostate tissue obtained from PCa patients following radical prostatectomy or in other patients requiring transurethral resection of the prostate (TURP) intervention. It was found that up to 94% of all inflammatory infiltrates expressed TP β , where its expression was associated with both B- and T-lymphocyte populations and with CD68-positive macrophages. In striking contrast to this, expression of the TP α isoform in the prostatic inflammatory infiltrates was much more variable and, where any TP α expression was present, it was mainly associated with macrophages and to a much lesser extent with either the B- and T-lymphocyte populations.

The monocytic THP-1 cell line can be readily induced to undergo differentiation into the macrophage lineage, such as in response to treatment with phorbol esters, and is frequently used as a cellular model in which to study monocyte-to-macrophage differentiation. Thus, as a means of obtaining molecular insight into the histological observations showing expression of both TP α and TP β in macrophage within the prostate tissue at the cellular level, expression of TP α and TP β was examined as a function of monocyte-to-macrophage cell differentiation in the THP-1 cell model. FOXP1 is a

tumour suppressor gene product implicated not only in prostate cancer (PCa) development but also acts as an essential regulator of monocyte-to-macrophage cell differentiation [43, 44]. Hence, in view of recent findings that expression of TP β in PCa is transcriptionally regulated by FOXP1 [33], the current study also explored the intriguing possibility that FOXP1 might play an additional role in the transcriptional regulation of TP β within the monocytic THP-1 lineage, accounting for the prominent histological expression of TP β in the tissue resident macrophage in the inflammatory infiltrates of the benign and tumour prostate specimens.

2. Materials and Methods

2.1 Materials

Mouse monoclonal *anti*-HDJ2 (DNAJ) antibody (#MS225-P1ABX) was from Neomarkers Inc. (Portsmouth, New Hampshire, USA). Mouse monoclonal *anti*-FOXP1 antibody (#MAB45341) was from R&D systems (Abingdon, UK). Monoclonal mouse *anti*-CD3 (#M7524), *anti*-CD4 (#M7310), *anti*-CD8 (#M7103), *anti*-CD20 (#M0755) and *anti*-CD68 (#M0876) antibodies were obtained from Dako (Agilent Technologies, Santa Clara, California, USA). Normal mouse immunoglobulin (Ig) G (sc-2025), biotinylated goat *anti*-rabbit IgG (sc-2040), biotinylated goat *anti*-mouse IgG (sc-2039) and horseradish peroxidase (HRP)-conjugated polyclonal goat *anti*-mouse IgG secondary antibodies (sc-2005) were obtained from Santa Cruz Biotechnology® (Santa Cruz, California, USA). TRIzol™ reagent, Superscript™ III reverse transcriptase (#18080-044), AlexaFluor® 594-conjugated goat *anti*-rabbit IgG secondary antibody (#A11012) and the streptavidin AlexaFluor® 594-conjugate (#S11227) were purchased from Invitrogen Life Technologies™ (Thermo Fisher Scientific, Waltham, MA, USA). The Dual-Luciferase® Reporter Assay System (#E1910) and RQ1 RNase-Free DNase were from Promega Corporation (Madison, Wisconsin, USA). Effectene® (#301425) was from Qiagen (Hilden, Germany). RPMI Medium 1640 (#31870-025), Opti-MEM 1 (#31985-047) and L-glutamine (#2530-024) were from Gibco® (Thermo Fisher Scientific, Waltham, MA, USA). DharmaFECT™ 2 (#11521741) transfection reagent was from ThermoFisher Scientific (Waltham, MA, USA). The Brilliant III Sybr Green QPCR kit (#600882) and the QuikChange™ site-directed mutagenesis (SDM) kit were from Agilent Technologies (Santa Clara, California, USA). Chromogen 3,3'-diaminobenzidine (DAB) substrate (#D4293), hydrogen peroxide (30% w/w) solution (#H1009), haematoxylin solution (Harris modified; #HHS16), DPX mounting medium (#44581), phorbol 12-myristate 13-acetate (PMA; #P8139), streptavidin-conjugated HRP (#S5512), Mowiol® 4-88 mounting medium and 4'-6-diamidino-2-phenylindole (DAPI; #D9542) were obtained from Sigma-Aldrich (Arklow, Wicklow, Ireland). The Avidin/Biotin Blocking Kit (#SP-2001) was from Vector Laboratories Ltd. (Peterborough, United Kingdom). Affinity-purified *anti*-TP α and *anti*-TP β antibodies have been previously described and extensively characterised [31, 45].

2.2 Cell Culture

The monocytic THP-1 cell line was obtained from the American Type Culture Collection (ATCC) and was cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine. THP-1 cells were grown at 37 °C in a humid environment with 5% CO₂ and were confirmed to be free from mycoplasma contamination. THP-1 cells were routinely passaged every 3-4 days to maintain cells in their exponential growth phase (typically between 5 × 10⁵ cells/ml and 2 × 10⁶ cells/ml).

2.3 Luciferase-based Genetic Reporter Plasmids

The luciferase reporter vectors pGL3Basic (pGL3B), pRL-Thymidine Kinase (pRL-TK) and the pGL3Control vector, encoding the SV40 promoter, were obtained from Promega Corporation. The plasmids pGL3B:Prm3, pGL3B:Prm3a and pGL3B:Prm3ab, and pGL3B:Prm3aab, encoding the full-

length Prm3 sequence (nucleotides -1394 to +1 of the human *TBXA2R* gene) and various subfragments thereof in the pGL3Basic reporter vector, have been previously described [22, 23].

The plasmids pGL3B:Prm3^{FOXP1*(-1298)}, pGL3B:Prm3^{FOXP1*(-943)}, pGL3B:Prm3^{FOXP1*(-597)} and pGL3B:Prm3^{FOXP1*(-496)} were generated by QuikChangeTM site-directed mutagenesis (SDM) of the consensus FOXP1 binding site (g/tTGGTTg/t to g/tTCCTTg/t,) at nucleotide -1298 (FOXP1^{#1}), -943 (FOXP1^{#2}), -597 (FOXP1^{#3}) and -496 (FOXP1^{#4}), respectively, within Prm3, as previously described [33]. In all cases, – designation indicates nucleotides 5' of the translational ATG start codon (designated +1), where the nucleotides that were mutated are underlined in bold.

2.4 Luciferase-based Genetic Reporter Assays

For luciferase gene reporter assays, immediately prior to preparing DNA transfection complexes, THP-1 cells were seeded onto 6-well plates (4.5×10^5 cells/35 mm well) in complete culture medium (1.5 ml RPMI, 10% FBS). Thereafter, cells were co-transfected with pGL3B-recombinant plasmids (2 μ g) and pRL-TK (25 ng), encoding firefly and renilla luciferases, respectively, using Effectene[®] transfection reagent (3 μ l/35 mm well).

Transiently-transfected THP-1 cell suspensions were harvested 48 hr post-transfection and assayed for firefly and renilla luciferases using the Dual-Luciferase[®] Reporter Assay System, essentially as previously described [32]. Results are expressed as a ratio of firefly to renilla luciferase (relative luciferase units, RLUs; n=4), where 'n' refers to the number of independent experiments and not to replicates within the same experiment.

2.5 Histology Tissue Specimens and Immunohistochemical Analysis

Included in this study were 15 full-face formalin-fixed and paraffin-embedded radical prostatectomy or transurethral resection of the prostate (TURP) tissue samples which were obtained through ethical consent from St. Vincent's University Hospital, Dublin, Ireland. **Table 1A** provides a summary of the 15 prostate tissue specimens examined in the current study, including details on the section type and histological diagnosis. In addition, control tissue including tonsil, kidney, urinary bladder and testis were also obtained through ethical consent from St. Vincent's University Hospital, Dublin, Ireland. Tissue samples were sectioned at 4 μ m thickness and baked onto slides at 50 – 56 °C for 60 min.

Sections were dewaxed in two changes of xylene (2 x 10 min incubations) and rehydrated through a series of decreasing alcohol solutions (100%, 3 min x 2; 95%, 1 min; 80%, 1 min) before being washed in double-distilled water (ddH₂O). Antigen retrieval was performed by boiling the slides in Tris-EDTA buffer, pH 9 (prior to incubation with *anti*-CD3, *anti*-CD8, *anti*-CD4, *anti*-CD20, and *anti*-CD68 antibodies), or in sodium citrate buffer, pH 6 (prior to incubation with *anti*-TP α and *anti*-TP β antibodies), in a pressure cooker for 5 min. Endogenous peroxidase activity was blocked by incubating the slides in hydrogen peroxide (3% v/v prepared in absolute methanol) for 10 min at room temperature (RT), followed by washing in 1X phosphate-buffered saline (PBS; ~500 μ l/slide). To block non-specific binding and endogenous biotin, the tissue sections were incubated for 30 min at RT with 5% goat serum in PBS (Blocking Buffer) containing Avidin D (4 drops/ml; Avidin/Biotin Blocking Kit). Sections were then incubated with the desired primary (1^o) antibody (~500 μ l/slide) for the required incubation period (30 min or overnight) in a humidified chamber (Refer to **Supplemental Table I**). All primary antibodies were diluted in Blocking Buffer, containing Biotin (4 drops/ml; Avidin/Biotin Blocking Kit). As a negative control, sections were also incubated with Blocking Buffer in the absence of primary antibody.

To investigate TP isoform expression in other tissue samples, including in associated regions of inflammation, the *anti*-TP α and TP β specific antibodies were also used to screen tissue sections from human kidney, urinary bladder and testis (**Supplemental Figure 2**). In addition, to confirm specificity of the antibodies directed to the various cluster of differentiation (CD) cell markers, *anti*-CD3, -CD8, -

CD4, -CD20, -CD68 histology staining was optimised and cross-evaluated in tissue sections from human tonsil (**Supplemental Figure 3**).

Following incubation with primary antibody, sections were washed in PBS (3 x 5 min washes; 500 μ l/slide) and incubated for 30 min with a biotinylated *anti*-rabbit (for detection of TP α and TP β) or *anti*-mouse (for detection of CD3, CD8, CD4, CD20, CD68) immunoglobulin (Ig) G secondary antibody prepared in 5% goat serum (1:500; Refer to **Supplemental Table 1**). Sections were then washed in PBS (3 x 5 min washes), followed by incubation with streptavidin-conjugated horseradish peroxidase (HRP; diluted 1:1500 in Blocking Buffer) for 30 min at room temperature. Thereafter, the slides were incubated with the chromogen 3,3'-diaminobenzidine (DAB) substrate for 5 min. Finally, tissue sections were counterstained for 1 min with haematoxylin (Harris modified), followed by washing in ddH₂O. Sections were then dehydrated through increasing alcohol series (80%, 1 min; 95%, 1 min; 100%, 3 min x 2) and xylene (2 x 10 min), prior to mounting in DPX.

Slides were digitally scanned at 20X or 40X using the Aperio® Slidescanner and supporting Aperio® ImageScope V12.2 software analysis package from Leica Biosystems or supported by QuPath (Version 0.1.2) software analysis package [46]. Histological patterns of chronic prostatic inflammation (peri-glandular, glandular and stromal) were identified based on the histopathological classification system described by Nickel *et al.* [47]. The number of inflammatory infiltrates was manually counted across the entire digitally scanned prostate sections and scored by at least two independent investigators for CD3, CD4, CD8, CD20, CD68, TP α & TP β staining. Scoring of infiltrates for each of the CD markers and TP α /TP β was based on the proportion of cells staining positive (brown staining, DAB) relative to the total number of cells within an infiltrate as follows: 0, negative; 1, up to 20 % positively stained cells; 2, up to 40 % positively stained cells; 3, up to 60 % positively stained cells; 4, up to 80 % positively stained cells and 5, up to 100 % positively stained cells. For correlation and linear regression analyses between TP α or TP β with each of the individual CD markers, scores for each of the 15 prostate cases were calculated as the mean of the scores for all the infiltrates within that case.

2.6 Disruption of FOXP1 expression in THP-1 cells by *small interfering (si)*RNA

*Small interfering (si)*RNA-mediated disruption of FOXP1 expression in THP-1 cells was achieved using DharmaFECT™ 2 transfection reagent and the reverse transfection method. The following specific *si*RNA sequences were used: *si*RNA_{FOXP1} (5'-CCACGTGGAAGAATGCAGT-3') or, as a control, a nonsense scrambled *si*RNA sequence (*si*RNA_{CONTROL}, 5'-AATTCTCCGAACGTGTCACGT-3'). Routinely, *si*RNA transfection complexes were prepared by diluting either the appropriate *si*RNA oligonucleotide (500 nM) or DharmaFECT™ 2 transfection reagent (5-10 μ l) in 200 μ l of OptiMEM® 1 reduced serum medium in separate microcentrifuge tubes. The contents of both tubes were then mixed by vortexing, incubated at room temperature for 5 min, and combined to give a total volume of 400 μ l.

Routinely THP-1 cells were passaged 24 hr prior to *si*RNA transfection to ensure the cells were in their exponential growth phase. On the day of transfection, immediately prior to preparation of *si*RNA transfection complexes, THP-1 cells were collected by centrifugation (1,200 rpm for 3 min at room temperature) and re-suspended in fresh culture medium (RPMI, 10% FBS) at a density of 4.5×10^5 cells/ml. Following incubation for 30 min, the *si*RNA-transfection complexes were added to 6-well plates (400 μ l/35 mm well, containing 500 nM *si*RNA). THP-1 cell suspensions (4.5×10^5 cells in 1 ml) were then added dropwise to the *si*RNA complexes in each 35 mm well and the contents were gently mixed. The volume was adjusted to 2 ml with complete culture medium to give a final concentration of 50 nM *si*RNA and the cells were incubated at 37 °C for 48-72 hr.

To confirm *si*RNA-mediated knock-down of FOXP1, cells were harvested 48–72 h post-transfection and whole cell lysates (typically, 10 - 30 μ g per lane) were resolved by SDS-PAGE on 10% acrylamide gels and proteins were transferred to polyvinylidene difluoride (PVDF) membranes, as per standard methodology. Membranes were blocked in 5% milk powder dissolved in 1X Tris-buffered

saline (TBS; 10 mM Tris-HCl, 100 mM NaCl, pH 7.4) for 1 h at room temperature and incubated overnight at 4°C with an *anti*-FOXP1 primary antibody, diluted 1:1500 in blocking solution (5% milk in TBS-T). Membranes were then washed in 1X TBS and screened with goat *anti*-mouse horseradish peroxidase (HRP) secondary antibodies, followed by chemiluminescence detection. To confirm uniform protein loading, membranes were screened with an *anti*-HDJ2 primary antibody (1:8000) for 1 hr at room temperature, followed by incubation with goat *anti*-mouse HRP secondary antibody and detection by chemiluminescence. Blots shown are representative of three independent experiments.

2.7 PMA-induced differentiation of THP1 cells

For differentiation of THP-1 monocytic cells toward the macrophage phenotype, a protocol was employed whereby the cells were incubated with phorbol 12-myristate 13-acetate (PMA) for 3 days, followed by 5 days culturing in growth medium without PMA [48]. To this end, THP-1 cells were routinely seeded onto 6-well plates (2×10^5 cells/35 mm well) in complete culture medium (2 ml RPMI, 10% FBS) or 10 cm diameter tissue culture dishes (1×10^5 cells/ml, 8 ml RPMI, 10% FBS). PMA was added to the cells at a final concentration of 200 nM and the cells were incubated at 37 °C for 3 days. Thereafter, the PMA-supplemented culture medium was removed and the attached cells were washed with 1X PBS-EDTA (1 ml). Fresh PMA-free culture medium (2 ml RPMI, 10% FBS) was added and the cells were incubated at 37 °C for a further 5 days in order to obtain phenotypic characteristics of macrophages [48].

2.8 Confocal Microscopy

THP-1 cells were seeded on to poly-L-lysine coated coverslips (2×10^5 cells/35 mm well) in complete culture medium and subjected to PMA-induced differentiation over an 8-day period (3 days plus PMA, 5 days without PMA), as previously described. At various time-points during the differentiation protocol (0, 1, 2, 3 and 8-days post-PMA stimulation), the culture medium was removed and the attached cells were washed with 1X phosphate-buffered saline (PBS; 1 ml). To fix the cells by cross-linking, the cells were incubated in 1 ml of 3.7% paraformaldehyde (pH 7.4; in PBS) for 15 min at room temperature (RT). Cells were washed three times in PBS (5 min/wash) and permeabilised by incubating in 1 ml of Triton X-100 (0.2% in PBS) for 10 min on ice. Cells were then washed three times in 1X TBS.

To block non-specific binding, the cells were incubated with 1% bovine serum albumin (BSA) dissolved in 1X TBS (1% BSA-TBS; 1 ml/35 mm well) for 1 hr at RT on a rotating platform shaker. For immunolabelling, cells were incubated overnight at 4 °C with affinity-purified isoform-specific *anti*-TP α (5 μ g/ml) or *anti*-TP β (5 μ g/ml) primary antibodies, prepared in Blocking Buffer (1% BSA-TBS; 200 μ l/well). As a negative control, cells were also incubated with Blocking Buffer in the absence of primary antibody. Cells were washed with 1 ml of TBS (3 x washes; 5 min/wash) and incubated with Blocking Buffer for a further 30 min, prior to secondary antibody incubation. Following incubation with *anti*-TP β antibody, cells were incubated with an AlexaFluor® 594-conjugated goat *anti*-rabbit immunoglobulin (Ig) G secondary antibody prepared in Blocking Buffer (1:2000; 500 μ l/well) for 45 min at RT. For detection of TP α , a streptavidin-based signal amplification method was used. To this end, cells were initially incubated with a biotinylated *anti*-rabbit IgG secondary antibody prepared in Blocking Buffer (1:2000; 500 μ l/well) for 45 min at RT. The cells were then incubated with a streptavidin AlexaFluor® 594-conjugate (1 μ g/ml; 500 μ l/well) for a further 15 min. Immunolabelled cells were washed in TBS and counterstained by incubating with 4'-6-diamidino-2-phenylindole (DAPI; 1 μ g/ml in H₂O) for 1 min at RT, followed by washing in H₂O. Cells were mounted in Mowiol® 4-88 mounting medium.

Images were captured at 63X magnification using a Carl Zeiss Laser Scanning LSM 510 UVMETA microscope and Zeiss LSM imaging software. Images presented are representative of three independent experiments (n = 3).

2.9 Real time quantitative-reverse transcriptase (qRT)-PCR Analysis

Total RNA was extracted from THP-1 cells using TRIzolTM reagent and converted to first-strand (1^o) cDNA, as previously described [33]. Briefly, RNA samples were initially subjected to DNase I treatment by incubating with RQ1 RNase-free DNase (1 U/5 μ g RNA). Thereafter, RNA (2 μ g/20 μ l reaction) was converted to 1^o cDNA using random hexamer primers (150 ng) and SuperscriptTM III reverse transcriptase (1 μ l; 200 U/ μ l). As negative controls, additional cDNA reactions were carried out in the absence of reverse transcriptase (-RT). The 1^o cDNA was then used as a template for quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis of TP α or TP β mRNA expression using the Brilliant III SYBR Green QPCR kit, as previous [33]. Gene-specific primers were used to selectively amplify a region of either the TP α (246 bp) or TP β (234 bp) mRNAs or, as a reference control, a 149 bp region of the human *18 s rRNA* gene [33]. To confirm that amplification was not due to the presence of genomic DNA contamination, -RT negative control samples were routinely included in the PCR reaction set-up. As additional negative controls, reactions were also carried out in the absence of template cDNA (No Template Control/NTC). Following amplification, PCR reactions were subjected to melting curve analysis (55-95 $^{\circ}$ C) to confirm the specificity of the qRT-PCR reaction products amplified. In addition, qRT-PCR products (5 μ l) were routinely analysed on 2% (w/v) agarose gels to confirm amplification of the correct PCR product size for each primer pair.

Levels of TP α and TP β mRNA were normalised using equivalent *18s rRNA* expression levels to obtain Δ Ct values and relative mRNA expression levels were calculated using the $\Delta\Delta$ Ct method [49]. For experiments analysing the effect of PMA-induced differentiation on TP α /TP β expression in THP-1 cells, results were calculated as mean changes in TP α or TP β mRNA expression following stimulation with PMA for 3/8 days relative to those levels in undifferentiated (Day 0) THP-1 cells, assigned a value of 1. Alternatively, for siRNA-mediated disruption experiments, data is presented as mean changes in TP α or TP β mRNA expression relative to the levels in siRNA_{CONTROL}-transfected cells, assigned a value of 1. In all cases, the qRT-PCR data presented is representative of three independent experiments/RNA isolations (n $\geq$ 3).

2.10 Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation (ChIP) was performed essentially as previously described [33]. Briefly, THP-1 cells ($\sim 1 \times 10^6$) were collected by centrifugation (1,200 rpm, 3 min, 4 $^{\circ}$ C), washed in ice-cold PBS and re-suspended in 50 ml of serum-free RPMI (0% FBS). Formaldehyde-cross-linked chromatin was sonicated to generate 350-1,000 bp fragments and re-suspended in a final volume of 6 ml immunoprecipitation (IP) Dilution Buffer (IPDB). Sheared chromatin was then pre-cleared by incubation overnight at 4 $^{\circ}$ C, with rotation, with 60 μ g of normal mouse immunoglobulin G (IgG). Salmon sperm DNA/protein A agarose beads (250 μ l) were added and incubation continued overnight at 4 $^{\circ}$ C with rotation. Chromatin was divided into aliquots (672 μ l each) and immunoprecipitated with an anti-FOXP1 antibody (R & D Systems; 10 μ g), or as controls, with equivalent amount of the normal mouse IgG or in the absence of primary antibody (-AB), overnight at 4 $^{\circ}$ C with rotation. An aliquot of the pre-cleared chromatin (270 μ l) was taken as an input control and stored at -80 $^{\circ}$ C for later use. ChIP samples were incubated with salmon sperm DNA/protein A agarose beads for 3 hr at 4 $^{\circ}$ C and immune complexes were eluted from the beads. Cross-links were reversed by incubation with RNase A (2 μ g) at 65 $^{\circ}$ C overnight; proteinase K (9 μ l at 10 mg/ml) was added and incubation continued at 45 $^{\circ}$ C for 7 hr. Thereafter, ChIP samples were purified by phenol: chloroform extraction, followed by ethanol precipitation and re-suspended in H₂O (50 μ l), as previous [33].

Real-time quantitative (qRT)-PCR analysis was carried out using the Agilent MX3005P QPCR system (typically for 40 cycles) to obtain cycle threshold (Ct) values, as previous [33]. Aliquots (typically 5 μ l) of the PCR products were analysed by agarose gel electrophoresis. The relative PCR product intensities generated due to FOXP1 binding, or the corresponding IgG and -AB controls, were calculated as a percentage relative to the input chromatin DNA analysed in parallel using the $\Delta\Delta$ Ct method [49]. The sequences of the primers used to amplify the Prm3 regions surrounding the putative FOXP1-binding elements, as well as the control regions (a non-specific region of Prm1 or of the unrelated *GAPDH* gene), have been previously described [33].

2.11 Statistical Analysis

Statistical analyses of differences were carried out using either paired or unpaired Student's t tests or one-way ANOVA, followed by post-hoc Bonferroni's or Dunnetts multiple comparison t-tests, employing GraphPad Prism (V6) throughout. All values are expressed as mean $\pm$ standard error of the mean (SEM). Regression analysis with Pearson's correlation coefficients was performed to determine linear associations between either TP α or TP β with each of CD3, CD4, CD8, CD20 and CD68 using GraphPad Prism (V6). P values $\leq$ 0.05 were considered to indicate statistically significant differences, where *, **, *** and **** denotes $p \leq$ 0.05, 0.01, 0.001, and 0.0001, respectively.

3. Results

3.1 TP α and TP β expression in chronic inflammation of the prostate

The initial aim of this study was to investigate the expression of the individual TP α and TP β isoforms of the T Prostanoid receptor (TP) in regions of chronic inflammatory cell infiltration in human prostate tissue obtained from PCa patients following radical prostatectomy or from patients undergoing transurethral resection of the prostate (TURP) where, in this case, the majority were benign and associated with other conditions of the prostate (**Table 1A**). Immunohistochemical (IHC) analysis of TP α and TP β expression was carried out on serial tissue sections using affinity-purified *anti*-TP α or *anti*-TP β isoform-specific antibodies [31, 45].

Consistent with our previous histological evaluations (Mulvaney *et al.*, 2016), expression of TP α and TP β in the benign regions of the prostate specimens was detected in the luminal epithelial cells lining the secretory glands and in the fibromuscular stromal smooth muscle cells (**Figure 1A; upper panels**). Strong positive staining of TP β and, to a lesser extent, TP α was also notable within the tumour epithelium (**Figure 1C; lower panels**). On scoring the tissues for regions of inflammation, a total of some 293 regions of chronic inflammatory cell infiltrates were identified within the 15 prostate specimens (**Table 1A**). Strikingly, strong expression of the TP β isoform was consistently found within the prostatic inflammatory infiltrates, with positive immune detection identified in 94% of all infiltrates and where all cell types within the infiltrates expressed TP β (**Figure 1, Table 1B**). In dramatic contrast to TP β , expression of the TP α isoform in the prostatic inflammatory infiltrates was much lower and more variable (**Figure 1, Table 1B**). In total, while weak expression of TP α was evident in 72% of all inflammatory infiltrates, the majority of cell-types within those infiltrates did not show any TP α expression (**Figure 1 & Table 1B**).

Notably, strong TP β expression was detected within immune cell infiltrates associated with each of the three primary histological patterns typical of chronic prostatic inflammation (**Figure 1**), including in regions of glandular, peri-glandular and stromal inflammation [47]. Foci of inflammatory cells lying within the fibromuscular stroma and surrounding either the benign or malignant epithelial

glands represented the most common pattern of chronic inflammation observed. This pattern of inflammation, namely peri-glandular inflammation, exhibited strong positive staining for TP β including in those infiltrates found within regions of the histologically benign prostate (**Figure 1A; upper panels**), and surrounding glands of prostate adenocarcinoma (intra-tumoral; **Figure 1C**). Furthermore, strong TP β expression was also observed within inflammatory infiltrates surrounding glands which exhibited morphological signs of epithelial cell atrophy indicated by loss of the basal glandular epithelial layer (**Figure 1A; lower panels**), a histological pattern which is typically indicative of proliferative inflammatory atrophy (PIA) precursor lesions [5]. Substantial TP β expression was also observed within foci of immune cells infiltrating either the benign, atrophic or malignant glandular epithelium and lumina (**Figure 1B & 1C**). This glandular inflammation represented a prevalent inflammatory pattern throughout the prostate specimens, often occurring alongside regions of peri-glandular inflammation (**Figure 1A**). In terms of the TP α isoform, as stated its expression in the prostatic inflammatory infiltrates was more variable than that of TP β (**Figure 1**). In those infiltrates where any positive expression of TP α was detected, it was mainly associated with isolated cell-types within regions of peri-glandular and/or glandular inflammation surrounding either the atrophic or malignant epithelium (**Figure 1A, lower panels; 1B & 1C**). Additional representative examples of TP α and TP β expression in regions of chronic prostatic inflammation are given in **Supplemental Figure 1**. Moreover, it was notable that the observed pattern of predominant expression of TP β , but not of TP α , in regions of chronic inflammation was found in other tissues including the kidney, urinary bladder and testes (**Supplemental Figure 2**).

To determine the specific immune cell populations within the prostatic infiltrates, tissue sections were also screened with antibodies to a range of leukocyte-specific cluster of differentiation (CD) cell markers (**Figures 2, 3, Table 2**). In the prostate specimens, the immune cell infiltrates mainly consisted of a mixed population of CD3⁺ T lymphocytes and CD20⁺ B lymphocytes (**Figure 2A**). Within the infiltrates identified, almost all were positive for CD3 expression (99% of infiltrates) with 86% of the infiltrates positive for CD20 (**Table 2**). Cytotoxic CD8⁺ T cells (94% of infiltrates) were the predominant cell type present in the T lymphocyte population, while CD4⁺ T-helper cells (74% of infiltrates) were present within the immune cell infiltrates in smaller numbers (**Figure 2A, Table 2**). Significant numbers of CD68⁺ macrophages were also detected within the majority of inflammatory infiltrates (**Figure 2A & 2B**) with 92% of the infiltrates showing positive CD68⁺ expression (**Table 2**). Moreover, TP β is expressed throughout the entire immune cell populations present in the prostatic infiltrates, where its expression did not correlate with either the T lymphocyte or macrophage populations, but positively correlated with increased numbers of CD20⁺ B lymphocytes (**Figure 2 & Supplemental Figure 4**).

In contrast, TP α expression, where present, typically exhibited a weaker focal staining pattern compared with that of TP β (**Figure 2A**). This focal staining of TP α within the infiltrates was mainly observed within macrophage (**Figure 2A & 2B**) where increased expression of TP α showed a strong positive correlation with increased CD68⁺ macrophage populations (**Supplemental Figure 4E**). In contrast to this, expression of TP α within the lymphocyte cell populations was more variable, with no expression of TP α detected within the majority of B- or T-lymphocytes present (**Supplemental Figure 4A-4D**).

Taken together, the above data reveal extensive differences in the expression pattern of the TP α and TP β receptor isoforms in regions of prostatic inflammatory cell infiltrate in tissue specimens from patients diagnosed with PCa and in others with benign conditions of the prostate. These findings are consistent with the independent expression/transcriptional regulation of the individual TP α and TP β isoforms in multiple cell/tissue-types, including in PCa and various other cancers [22, 25, 29, 31]. It was also notable that the observed differences in TP α and TP β expression within the inflammatory

infiltrating regions occurred irrespective of whether it was associated with PCa or in patients presenting with a benign condition of the prostate undergoing TURPs (data not shown).

3.2 TP α and TP β expression is up-regulated during THP-1 Monocyte-to-Macrophage Differentiation

Thus, expression of TP α and TP β was evident in tissue-resident macrophages both within the benign and *intra*-tumoral regions of the prostate tissue specimens. Macrophages are a major component of the inflammatory infiltrate observed in many tumours, including within the prostate, and contribute to various aspects of cancer progression [50, 51]. Human macrophages have long been known to produce TXA₂ and, along with platelets, represent the predominant source of TXA₂ generated within the inflammatory environment [52, 53]. The human monocytic THP-1 cell lineage is frequently employed as a model system for the study of monocyte-to -macrophage biology/function as it can be induced to undergo differentiation towards the macrophage phenotype such as in response to treatment with the phorbol ester phorbol-12-myristate-13-acetate (PMA) [54]. Hence, to gain a greater molecular insight into observations from the histology studies at the cellular level, the expression profiles of the TP α and TP β isoforms in non-differentiated and PMA-differentiated THP-1 cells was examined.

Initially, quantitative real-time RT-PCR (qRT-PCR) confirmed expression of both the TP α and TP β mRNAs in non-differentiated THP-1 monocytes where there was a 2.5-fold higher level of TP α than TP β mRNA expression (**Figure 3A**). To induce monocyte-macrophage differentiation, THP-1 cells were incubated with PMA for 3 days, followed by a period of culturing in the absence of PMA for a further 5 days [48]. While qRT-PCR analysis (**Figure 3B**) showed that there was no significant change in the expression of either TP α or TP β mRNA during the earlier stages of differentiation (Day 0-3), the fully-differentiated THP-1-derived macrophage-like cells (Day 8) showed a substantial increase in both TP α (3.9-fold) and TP β (5.9-fold) mRNA expression compared to their levels in the non-differentiated THP-1 monocyte precursor cells (Day 0; **Figure 3B**).

Confocal immunofluorescence microscopy was then used to analyse and quantify any changes in endogenous TP α and TP β receptor expression during THP-1 monocyte-to-macrophage cell differentiation at the protein level (**Figure 3C**). In the non-differentiated THP-1 monocytic cells, TP α and TP β expression was found to be predominantly intracellular where basal levels of TP α protein expression tended to be weaker than those of TP β (Day 0; **Figure 3C**). Both the *anti*-TP α and *anti*-TP β immunostaining was mainly evident in the cytosolic and nuclear fractions, with some minor evidence of plasma membrane staining for TP β (Day 0; **Figure 3C**). PMA- treatment led to a subtle, but differential, change in the expression patterns of TP α and TP β during the early stages of differentiation (Day 1-3; **Figure 3C**). Specifically, there was a shift towards more prominent staining of TP β at the plasma membrane, while the TP α isoform was associated with/recruited into punctuate vesicular structures during the early stages of THP-1 differentiation suggestive of its recruitment into early endosomes (Day 1–3; **Figure 3C**).

At day 8, the fully-differentiated THP-1 cells displayed macrophage-like morphological characteristics, including enhanced cell adherence/spreading and an increase in cytoplasmic volume compared with their monocytic precursor cells (Day 0 *versus* Day 8; **Figure 3C**). Strikingly, this was accompanied by dramatic alterations in both the pattern/localisation and the intensity of TP α and TP β receptor expression in the fully differentiated macrophage-like THP-1 cells (**Figure 3C**). Specifically, there was a dramatic shift towards predominant plasma membrane staining of both the TP α and TP β receptor isoforms (Day 8; **Figure 3C**).

Taken together, the above data show that TP α and TP β mRNA and protein expression is up-regulated following PMA-induced monocyte-to-macrophage differentiation in THP-1 cells, and this up-regulation is accompanied by corresponding re-distributions of both TP α and TP β to a predominantly plasma membrane localisation.

3.3 Down-regulation of FOXP1 increases TP β expression in THP-1 monocytes

While TP α and TP β are encoded by the same *TBXA2R* gene, the two receptor isoforms are differentially expressed being transcriptionally regulated by distinct promoters within the *TBXA2R* [22]. More specifically, promoter (Prm)1 which is located 5' of exon 1 within the *TBXA2R* regulates TP α expression, while Prm3, located within exon 2, exclusively regulates TP β expression [22-24]. Aside from its role in various cancers, including in PCa, the tumour suppressor gene product FOXP1 plays an essential role as a regulator of monocyte differentiation, such as through its transcriptional repression of the macrophage colony stimulating factor (M-CSF) receptor (Shi *et al.*, 2004). As previously reported by us [33], bioinformatic analysis of Prm3 identified four highly-ranked consensus *cis*-acting elements for the forkhead transcription factor FOXP1, and which were confirmed to be involved in the transcriptional regulation of TP β in PCa. These four *cis*-acting FOXP1 elements are designated herein as FOXP1^{#1} (at -1298), FOXP1^{#2} (at -943), FOXP1^{#3} (at -597) and FOXP1^{#4} (at -496). Hence, in view of the prevalence of these *cis*-acting FOXP1 elements combined with their established roles in regulating TP β expression through Prm3 in the PCa setting at least, it was next sought to investigate whether FOXP1 might play a role in the transcriptional repression of Prm3-directed TP β expression in THP-1 monocytes and in their fully differentiated macrophage variants.

In keeping with previous reports [43], immunoblot analysis confirmed that FOXP1 is abundantly expressed in non-differentiated/monocytic THP-1 cells and that its expression is down-regulated following PMA-induced monocyte-to-macrophage differentiation (**Supplemental Figure 5**). To investigate whether FOXP1 transcriptional repressor might represent a candidate factor regulating TP β expression in THP-1 monocytes, the effect of *siRNA*-mediated down-regulation of FOXP1 on TP α and TP β mRNA expression was examined. Immunoblot analysis confirmed effective disruption of FOXP1 expression (on-average, 57.6%) in THP-1 cells, relative to the *siRNA*_{CONTROL} (**Figure 4Ai & 4Aii**). Furthermore, real-time quantitative reverse transcriptase (qRT)-PCR analysis confirmed that TP β mRNA expression levels were significantly increased (2.7-fold) following *siRNA*-mediated disruption of FOXP1 in THP-1 cells, relative to those levels in cells transfected with the *siRNA*_{CONTROL} sequence (**Figure 4B**). In contrast, there was no change in TP α mRNA expression following disruption of FOXP1 in THP-1 cells (**Figure 4B**). Hence, these data confirm a role for FOXP1 in the transcriptional regulation of TP β , but not of TP α , expression in THP-1 monocytic cells.

3.4 Role of FOXP1 *cis*-acting elements in regulating Prm3-directed expression in THP-1 cells

Next, the role of the candidate FOXP1 *cis*-acting elements, designated FOXP1^{#1}, FOXP1^{#2}, FOXP1^{#3} and FOXP1^{#4}, in the transcriptional regulation of TP β through Prm3 was investigated through luciferase-based gene reporter assays. Noteworthy, due to the nature of the differentiation process involving loss of the cells' ability to undergo proliferation [48], all studies were carried out in the non-differentiated monocytic THP-1 as opposed to in cells induced to undergo differentiation to the macrophage phenotype. Initially, Prm3 was confirmed to direct reporter gene expression in THP-1 cells at levels 2.9-fold higher than those directed by pGL3Basic alone (**Figure 5A**).

Thereafter, 5' deletion analysis of Prm3 was used to map the key transcriptionally responsive repressor and activator regions within Prm3 in the non-differentiated/monocytic THP-1 cell line. Notably, 5' deletion of nucleotides from Prm3 to generate Prm3a resulted in a significant increase (~3-fold) in luciferase expression in THP-1 cells (**Figure 5B**). Hence, sequences/nucleotides between -1394 and -404 exerted a strong repressive effect on Prm3-directed gene expression in THP-1 cells, potentially through binding of transcriptional repressors to this region and thereby affecting the overall levels of Prm3-directed transcription. Further 5' deletion of the Prm3a sub-fragment to generate Prm3ab resulted in a decrease in luciferase expression and a return of luciferase activity to basal levels, equivalent to that directed by the full-length Prm3. These data are indicative of the presence of positive regulatory or transcriptional activator sequences between nucleotides -404 and -320, which can also alter the

transcriptional activity of Prm3 in THP-1 cells (**Figure 5B**). Additional 5' deletion of Prm3ab to generate Prm3aab did not significantly affect Prm3-directed luciferase activity in THP-1 cells (**Figure 5B**).

Furthermore, site-directed mutagenesis (SDM) along with gene reporter analysis established that each of the individual FOXP1 *cis*-elements, designated FOXP1^{#1}, FOXP1^{#2}, FOXP1^{#3} and FOXP1^{#4}, play a role in the transcriptional regulation of Prm3 in THP-1 cells. Specifically, mutation of each FOXP1 *cis*-element yielded significant increases in Prm3-directed luciferase activity in THP-1 cells, where the largest increase (1.6-fold) was observed on mutation of the FOXP1^{#2} element (**Figure 5C**). Hence, these data suggest that FOXP1 may mediate transcriptional repression of TP β in THP-1 cells through its binding to the FOXP1^{#1}, FOXP1^{#2}, FOXP1^{#3} and FOXP1^{#4} *cis*-acting elements within Prm3 of the *TBXA2R* gene.

3.5 FOXP1 binds to specific *cis*-acting elements within Prm3 in monocytic THP-1 cells

To investigate whether FOXP1 actually binds *in vivo* to the candidate FOXP1 *cis*-elements within Prm3 in the non-differentiated/monocytic THP-1 cells, chromatin immunoprecipitation (ChIP) analyses were performed employing an *anti*-FOXP1 specific antibody. PCR primers surrounding the Prm3 region containing the FOXP1^{#2} element (**Figure 6Ai**) specifically generated amplicons from the input chromatin and *anti*-FOXP1 immunoprecipitates, but not from either the control/non-immune IgG or minus antibody (-AB) control precipitates in monocytic THP-1 cells (**Figure 6B & 6C**). Similarly, primers specific to the region surrounding the adjacent FOXP1^{#3} and FOXP1^{#4} elements within Prm3 (**Figure 6Ai**) generated amplicons from the input chromatin and *anti*-FOXP1 immunoprecipitates in THP-1 cells, but not from the control IgG or -AB chromatin precipitates (**Figure 6B & 6C**). Hence, these data confirmed specific binding of FOXP1 to the FOXP1^{#2}, FOXP1^{#3}, and FOXP1^{#4} elements within Prm3 in THP-1 cells. Notably, despite repeated attempts at PCR amplifications, specific binding of FOXP1 to the putative FOXP1^{#1} element could not be confirmed by ChIP analysis, most likely due to local secondary structures within the region surrounding this FOXP1^{#1} element. However, through mutational analysis (**Figure 5C**), it was established that this FOXP1^{#1} element is functionally active and plays a role in the transcriptional repression of Prm3 in THP-1 cells. The specificity of FOXP1 binding to Prm3 in THP-1 cells was confirmed whereby primers surrounding a control region of Prm1 within the *TBXA2R* (**Figure 6Ai**) or of the unrelated *GAPDH* gene (**Figure 6Aii**) specifically generated an amplicon from the input chromatin only, but not from the *anti*-FOXP1, *anti*-IgG or -AB ChIP complexes (**Figure 6B & 6C**).

Collectively, the above data establish that the forkhead transcription factor FOXP1 can bind to specific *cis*-acting FOXP1 elements within Prm3 to mediate transcriptional repression of TP β in monocytic THP-1 cells where, through a combination of mutational analyses and luciferase-based gene reporter studies in addition to *in vivo* ChIP studies, it was confirmed that all four FOXP1 *cis*-elements within Prm3 are functionally important. Furthermore, PMA-induced differentiation of the monocytic THP-1 cell line to the fully differentiated macrophage phenotype coincides with almost complete down-regulation of FOXP1 and with a concomitant increase in TP β mRNA and protein expression. It is therefore proposed that such down-regulation of FOXP1 in the fully differentiated THP-1 macrophage lineage coincides with decreased binding of the repressor FOXP1 to the *cis*-elements within Prm3 and, in turn, accounting for the increased expression of TP β in the fully differentiated macrophage lineage.

4 Discussion

A relationship between cancer and inflammation was first proposed by Virchow in 1863 and chronic inflammation is now recognised as a major driver of tumorigenesis [9]. In fact, recent epidemiological data estimates that 20% of all human cancers are related to chronic infections or to other unresolved inflammatory states [9, 10]. The origin of chronic prostatic inflammation, be it associated with PCa or other benign prostate conditions, remains a subject of debate and possible causative factors include infectious agents, urine reflux, dietary factors, and hormones, or a combination of two or more of these [16]. Regardless of aetiology, inflammation is a risk factor for prostate cancer (PCa) and the probability of disease relapse following prostatectomy is increased in patients with high-grade inflammation surrounding their malignant glands [8]. Thus, determining the components of the innate immunity and inflammatory process that can contribute to the pathogenesis of PCa is of vital importance [9].

The pro-inflammatory prostanoid thromboxane (TX) A₂ signals through the TP α and TP β isoforms of the human T Prostanoid receptor (TP) and is implicated in the pathophysiology of PCa [31, 55, 56]. TP α and TP β are encoded by the same *TBXA2R* gene but are differentially expressed in numerous cell/tissue types due to their transcriptional regulation by distinct promoters (Prm), referred to as Prm1 and Prm3, respectively [22, 24, 25]. Moreover, TP α and TP β display several key differences in their signalling and regulatory mechanisms which may influence their relative contribution to disease processes, including in various cancers [26-28, 40]. Mechanistically, TP α and TP β can activate signalling by several key oncogenic pathways, including both ERK- and RhoA-mediated cascades [27, 34, 45]. More recent studies by us have also revealed that the TPs can both mimic and augment the action of androgens (testosterone) within the prostate by complexing with and activate signalling by the protein kinase C-related kinases (PRKs), AGC kinases and RhoA effectors that play an essential role in the epigenetic regulation of androgen receptor (AR)-dependent gene expression in the prostate [57, 58]. Furthermore, as stated, TXA₂ is also a potent pro-inflammatory and immune-regulatory agent [59]. Hence, it is possible that the influence of TXA₂ on neoplastic disease may also be partly explained due to its ability to regulate local inflammation and immunity, including within the tumour microenvironment [40].

Thus, to gain insight into the role of the TXA₂/TP axis in the regulation of local inflammation/immunity within the prostate, the current study investigated the expression profile of both TP α and TP β in regions of prostatic inflammatory cell infiltration through IHC screening of prostate tissues obtained from patients diagnosed with PCa or in other patients presenting with a benign condition of the prostate. In each of these prostate sections, both benign and diseased (e.g tumour) tissue was evident. In the benign prostate regions or specimens, TP α and TP β were expressed in the epithelial cells lining the prostatic glands and in the fibromuscular stromal smooth muscle cells, consistent with the contractile role of TXA₂ in the prostate [60]. Strong expression of TP β , and to a lesser degree TP α , was also notable within the tumour epithelium. Strikingly, TP β expression was also consistently detected within regions of inflammatory cell infiltrate throughout the prostate specimens, with intense positive TP β immunoreactivity identified in ~94% of all infiltrates present. In dramatic contrast to TP β , expression of the TP α isoform in the prostatic inflammatory infiltrates was more variable, whereby some patients showed positive TP α staining in less than one-fifth (e.g. 18%) of their infiltrates, while others displayed a higher frequency (e.g. 67% or 81%) of infiltrates showing positive expression of TP α . Moreover, within the inflammatory infiltrates, expression of TP β was associated with both B- and T-lymphocyte populations and with CD68-positive macrophage. In striking contrast to this, in the case of the TP α isoform, where its expression in the prostatic inflammatory infiltrates was present, it was mainly associated with macrophage populations. It should be acknowledged as a limitation of this immunohistochemical analysis that TP and CD marker immunohistochemical analysis was performed in serial tissue sections and did not use dual staining of the same slide as a means of protein colocalization. None the less, these data reveal extensive differences in the expression pattern of the

individual TP isoforms in regions of prostatic immune cell infiltration and thereby provides the first detailed insight into TP α *versus* TP β expression in the context of chronic inflammation of the prostate.

The significance of the observed differences in the expression of the TP isoforms in B- and T-lymphocytes is currently unclear, requiring additional exploration beyond the scope of the current study. None-the-less, it may reflect an ability of TP α and TP β to differentially regulate immunity and/or inflammation such as in diseases of the prostate, including in PCa. Notably, it has recently been established that PRK1 plays an essential role in the migration and trafficking of B- and T-lymphocytes [61]. More specifically, that study showed that while lymphocytes from knock-in mice expressing a kinase inactive mutant of PRK1 (PRK1^{T778A}, corresponding to Thr⁷⁷⁴ of human PRK1) developed normally, they sequestered in the spleen and lymph nodes and failed to egress resulting in reduced lymphocyte numbers in the peripheral circulation [61]. As stated, through a series of recent studies, we demonstrated that both the TP α and TP β isoforms complex with and regulate signalling by PRK1 and PRK2 to both mimic and regulate androgen/AR-signalling within the prostate but that this occurs in a TP isoform-specific manner [57, 58]. Moreover, agonist-activation of both TP α or TP β leads to rapid and sustained T-loop phosphorylation of the PRK1 at Thr⁷⁷⁴ through a PI3'K/PDK1-dependent mechanism [57]. Hence, it is indeed possible that TP-mediated PRK1 or PRK2 signalling through TP α &/or through TP β may play an essential role in the trafficking of B- and T-lymphocytes.

Macrophages are cells of the innate immune response that are critical in the pathogenesis of many diseases, including cancer progression [50, 51]. In the prostate for instance, many studies found that higher numbers of tumour-associated macrophages (TAMs) correlate with a worse prognosis [51]. Macrophages arise from precursor blood monocytes which are recruited to the tissue/tumour by specific inflammatory signals, such as MCP-1 [50]. Following differentiation, macrophages acquire a large repertoire of functions through the co-ordinated expression of numerous genes, allowing them to participate in inflammatory and immune responses [62]. Macrophages have long been known to produce TXA₂ [52, 53]; however, the relative roles of TP α and TP β in human monocyte and macrophage biology/function is unknown.

Real time quantitative PCR (qRT-PCR) analysis confirmed TP α and TP β mRNA expression in monocytic THP-1 cells, where expression of both mRNAs was substantially up-regulated following PMA-induced monocyte-to-macrophage differentiation. Confocal microscopy confirmed that PMA-induced THP-1 differentiation also led to significant upregulation of both TP α and TP β expression and re-localization at the plasma membrane. Collectively, these data reveal that differentiated macrophages express TP α and TP β at high levels compared with their monocytic precursors, highlighting the TXA₂-TP axis as a novel pathway that is up-regulated during the process of monocyte-to-macrophage differentiation.

Macrophages display striking phenotypic heterogeneity [63]. Following differentiation, there is considerable plasticity in the phenotype of tissue-resident macrophages which integrate signals from their surrounding microenvironment and undergo activation to perform specific functional properties [63]. The classical version of macrophage activation is induced by IFN- γ , which stimulates a pro-inflammatory phenotype required for the killing of intracellular pathogens [64]. Classically activated (M1) macrophages are typified by their release of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-12 [65]. Macrophages also undergo alternative activation in response to IL-4 and IL-13, which induce a different phenotype known to be important for immunity, hypersensitivity, allergy and parasitic infections [64]. Alternatively activated (M2) macrophages are also implicated in a range of (patho)physiologic processes, such as homeostasis, inflammation, repair, metabolic functions, and malignancy [64]. Monocytic cell lines such as THP-1, HL-60 and U937 are frequently employed for the study of human macrophage function as they can overcome many of the barriers encountered when working with primary tissue-resident macrophages which cannot be easily expanded *ex vivo* [48, 54]. Herein, studies were performed using a specific differentiation protocol whereby THP-1 cells were

continuously stimulated with the phorbol ester PMA for three days, followed by a period where the differentiating cells were cultured in the absence of PMA for a further 5 days. PMA-stimulation of THP-1 cells by this method has been shown to induce a macrophage-like phenotype which closely resembles the morphological, biochemical, and physiological characteristics of primary differentiated tissue macrophages and thus, represents a relevant model in which to study macrophage biology/function [48].

Using this model, data herein have provided the first insight into the expression pattern of the TP α and TP β isoforms in the context of human monocyte/macrophage biology. A noted limitation of this approach was that observations were all drawn from experimentation in the immortalized THP-1 cell line, rather than in primary monocytes/macrophages. Given the inherent plasticity of macrophages, it will now be of interest to advance our findings to additional cellular models of human monocyte-to-macrophage differentiation and as a function of their subsequent polarized activation states (M1- *versus* M2-like) with respect to their expression of the TPs. Moreover, it will be critical to translate these findings to the *in vivo* setting through further histological examination of the precise expression profile of both TP α and TP β in the tissue-resident macrophages found within various tissue/inflammatory environments, including within the tumour setting. Such studies should lead to a better understanding of the role of TXA₂ and its receptors in macrophage biology and function and the respective impact on human health and disease. Furthermore, we found that the immune cell types expressing high levels of TP β within the prostate consisted of a mixed leukocyte population, including not only CD68⁺ macrophages, but also T cells (CD3⁺, CD4⁺ and CD8⁺) and B cells (CD20⁺).

FOXP1 belongs to the P-subfamily (FOXP1-4) of the broader forkhead box (FOX) superfamily which play crucial roles in developmental processes and are known to regulate immune cell function [66]. As with other members of the family, FOXP1 has a diverse repertoire of functions, where it largely serves as a transcriptional repressor [67]. For instance, FOXP1 functions as an essential transcriptional regulator of B cell development and is involved in the regulation of cardiac valve and lung development in mice [68-70]. FOXP1 also plays an important role in malignancy [67], serving as a tumour repressor gene and now also known to regulate TP β expression in prostate adenocarcinoma LNCaP and PC-3 cells through its binding to the four FOXP1 *cis*-elements within Prm3 of the *TBXA2R* [33]. In addition, FOXP1 operates as a key transcriptional repressor of the *c-fms* gene that encodes the M-CSF receptor required for the differentiation, proliferation, and survival of macrophages [43]. Down-regulation of FOXP1 by integrin engagement leads to up-regulated M-CSF receptor expression and is, therefore, an essential step in the control of monocyte differentiation and macrophage function [43, 44].

In keeping with this, data herein confirmed that PMA-induced differentiation of THP-1 cells was associated with down-regulation of FOXP1 expression in the fully-mature (Day 8) macrophage-like cells [43]. Furthermore, targeted *siRNA*-disruption of FOXP1 significantly up-regulated TP β mRNA expression in THP-1 cells, confirming a role for FOXP1 in the transcriptional regulation of TP β expression in THP-1 monocytes. Genetic reporter assays, combined with mutational analyses, and chromatin immunoprecipitation (ChIP) analyses in the undifferentiated/monocytic THP-1 lineage established that FOXP1 exerts its repressive effect on TP β through binding to key *cis*-acting elements (at nucleotide -1298, -943, -597 and -496) within its promoter (Prm) 3 regulatory region of the *TBXA2R* gene. Moreover, 5' deletion analysis revealed that these candidate FOXP1 *cis*-binding sites localise to a region of Prm3 (i.e. nucleotides -1394 to -404) which exerts a strong repressive effect on Prm3-directed luciferase gene expression in THP-1 cells. Furthermore, PMA-induced differentiation of THP-1 cells to the fully differentiated macrophage phenotype coincided with a decrease in the expression of FOXP1 to undetectable levels, and with a concomitant increase in TP β mRNA and protein expression. Hence, as proposed in the model in **Figure 7**, these data reveal a role for FOXP1 not only in PCa [33] but also in the transcriptional repression of TP β expression in monocytes. Mechanistically, this FOXP1-mediated repression is lifted or lost in differentiated macrophages due to the down-regulation of FOXP1

allowing for upregulation of TP β expression, and possibly accounting for the prominent expression of TP β in the tissue-resident macrophages of the prostate tissue (**Figure 7**). In turn, the increased expression of TP β may drive autocrine or paracrine signalling by the TXA₂-TP β signalling axis, such as within the intra-tumoral regions of prostate microenvironment to further augment the already established role of TP β in PCa [31]. Notably, siRNA-disruption of FOXP1 had no effect on TP α mRNA expression. Thus, the transcriptional regulatory effects of FOXP1 in THP-1 cells are specific for TP β . While the mechanism regulating TP α expression in monocytes/macrophages remains to be fully investigated, it may involve an established regulatory transcriptional pathway of WT1-mediated repression and Egr1-mediated activation as previously reported for the *TBXA2R* [71] and for numerous other cell/tissue types including in several different neoplastic settings [72]. Therefore, these data greatly extend knowledge of the FOXP1 transcriptional repressor in the context of the TXA₂-TP signalling axis and monocyte/macrophage biology, including in PCa. It is proposed that loss of FOXP1, either due to genome copy number alterations or chromatin rearrangements of the *FOXP1* gene at Chr 3p [33, 73] or due to increased numbers of tumour associated macrophages (TAMs) in regions of chronic inflammation (**Figure 7**), may lead to increased TP β expression to drive TXA₂-TP signalling within the PCa tumour microenvironment (**Figure 7** and [33]). Notably as previously stated, in addition to FOXP1, expression of TP β in the prostate was also confirmed to be a *bona fide* target of the tumour suppressor NKX3.1, a transcriptional regulator widely implicated in PCa development. Specifically, in the case of the *TBXA2R*, siRNA-disruption of NKX3.1 coincided with up-regulation of TP β expression, while genetic-reporter and ChIP analyses confirmed that NKX3.1 bound to *cis*-elements within *Prm3* to transcriptionally repress TP β in PCa lines [33]. Interestingly, a recent study correlating immunoregulatory expression data from engineered mouse models and human prostate tumours has demonstrated that loss of function of NKX3.1 accelerates inflammation-driven PCa initiation [74]. While NKX3.1 does not appear to be expressed in THP-1-derived monocytes/macrophages (unpublished observations), whether it might play a role in regulating TP β expression in other immune cell types within inflammatory infiltrates, such as in TAMs, B- or T- lymphocyte populations, may indeed be worthy of further investigation.

The molecular mechanisms responsible for priming the pathogenesis of cancer-related inflammation are complex and rely on a delicate interplay between the tumour and its microenvironment [10]. Tissue-resident macrophages, including TAMs, secrete large quantities of pro-inflammatory mediators including cytokines and prostanoids (mainly TXA₂ and PGE₂), as well as various proteases, growth factors and angiogenic factors, all of which are important mediators in tissue remodelling, but can also promote neoplastic progression [10]. Furthermore, along with TAMs, enhanced platelet activation also leads to the release of pro-tumorigenic factors in the tumour microenvironment, particularly TXA₂, the major product of COX-1 in platelets [75]. Furthermore, COX-2 expression is dramatically increased in response to various inflammatory stimuli in both tumour and immune cells, also potentiating prostanoid biosynthesis within the tumour environment [13]. In turn, TXA₂ acts as an autocrine or paracrine mediator within its tissue microenvironment, and has potent effects leading to the activation of several tumour promoting pathways including ERK-, RhoA- and PRK-mediated cascades [27, 34, 57]. In addition, autocrine signalling by the TXA₂-TP axis can contribute to macrophage production of pro-inflammatory mediators, such as TNF- α , IL-1 β and MMP-9, supporting the development of a persistent inflammatory state [76, 77].

Critically, findings herein have demonstrated that elevated TP β expression in the prostate microenvironment is found not only within the tumour epithelium but also correlates with areas of chronic inflammatory cell infiltration, including in macrophages and B and T lymphocytes. Furthermore, the study highlights further critical differences in the expression profile of the TP α -relative to that of the TP β - isoform, which is likely to translate into key functional differences between the two isoforms in the context of tissue immunity and inflammation, be it associated with PCa or other

benign conditions of the prostate. Such findings add further credence to the proposed mechanism by which the TXA₂-TP β axis might drive cancer development and progression, through persistent autocrine signalling by TXA₂ generated within the inflammatory tumour environment. These findings also provide a further mechanistic explanation for the prophylactic benefits of Aspirin and other NSAIDs in reducing PCa risk through continuous suppression of TXA₂ biosynthesis [78-80]. Overall, our data reveal that TP β expression strongly correlates with regions of chronic inflammation in human prostate tissue, highlighting this isoform as a potential target in the treatment of inflammation-driven PCa. Moreover, we have identified the TXA₂-TP α /TP β axis as a novel pathway which is up-regulated during the process of monocyte-to-macrophage differentiation.

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Conflict of Interest:

The authors declare that they have no competing interests.

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Table 1A: Summary of prostate tissue specimens used for immunohistochemical (IHC) analysis.

Prostate Cases	15
RP / TURP Cases*	9 / 6
PCa / Benign Cases[#]	10 / 5
Total Inflammatory Infiltrates	293
Infiltrates/Case (Mean $\pm$ SEM^{\$})	17 $\pm$ 4.4

* RP, Radical Prostatectomy; TURP, Transurethral Resection of the Prostate.

[#] Prostate cancer (PCa) cases contained regions of both benign prostate tissue and prostate adenocarcinoma.

^{\$} SEM, Standard Error of the Mean.

Table 1B: Expression of TP α and TP β in inflammatory infiltrates within prostate tissue specimens.

Staining Marker	Infiltrates Scored*	Infiltrates with Positive Staining[#]
TPα	260	187 (72%)
TPβ	242	227 (94%)

* Total number of inflammatory infiltrates located on respective stained section and scored for the respective marker.

[#] Number (and percentage) of infiltrates containing inflammatory cells that displayed any positive staining for the respective marker. Not all cells within a given infiltrate showed positive staining.

Table 2: Expression of Inflammatory Markers in inflammatory infiltrates within prostate tissue specimens.

Staining Marker	Infiltrates Scored*	Infiltrates with Positive Staining[#]	Inflammatory Cell Type^{\$}
CD3	293	290 (99%)	T Lymphocytes
CD4	290	215 (74%)	Helper T Lymphocytes
CD8	291	274 (94%)	Cytotoxic T Lymphocytes
CD20	291	250 (86%)	B Lymphocytes
CD68	291	268 (92%)	Macrophages

* Total number of inflammatory infiltrates located on stained section and scored for the respective marker

[#] Number (and percentage) of infiltrates containing inflammatory cells that displayed any positive staining for the respective marker. Not all cells within a given infiltrate showed positive staining.

^{\$} Specific inflammatory cell indicated by respective cluster of differentiation (CD) marker.

FIGURES & FIGURE LEGENDS

Figure 1. TP α and TP β expression in histological patterns of chronic prostatic inflammation.

Panel A-C: Representative immunohistochemical (IHC) images of TP α and TP β expression in histological areas of chronic prostatic inflammation in (A) peri-glandular, (B) glandular and (C) intra-tumoral regions are shown. In total, 15 full-face prostate tissue specimens were screened with affinity-purified *anti*-TP α or *anti*-TP β isoform-specific antibodies as indicated, where matched serial sections were used throughout as a means of comparing expression between slides. Sections were counterstained with haematoxylin. Images were captured at 200X magnification using the Aperio® ScanScope XT and supporting ImageScope V12.2 software (left-hand *Panels*). The region of inflammation that is boxed in the left-hand panels is depicted at higher magnification (1000X) in the corresponding right-hand panels. The boxed regions in the lower left (200X) and lower right (1000X) panels of **Figure 1A** highlight regions of glandular epithelial atrophy.

Figure 2. Immunohistochemical (IHC) analysis of immune cell populations infiltrating the prostate. **Panel A(i)-(iii):** Representative IHC images of TP α and TP β expression in histological patterns of chronic prostatic inflammation are shown. In total, 15 full-face prostate sections were screened with affinity-purified *anti*-TP α or *anti*-TP β isoform-specific antibodies. For characterisation of immune cell types within the prostatic infiltrates, matched serial sections were also screened with antibodies directed to a range of leukocyte-specific cluster of differentiation (CD) cell marker proteins, including CD3⁺ (T lymphocyte), CD4⁺ (T-helper cell), CD8⁺ (cytotoxic T cell), CD20⁺ (B lymphocyte) and CD68⁺ (macrophage). All sections were counterstained with haematoxylin. Images were captured at 200X magnification using the Aperio® ScanScope XT and supporting ImageScope V12.2 software. **In Panel B(i)-(iii),** matched serial sections screened with *anti*-TP α , *anti* TP β and *anti* CD68 antibodies are shown at 1000X magnification. The red arrowheads indicate the presence of macrophage within the prostate tissue displaying positive TP α , TP β or CD68 expression.

Figure 3. Analysis of TP α and TP β expression in monocytic THP-1 cells.

Panel A: The relative mRNA expression levels of the TP α and TP β in THP-1 cells were measured by qRT-PCR, where levels of TP α and TP β mRNA were normalised using relative *18s rRNA* expression levels. Results are presented as mean relative TP α or TP β mRNA expression ($\pm$ SEM; $n \geq 3$), calculated using the $\Delta\Delta C_t$ method. The asterisks (*) show that there were significantly higher levels of TP α than TP β mRNA in the THP-1 cells ($p < 0.001$). **Panel B:** THP-1 cells were treated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 3 days followed by 5 days in the absence of PMA to induce monocyte-to-macrophage differentiation. Data is presented as mean changes in TP α or TP β mRNA expression following stimulation with PMA for 3 and 8 days relative to those levels in undifferentiated THP-1 cells (Day 0), assigned a value of 1 ($n = 3$; $\pm$ SEM). All data was normalised relative to *18s rRNA* levels. The asterisks (*) indicate that the fully-differentiated THP-1-derived macrophages (Day 8) showed significant increases in TP α and TP β mRNA expression, compared to the undifferentiated THP-1 monocytes (Day 0; $p < 0.05$). **Panel C:** THP-1 cells, seeded onto poly-L-lysine coated coverslips (2×10^5 cells/35 mm well), were treated with 200 nM PMA for 3 days followed by 5 days in the absence

of PMA to induce monocyte-to-macrophage differentiation. Cells were fixed at the indicated timepoints and immunolabelled, under permeabilising conditions, with anti-TP α or anti-TP β isoform-specific antibodies (5 μ g/ml in 1% BSA-TBS), followed by detection with AlexaFluor® 594 (green) and counterstaining with DAPI (1 μ g/ml; blue). Images were captured at 63X magnification using a Carl Zeiss Laser Scanning LSM 510 UVMETA microscope and Zeiss LSM imaging software. Scale bars (10 μ m) are shown on the right of each image. The upper panels show anti-TP α and anti-TP β immunostaining (green) while lower panels of each image show the overlay with DAPI nuclear staining (blue). Data presented is representative of at least three independent experiments ($n \geq 3$).

Figure 4. Effect of siRNA- disruption of FOXP1 on TP α and TP β expression in THP-1 cells.

Panel A: Immunoblot analysis of FOXP1 expression in THP-1 cells (30 μ g whole cell lysate/lane) following transfection for 48 hr with siRNA_{FOXP1} or siRNA_{CONTROL} (50 nM each). The bar chart (ii) shows the mean relative level of FOXP1 expression in THP-1 cells, as determined by quantitative densitometry, where levels in siRNA_{CONTROL}-transfected cells are assigned a value of 100% ($n = 3$; $\pm$ SEM). ##### signifies that transfection with siRNA_{FOXP1} resulted in significant knockdown of FOXP1 expression in THP-1 cells, relative to siRNA_{CONTROL} ($p \leq 0.0001$), where the mean/nett knockdown was 57.6%. **Panel B:** THP-1 cells were transfected for 48 hr with siRNA_{FOXP1} or siRNA_{CONTROL}, prior to real-time quantitative reverse transcriptase (qRT)-PCR analysis of TP α and TP β mRNA expression. Data is presented as mean changes in TP α or TP β mRNA expression relative to levels in siRNA_{CONTROL}-transfected cells, set to a value of 1 ($\pm$ SEM; $n = 3$). All data was normalized relative to 18s rRNA levels. **** indicates that siRNA-mediated disruption of FOXP1 expression significantly increased TP β mRNA levels in THP-1 cells ($p \leq 0.0001$; $n = 3$).

Figure 5. Role of FOXP1 cis-elements in regulating Prm3-directed expression in THP-1 cells.

Panels A – C: THP-1 cells were co-transfected with pRL-TK (25 ng) along with the recombinant pGL3Basic plasmids (2 μ g) encoding Prm3, its 5' deletion subfragments Prm3a (-404), Prm3ab (-320) and Prm3aab (-154), its mutant variants Prm3^{FOXP1^{#1}} (-1298), Prm3^{FOXP1^{#2}} (-943), Prm3^{FOXP1^{#3}} (-597) and Prm3^{FOXP1^{#4}} (-496) or as control, with the empty pGL3Basic (pGL3B) vector. Firefly and renilla luciferase activity was measured at 48 hr post-transfection. **Panel A:** Results are expressed as mean firefly relative to renilla luciferase activity (RLU $\pm$ SEM; $n \geq 3$). The asterisks (*) show that Prm3-directed reporter gene expression in THP-1 cells at levels that were significantly higher than those directed by the empty pGL3Basic vector ($p < 0.0001$). **Panel B:** Schematic of the human *TBXA2R* genomic region, spanning nucleotides -8500 to +786, encoding promoter (Prm)1 and Prm3 located 5' of exon (E)1 and E2, respectively, is shown where nucleotide +1 corresponds to the translational start site (ATG). Results are presented as mean firefly relative to renilla luciferase activity (RLU), expressed as a fold change in RLU where the full-length Prm3 (-1394 to +1) was assigned a value of 1 ($\pm$ SEM; Data $n \geq 3$). Asterisks ** indicates that 5' deletion of Prm3 sequences to generate Prm3a significantly increased luciferase reporter gene expression in THP-1 cells ($p \leq 0.01$). **Panel C:** A schematic of Prm3 shows the position of the FOXP1^{#1} (at -1298), FOXP1^{#2} (at -943), FOXP1^{#3} (at -597) and FOXP1^{#4} (at -496) cis-elements, where the number given refers to the 5' nucleotide of each cis-acting element and the star symbol indicates the mutated elements. Results are presented as mean fold change of luciferase activity (RLU) directed by the wild-type Prm3, assigned a value of 1 ($\pm$ SEM; Data $n = 3$). Mutation of the FOXP1 elements at -1298, -943 -597 and -496 resulted in significant increases in levels of Prm3-directed expression in THP-1 cells ($p \leq 0.05/0.0001$, respectively), where * and **** signify $p \leq 0.05$ and 0.0001, respectively.

Figure 6. Chromatin immunoprecipitation (ChIP) analysis of FOXP1 binding to Prm3 in THP-1 monocytes.

Panel A: (i) A schematic of the region spanning -8500 to +1 of the *TBXA2R* showing the relative positions of the promoter (Prm) 1 and Prm1 that transcriptionally regulate TP α and TP β , respectively, where the nucleotide positions of the four FOXP1 cis-elements, designated FOXP1^{#1}, FOXP1^{#2}, FOXP1^{#3} and FOXP1^{#4}, within Prm3 are also indicated. The arrows show the primers used to amplify the test regions of Prm3 surrounding either the putative FOXP1^{#2} site (nucleotides -1032 to -835; black arrows) or the adjacent FOXP1^{#3} and FOXP1^{#4} sites (nucleotides -634 to -355; light grey arrows), as indicated. As a control, primers were used to amplify a non-specific region of (i) Prm1 (nucleotides -7718 to -7610 of the *TBXA2R*; white arrows) or (ii) *GAPDH* (nucleotides -810 to -705; dashed arrows).

Panel B: For chromatin immunoprecipitation (ChIP) analysis of FOXP1 binding to Prm3 in THP-1 cells, chromatin extracted from anti-FOXP1, control/non-immune mouse immunoglobulin (Ig) G or no primary antibody (-AB) control immunoprecipitates, was subjected to real time quantitative PCR (qRT-PCR) analysis. Primers were used to amplify the test regions of Prm3 surrounding either the FOXP1^{#2} element (black bars), or the FOXP1^{#3} and FOXP1^{#4} elements (grey bars), or the control regions of Prm1 (pattern bars), or *GAPDH* (white bars), as indicated in the graph. Results are presented as relative levels of PCR amplicons generated from the anti-FOXP1 or control immunoprecipitates, expressed as a percentage of those derived from the corresponding input chromatin (Data $n = 3$; $\pm$ SEM). The asterisks (*) indicate that significant levels of FOXP1 were found associated with the test regions of Prm3 in the anti-FOXP1 chromatin immunoprecipitates in THP-1 cells compared to the IgG or -AB controls, where *** and **** signify $p \leq 0.001$ and 0.0001 , respectively.

Panel C: Agarose gel electrophoresis of PCR products derived from THP-1 chromatin extracted from anti-FOXP1, normal mouse IgG or no 1° antibody (-AB) immunoprecipitates, or the corresponding input controls. For ChIP analysis of FOXP1 binding to Prm3, PCR primers were used to amplify the test regions of Prm3 surrounding the FOXP1^{#2} cis-binding element (at -943), or the FOXP1^{#3} and FOXP1^{#4} elements (at -597 and -496, respectively) or the control Prm1 region of the *TBXA2R* or control region of the *GAPDH* gene, as indicated. Data shown are representative of three independent experiments ($n = 3$).

Figure 7. Proposed model of FOXP1-mediated transcriptional regulation of Prm3-directed TP β expression during monocyte-to-macrophage differentiation.

In the undifferentiated THP-1 monocytes, the forkhead transcription factor FOXP1 binds to four *cis*-acting regulatory elements (forkhead binding sites/FHBS) within promoter (Prm)3 of the *TBXA2R* gene, keeping TP β mRNA and protein expression in a transcriptionally repressed state. The four forkhead *cis*-elements or binding sites (FHBS) are designated as FHBS^{#1} (at -1298), FHBS^{#2} (at -943), FHBS^{#3} (at -597) and FHBS^{#4} (at -496). Phorbol-12-myristate-13-acetate (PMA)-induced differentiation of the monocytic THP-1 cell line to the fully differentiated macrophage phenotype coincides with a decrease in the expression of FOXP1 and with a concomitant increase in TP β mRNA and protein expression. In turn, increased expression of TP β within regions of chronic inflammatory infiltration within the stromal, peri-glandular or glandular microenvironment may drive autocrine or paracrine signalling by the TXA₂-TP β signalling axis, such as within the intra-tumoral regions of prostate to further augment the role of TP β in PCa. It is therefore proposed that down-regulation of FOXP1 expression during monocyte-to-macrophage differentiation results in a loss of FOXP1 binding to its *cis*-acting elements within Prm3 which, in turn, leads to increased Prm3-directed expression of TP β in the fully differentiated macrophage lineage. Hence, FOXP1 plays a key role in the transcriptional repression of TP β in monocytes which is lifted in the differentiated macrophages allowing for upregulation of TP β expression, and possibly accounting for the prominent expression of TP β in the tissue-resident macrophages found within the prostate tissue microenvironment.

SUPPLEMENTAL DATA

Supplemental Table 1: Primary antibodies used for immunohistochemical (IHC) analysis

Primary Antibody [†]	Optimal Concentration*	Incubation	Secondary Antibody ^{\$}
Affinity purified rabbit <i>anti</i> -TP α	12 μ g/ml	Overnight at 4 °C	Biotinylated <i>anti</i> -rabbit IgG (1:500)
Affinity purified rabbit <i>anti</i> -TP β	1.3 μ g/ml	Overnight at 4 °C	Biotinylated <i>anti</i> -rabbit IgG (1:500)
Monoclonal mouse <i>anti</i> -CD3	2.82 μ g/ml (1:50)	30 min at room temperature	Biotinylated <i>anti</i> -mouse IgG (1:500)
Monoclonal mouse <i>anti</i> -CD4	7.36 μ g/ml (1:25)	30 min at room temperature	Biotinylated <i>anti</i> -mouse IgG (1:500)
Monoclonal mouse <i>anti</i> -CD8	1.2 μ g/ml (1:150)	30 min at room temperature	Biotinylated <i>anti</i> -mouse IgG (1:500)
Monoclonal mouse <i>anti</i> -CD68	0.2 μ g/ml (1:150)	30 min at room temperature	Biotinylated <i>anti</i> -mouse IgG (1:500)
Monoclonal mouse <i>anti</i> -CD20cy	0.3 μ g/ml (1:600)	30 min at room temperature	Biotinylated <i>anti</i> -mouse IgG (1:500)

[†] The affinity-purified isoform-specific *anti*-TP α and *anti*-TP β antibodies used for detection of TP α and TP β expression have been previously described and extensively characterised [31, 45]. Monoclonal mouse *anti*-CD3, *anti*-CD4, *anti*-CD8, *anti*-CD20 and *anti*-CD68 antibodies were obtained from Dako (Agilent Technologies, Santa Clara, California, USA).

* The optimal concentration of each primary antibody used for immunohistochemical staining is given.

^{\$} Tissue sections were incubated for 30 min with a biotinylated *anti*-rabbit or *anti*-mouse immunoglobulin (Ig) G secondary antibody from Santa Cruz Biotechnology® (Santa Cruz, California, USA), as indicated.

Supplemental Figure 1. TP α and TP β expression in histological patterns of chronic prostatic inflammation.

Panel A-C: Representative IHC images of TP α and TP β expression in histological areas of chronic prostatic inflammation in (A) peri-glandular, (B) glandular and (C) intra-tumoral are shown. In total, 15 full-face prostate tissue specimens were screened with affinity-purified *anti-TP α* (12 μ g/ml) or *anti-TP β* (1.3 μ g/ml) isoform-specific antibodies, as indicated, where matched serial sections were used throughout as a means of comparing expression between slides. Sections were counterstained with haematoxylin. Images were captured at 200X magnification using the Aperio® ScanScope XT and supporting ImageScope V12.2 software (left-hand *Panels*). The region of inflammation that is boxed in the left-hand panels is depicted at higher magnification (1000X) in the corresponding right-hand panels.

Supplemental Figure 2. IHC analysis of TP α and TP β expression in human kidney, urinary bladder and testes, including in associated regions of chronic inflammation.

Representative IHC images of TP α and TP β expression in tissue sections from human kidney, urinary bladder and testes and in histologically defined areas of chronic prostatic inflammation. Representative full-face tissue slices (4 μ m) were screened with affinity-purified *anti*-TP α (12 μ g/ml) or *anti*-TP β (1.3 μ g/ml) isoform-specific antibodies, as indicated, where matched serial sections were used throughout as a means of comparing expression between slides. Sections were counterstained with haematoxylin. Images were captured at 200X magnification using the Aperio® ScanScope XT and supporting ImageScope V12.2 software. Prominent expression of TP β , but not of TP α , within regions of inflammation in the human kidney, urinary bladder and testes tissue sections were evident throughout the full-face sections. For each tissue, two representative images for TP α and TP β expression are shown.

Supplemental Figure 3. Confirmation of specificity of antibodies to CD cell surface markers.

Serial sections of human tonsil were screened with antibodies directed to a range of leukocyte-specific cluster of differentiation (CD) cell marker proteins, including CD3⁺ (T lymphocyte), CD4⁺ (T-helper cell), CD8⁺ (cytotoxic T cell), CD20⁺ (B lymphocyte) and CD68⁺ (macrophage). All sections were counterstained with haematoxylin. Images were captured at 200X magnification using the Aperio® ScanScope XT and supporting ImageScope V12.2 software. In all cases, two representative images for the specific antibodies are shown.

Supplemental Figure 4. Correlation of TP α & TP β expression with immune cell markers in inflammatory infiltrates.

Panel A-E: The extent of expression of TP α , TP β and of each of the specific CD marker proteins (CD3, CD4, CD8, CD20 and CD68) in the inflammatory cell infiltrates present in the 15 prostate tissue specimens was scored based on the proportion of cells staining positive (DAB, brown) relative to the total number of cells within an infiltrate as follows: 0, negative, no staining; 1, up to 20 % positively stained cells; 2, up to 40 % positively stained cells; 3, up to 60 % positively stained cells; 4, up to 80 % positively stained cells and 5, up to 100 % positively stained cells. Scores for each of the 15 prostate cases were calculated as the mean of the scores for the infiltrates within each tissue specimen with correlation and linear regression analyses between either of TP α or TP β and with each of the individual CD markers shown in **Panels A-E**. The best-fit linear regression is displayed as a solid line on each correlation plots and Pearson's correlation coefficients, 95% confidence intervals (CI), best-fit linear regression R-squared (R²) values, and two-tailed correlation P values are presented for each statistical comparison. P values ≤ 0.05 were considered to indicate statistically significant correlations, and where *, ** and **** denotes $p \leq 0.05$, 0.01 and 0.0001, respectively.

Supplemental Figure 5. Down-regulation of FOXP1 expression during THP-1 monocyte-to-macrophage differentiation.

Panels A & B: Influence of phorbol-12-myristate-13-acetate (PMA) -induced differentiation on FOXP1 expression in THP-1 cells. To induce differentiation towards a macrophage-like phenotype, THP-1 cells were incubated with PMA (200 nM) for 3 days, followed by incubation in the absence of PMA for a further 5-day period. Cells were harvested every 24 hr and aliquots (10 μ g whole cell lysate) were resolved by SDS-PAGE and immunoblotted with anti-FOXP1 antibody. Membranes were re-screened for HDJ2 expression to confirm uniform protein loading. Data presented are representative of three independent experiments ($n = 3$). The bar chart in **Panel B** shows the mean relative level of FOXP1 expression (85 kDa isoform) in THP-1 cells, as determined by quantitative densitometry, where levels in the undifferentiated cells (Day 0) are assigned a value of 100% ($n = 3$; $\pm$ SEM). The asterisks (*) show that FOXP1 expression levels were significantly down-regulated in the fully-differentiated THP-1-derived macrophages (Day 7 & 8), compared with the undifferentiated THP-1 monocytes (Day 0), where ** and *** represent $p \leq 0.01$ and 0.001, respectively.