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Increased T cell infiltration elicited by *Erk5* deletion in a *Pten*-deficient mouse model of prostate carcinogenesis

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Abstract

Prostate cancer (PCa) does not appear to respond to immune checkpoint therapies where T cell infiltration may be a key limiting factor. Here we report evidence that ablating the growth regulatory kinase *Erk5* can increase T cell infiltration in an established *Pten*-deficient mouse model of human PCa. Mice that were doubly mutant in prostate tissue for *Pten* and *Erk5* (prostate DKO) exhibited a markedly increased median survival with reduced tumor size and proliferation compared to control *Pten*-mutant mice, the latter of which exhibited increased *Erk5* mRNA expression. A comparative transcriptomic analysis revealed upregulation in prostate DKO mice of the chemokines *Ccl5* and *Cxcl10*, two potent chemoattractants for T lymphocytes. Consistent with this effect, we observed a relative increase in a predominantly CD4⁺ T cell infiltrate in the prostate epithelial and stroma of tumors from DKO mice. Collectively, our results offer a preclinical proof of concept for ERK5 as a target to enhance T cell infiltrates in prostate cancer, with possible implications for leveraging immune therapy in this disease.

Keywords

Erk5; Pten; Prostate Cancer; Cytokines; T Cells

Introduction

Prostate cancer (PCa) is a significant public health problem. In males worldwide, PCa is second in incidence to skin cancer and it is the second most common cause of cancer related deaths in men after lung cancer in the United States and the UK (1,2). Recent progress has been made in the treatment of PCa, through the development of novel inhibitors of the androgen receptor pathway and chemotherapy, which have led to improved patient survival. However, advanced PCa remains incurable, indicating a clear need to better understand the

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molecular events associated with disease progression to facilitate the development of novel therapies.

ERK5 (MAPK7) is a member of the mitogen-activated protein kinase (MAPK) family. MAPKs function to regulate and integrate cellular signalling cascades in order to control multiple intracellular events, including cell proliferation, differentiation, migration and survival. ERK5 is activated in response to extracellular stimuli, including mitogens and cellular stress, via sequential activation of MEK2/3 and then MEK5 which is the sole known MAPK kinase (MAP2K) of ERK5. Phosphorylation of ERK5 by MEK5 in turn causes translocation of ERK5 to the nucleus, where it can regulate transcription of its target genes. The uniquely large C-terminus of ERK5 also possesses a transcriptional activation domain and contains the nuclear localisation and export signals required for dynamic shuttling of ERK5. Despite its unique domain, it is noteworthy that the N-terminus of ERK5 shares ~66% sequence homology with ERK1/2 (3–6).

The ERK5 pathway has a critical role in development, as demonstrated by the identical embryonic lethal phenotype of *Mek5* and *Erk5* knockout mice due to cardiovascular defects (7–10). Furthermore, ERK5 has been implicated in endothelial cell function (9,11), skeletal muscle cell fusion (12), neuronal cell differentiation (13), the survival and proliferation of immune cells (14,15) and carcinogenesis (16). *In vivo* and clinical data suggest that aberrant or altered ERK5 signalling is implicated in skin cancer (17), colorectal cancer (18), squamous cell lung and esophageal cancer (19). *Erk5* is amplified in hepatocellular carcinoma (20) and is considered a potential therapeutic target in triple negative breast cancer (21).

Previous studies have demonstrated that MEK5 and ERK5 signalling are upregulated in PCa, and associated with metastases and reduced patient survival (22–24). Therefore, we investigated the *in vivo* role of *Erk5* in a genetically engineered mouse (GEM) model of PCa. In the *Pbsn* (PB)-Cre4:*Pten*^{fl/fl} (*Pten* null) (herein referred to as *Pten*^{fl/fl}) model of PCa (25,26), we show for the first time that *Erk5* deletion delayed PCa formation, extending the survival of these mice and reducing tumour size. We also observed elevated *Ccl5* and *Cxcl10* expression, along with enhanced tumoral T-cell infiltration.

Materials and Methods

Mouse strains and breeding

Pten^{fl/fl} [*Pbsn* (PB)-Cre4:*Pten*^{fl/fl} (*Pten* null)] mice (25,26) were intercrossed with mice harbouring the conditional inactivatable *Erk5* allele (where Exon 4 is flanked by LoxP sites) (27) to generate double mutant *Pten*^{fl/fl} *Erk5*^{fl/fl} (PB-Cre4:*Pten*^{fl/fl} *Erk5*^{fl/fl}) mice. Mice were genotyped by PCR by Transnetyx™. Mice were of a mixed background (C57BL/6J) with strain-matched littermates (either Cre^{+/ve} WT or mice not expressing Cre) used as control mice. Mice were taken at specific time points or aged until end point, which was defined as two of the following 3 criteria being met: palpable tumour; evidence of blood in the urine; mouse becoming thin/weight loss and ‘hunched’. All small animal (murine) experiments were approved by the Animal Care and Use Committee at the University of Glasgow.

Sample processing and histological analysis

Samples for histological analysis were fixed in 10% formalin for 48 hr, then transferred to 70% ethanol before being processed. Murine prostates were divided into two halves, with one half fixed and processed for histology while the other half was snap frozen by placing on dry ice for storage at -80°C. Routine haematoxylin and eosin staining was performed on formalin-fixed, paraffin-embedded (FFPE) prostate samples.

Immunohistochemistry (IHC)

IHC for Ki67 and CD3 was performed on FFPE sections from a minimum of three individual murine prostate samples for each genotype on Dako Autostainer. Briefly, samples were de-waxed in Xylene then re-hydrated through graded alcohols to dH₂O. Antigen retrieval was performed using sodium citrate (pH6) buffer in a PT module (Thermo Fisher Scientific) at 98°C for 25 min (Ki67, CD45, CD3). Slides were then washed in Tris-buffered TWEEN (TbT) prior to incubation in 3% H₂O₂ for 5 minutes to block endogenous peroxidases. Next, after washing in TbT, slides were either blocked with ImmPRESS normal goat blocking serum (Vector Laboratories) for 30 min then directly incubated with appropriate primary antibody for 35 min. Following antibody incubation, slides were washed twice with TbT then staining was visualised using DAKO envision HRP-tagged secondary antibodies and DAB chromogen kit. Slides were counterstained with Haematoxylin Z prior to mounting in DPX media. Antibodies used were: Ki67 (Thermo Fisher Scientific, (SP6) MA5-14520, 1:100) and CD3 (Abcam (SP7), ab16669, 1:50).

In situ hybridization (ISH)

Single ISH detection for *Erk5* (Advanced Cell Diagnostics (ACD) Probe: 433911) was performed manually using RNAscope® 2.5 HD reagent kit (brown) (ACD, 322310) according to manufacturer's instructions. Single ISH detection for *Ccl5* (ACD Probe: 469608), *Cxcl10* (ACD Probe: 408928), *Ppib* (ACD Probe: 313911) and *Dapb* (ACD Probe: 310043) was performed using RNAscope® 2.5 LS reagent kit (brown) (ACD, 322100) on the Lecia Bond Rx Autostainer according to manufacturer's instructions. For all single stains, positive staining was indicated by brown punctate dots present in the nucleus and/or cytoplasm.

Dual ISH detection for *Cd4* (fast red) and *Cd8* (DAB) mRNA was performed by an automated method using RNAscope® 2.5 HD Duplex Assay Kit (ACD, 322440) according to the manufacturer's instructions on the Lecia Bond Rx Autostainer. Positive staining was indicated by red and brown punctate dots present in the nucleus and/or cytoplasm.

Microscopy

Light microscopy was carried out using the Olympus BX51 microscope using Olympus UPlanFLN UIS2 10x and 40x objectives, Olympus DP71 camera and Cell^F software or Zeiss Axio Imager.A1 microscope using Zeiss EC Plan-NEOFLUAR 10x and 40x objectives, Zeiss AxioCam 10S camera and ZEN software.

Image analysis of IHC and ISH staining

Automated scoring of Ki67 IHC staining was performed using an algorithm designed within SlidePath's (Leica Biosystems) Tissue IA system to count nuclear Ki67 positive cells in scanned 'virtual' slides (20x magnification). Prior to image analysis, 12 representative regions of prostate epithelium per slide were annotated. A minimum of 3 slides per genotype being studied were assessed. All annotated regions on each slide were subsequently submitted for batch image analysis. The image analysis results for these slides were exported to Microsoft Excel. The overall % Ki67 for each sample was calculated by dividing the total number of Ki67 positive cells identified in the 12 annotated areas by the total number of cells identified in the 12 annotated areas on each slide.

For scoring of *Ccl5* and *Cxcl10* RNA ISH (RNA Scope), an algorithm within HALO software was designed and used to count the number of probe copies (dots) and number of cells in annotated areas of scanned slides (40x magnification). A minimum of 12 representative areas of prostate epithelium per slide were annotated and 4 (*Pten^{fl/fl}*) or 5 (*Pten^{fl/fl} Erk5^{fl/fl}*) slides were assessed for each genotype. The image analysis results for these slides were exported to Microsoft Excel, which included a value for the average number of probe copies per cell across the entire areas studied.

For epithelial CD3 scoring, SlidePath software was used to design an algorithm to automatically count the number of cells in annotated areas of scanned slides (20x magnification). Again, 12 representative regions of prostate epithelium per slide were annotated and 3 slides per genotype were assessed. The number of CD3 positive cells within each annotated area was counted manually. The % of CD3 positive cells for each sample was calculated by dividing the total number of CD3 positive cells in the 12 annotated areas by the total number of cells identified by the software in the 12 annotated areas on each slide.

For stromal CD3 scoring, lymphoid aggregate areas where intense CD3 staining was present were annotated and their area calculated using SlidePath Software in scanned slides (20x magnification). The total area of prostate present on each slide was also evaluated using Leica software. The total combined area of stained lymphoid aggregates on an individual slide per total prostate area on that slide was calculated for each sample. 3 slides were evaluated for each genotype.

Deconvolution of *Cd4/Cd8* dual ISH (RNA Scope) staining in scanned slides (40x magnification) was performed using HALO software.

Laser capture microdissection (LCM)

Frozen sections of mouse prostate tissue were cut using a cryostat (2 x 20 µm sections per slide) and put onto Leica FrameSlides (PET-membrane) – 5 slides were prepared for each sample; 3 independent samples for each genotype. Slides were stored at -80°C until needed.

Prior to LCM, slides were stained with cresyl violet immediately after being taken out of -80°C. The staining procedure was as follows: 70% ethanol (EtOH) (30 sec), 50% EtOH (30 sec); Cresyl violet (1% in 50% EtOH, filtered) (45 sec), wash twice in DEPC-treated dH₂O, 50% EtOH (30 sec), 70% EtOH (30 sec), 95% EtOH (30 sec), 100% EtOH (30 sec), 100%

EtOH (30 sec). N.B. 1x RNase Inhibitor (Sigma) was added to all solutions after the cresyl violet stain.

LCM was carried out using Leica LMD6000 Laser Microdissection System Z6018. Captured prostate epithelial material was collected into the lids of 0.5 ml Eppendorf tubes containing 20 µL RLT lysis buffer (Qiagen). After cutting (for a maximum of 30 min per slide to minimise RNA degradation), a further 30 µL RLT lysis buffer [containing β-mercaptoethanol (1%)] was added to the lysates before they were stored at -80°C until needed.

RNA isolation and quantitation

For LCM samples, RNA was isolated using RNeasy Micro Kit (Qiagen) as per the manufacturer's instructions including a DNase treatment step. RNA was then quantified using Qbit Assay (Invitrogen) according to the manufacturer's instructions, and quality assessed by running on 2100 Bioanalyzer (Agilent) to generate RNA electropherograms, with calculation of RNA integrity number (RIN) (range 7-10).

For non-LCM samples, RNA was isolated using RNeasy Mini Kit (Qiagen) as per manufacturer's instructions including a DNase treatment step. RNA samples were quantified by spectrophotometry using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

RNA sequencing (RNA-Seq) and bioinformatic analysis

200 ng LCM RNA samples (3 samples for each genotype) were processed and libraries prepared for sequencing using the TruSeq Stranded mRNA kit (Illumina, RS-122-2101) with Poly(A) selection according to the manufacturer's instructions. The amplified libraries were sequenced on the Nextseq 500 (Illumina) with a paired-end sequencing strategy. The read length was set at 75 bp with an expected total library size of ~330 bp. The mean number of reads per sample was over 30 million.

Quality checks on the raw RNASeq data files were done using fastqc (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>) and fastq_screen (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen). RNASeq reads were aligned to the GRCm38 (28) version of the mouse genome using tophat2 version 2.0.10 (29) with Bowtie version 2.1.0 (30). Expression levels were determined and statistically analysed by a combination of HTSeq version 0.5.4p3 (<http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html>), the R 3.1.1 environment, utilizing packages from the Bioconductor data analysis suite and differential gene expression analysis based on a generalized linear model using the DESeq2 (31). Pathway analysis of genes with a fold change >1.5 and an adjusted P value <0.05 was performed using GeneGo Pathways Software (MetaCore; <https://portal.genego.com/version6.22.67265>).

Quantitative real-time PCR (QPCR)

First strand cDNA was prepared from RNA samples using the High Capacity cDNA Transcription Kit (Applied Biosystems). QPCR was performed to evaluate relative transcript expression levels in cDNA samples using the Taqman technique. Primers were designed

using the Universal Probe Library (UPL) Assay design centre (Roche). Specific primer/probe combinations (Table S7) and Taqman Universal PCR Mastermix (Applied Biosystems) were added to cDNA samples (according to the manufacturer's instructions) before being run on an Applied Biosystems 7500 Fast Real-Time PCR System machine. The QPCR programme conditions were: 20sec at 50°C, 10min at 95°C, followed by 40 cycles of 15sec at 95°C and 1min at 60°C. Technical replicates (2 or 3 wells) for each sample and three independent samples for each genotype/cell line were included. *Casc3* was used as the reference gene (chosen as it had similar expression level across various genotypes) and 7500 Software v2.0.5 (Applied Biosystems) used 2^{-CT} method to determine the relative gene expression.

Culture of human prostate cancer cell lines and generation and culture of murine tumour-derived cell lines

The human prostate cancer cell line, CWR22, was obtained from the laboratory of Dr Thomas Pretlow, Case Western University, Cleveland, Ohio in 2002. CWR22 cells were maintained in RPMI medium containing 10% FBS. These cells were last authenticated in-house using Promega GenePrint® 10 Kit, according to manufacturer's instructions, in January 2016 and had a >95% match with American Type Culture Collection (ATCC) and DSMZ (German collection of cell cultures) databases. All cell lines in the laboratory are routinely tested every 6 months for mycoplasma in-house using Lonza MycoAlert™ Mycoplasma Detection Kit, according to manufacturer's instructions. The length of time between thawing of cells and their use in the experiments described was within 6 weeks.

Tumour-derived cell lines from *Pten^{fl/fl}* (P1) and double mutant PB-Cre4:*Pten^{fl/fl} Erk5^{fl/fl}* (PE) prostate tumours were generated from a small piece of murine prostate tumour tissue. Prostate tumours taken at the point of dissection were dipped in 70% EtOH, rinsed with dH₂O then placed into DMEM (Gibco) medium containing 20% foetal bovine serum (FBS) (Gibco). The tumour tissue was chopped as finely as possible then incubated at 37°C for 30 min with the addition of collagenase (1 mg/ml) (Sigma) and hyaluronidase (2 mg/ml) (Sigma) to the medium at 37°C for 1hr. Tissue was then pelleted by centrifugation at 241 x *g* for 5 min prior to re-suspension in fresh DMEM medium containing 20% FBS and antibiotics [Penicillin/Streptomycin (1%) (Gibco) and gentamicin (33 µg/ml) (Sigma)]. The following day, adherent cells were washed with PBS then cells were maintained in DMEM medium containing 10% FBS N.B. antibiotics were removed after 1 week of culture.

Statistical analysis

All statistical analyses (namely Mann Whitney, Pearson correlation coefficient, t-test and Kaplan Meier survival analysis) were performed using GraphPad Prism v5.0c. For all graphs, mean ± SEM (error bars) are presented.

Results

Erk5 deletion extended the survival of *Pten* null mice and reduced prostate tumour weight

Consistent with published literature implicating elevated ERK5 signalling in tumorigenesis, recent TCGA sequencing data (32) reported on cBioportal (33,34) demonstrate altered

(predominantly up-regulated) *Mek5* and *Erk5* status in around 11% of 333 human PCa cases (Figure 1A). Therefore, we sought to investigate whether ablating *Erk5* expression in the prostate could have an anti-tumorigenic effect *in vivo* using a murine model of PCa. The *Pten*^{fl/fl} model (26) is widely used in PCa research. *Pten*^{fl/fl} mice develop pre-neoplastic prostatic intraepithelial neoplasia (PIN) at around 3 months of age, followed by slow progression to prostate adenocarcinoma at >10 months, with no appreciable evidence of metastases up to 18 months of age (35).

We targeted ablation of *Erk5* expression to the prostate by crossing mice harbouring the conditional inactivatable *Erk5* allele (where Exon 4 is flanked by LoxP sites) (27) to *Pten*^{fl/fl} mice to generate double mutant PB-Cre4:*Pten*^{fl/fl} *Erk5*^{fl/fl} (herein referred to as *Pten*^{fl/fl} *Erk5*^{fl/fl}) mice. Analysing isolated prostatic epithelium by LCM from end point mice, QPCR assay for *Erk5* confirmed significantly reduced *Erk5* expression in prostates null for *Erk5* (PB-Cre4:*Erk5*^{fl/fl}, herein referred to *Erk5*^{fl/fl}) compared to WT and in *Pten*^{fl/fl} *Erk5*^{fl/fl} compared to *Pten*^{fl/fl} prostates (Figure 2C, left). Similarly, we found reduced expression of *Pten* mRNA in *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostates compared to WT (Figure 2C, right).

Compared to *Pten*^{fl/fl} mice, *Pten*^{fl/fl} *Erk5*^{fl/fl} mice have significantly prolonged median survival (Figure 1B, Table S1). At study end point (*Pten*^{fl/fl}: age range 9-15 months, average 11.54 months; *Pten*^{fl/fl} *Erk5*^{fl/fl}: age range 9.5-15.5 months, average 14.67 months), prostate tumours in *Pten*^{fl/fl} mice looked similar to our previous report (35) whereas prostate tumours in double mutant *Pten*^{fl/fl} *Erk5*^{fl/fl} mice were visibly smaller in appearance compared to *Pten*^{fl/fl} control mice (Figure 1C). Furthermore, throughout the study period at 3 and 9-10 months of age and at end point, *Pten*^{fl/fl} *Erk5*^{fl/fl} prostates weighted significantly less than those of the *Pten*^{fl/fl} control mice (Figures 1D-F, Tables S2-S4).

***Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours were less proliferative than *Pten*^{fl/fl} prostate tumours**

To further investigate the phenotypes of isolated prostates from *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} mice, histopathological analysis was carried out. Significantly, no adverse prostate phenotype was observed in *Erk5*^{fl/fl} mice – these prostates had similar histology to WT control mice (Figure 2A). At 3 months of age, prostates from both *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} mice developed pre-neoplastic PIN lesions, with intact glandular structures (Figure S1A). By 9-10 months of age (Figure S1B) and at study end point (Figure 2A), adenocarcinoma were present in both *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostates with glandular invasion into the surrounding stroma and merging of glands. There was no evidence of proliferative inflammatory atrophy in any of the samples studied.

At the three time points studied, *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours are highly proliferative, as evidenced by Ki67 immunohistochemistry (IHC) staining [Figures S1A (3 months), S1B (9-10 months) and 2A (end point)]. Although we did not see a difference in the percentage of Ki67 positive cells at 3 months in *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostates (Figure S1C), importantly at 9-10 months (Figure S1D) and study end point (Figure 2B), we observed significantly fewer Ki67 positive cells in *Pten*^{fl/fl} *Erk5*^{fl/fl} when compared to *Pten*^{fl/fl} prostate tumours. Collectively, double mutant *Pten*^{fl/fl} *Erk5*^{fl/fl} mice survived longer and harboured smaller prostate tumours with reduced proliferation than *Pten*^{fl/fl} mice.

***Erk5* Expression is increased in *Pten*^{fl/fl} prostate tumours compared to WT prostate**

Interestingly, when analysing LCM isolated prostatic epithelium by QPCR for *Erk5* expression, we discovered that *Erk5* expression is significantly increased in *Pten*^{fl/fl} prostate tumours compared to WT prostate at end point (Figure 2C). As an additional confirmation of this result, *In-situ* hybridisation (ISH) analysis using RNA scope for *Erk5* mRNA was performed and we saw increased ISH signal for *Erk5* in *Pten*^{fl/fl} prostate tumours compared to WT prostate (Figure 2D).

Elevated expression of the cytokines *Ccl5* and *Cxcl10* in *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours

To gain insight into the molecular differences between *Pten*^{fl/fl}, and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours, RNA-Seq was carried out using RNA samples prepared from prostatic epithelium isolated by LCM from the respective end point tumours (n=3 for each genotype). Bioinformatic analysis of the data revealed that the expression of 70 genes was significantly altered (p 0.05) between *Pten*^{fl/fl} *Erk5*^{fl/fl} and *Pten*^{fl/fl} prostate tumours. Furthermore, 67 of these genes had >1.5 fold change in expression (Figure 3A). Among these genes, pathways relating to inflammatory responses, immune responses and regulation of cytokine and chemokine expression were over-represented (Table S6).

Besides being members of the over-represented pathways, the cytokines Chemokine (C-C motif) ligand 5 (*Ccl5*) (Rantes) and C-X-C motif chemokine 10 (*Cxcl10*) were among the most highly up-regulated genes in *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours (Figure 3A, Table S5). Using prostate epithelial RNA samples prepared using LCM, we found a trend for increased *Ccl5* and *Cxcl10* expression at 9-10 month time point in *Pten*^{fl/fl} *Erk5*^{fl/fl} compared to *Pten*^{fl/fl} prostate tumours by QPCR (Figure 3B). At end point, *Cxcl10* expression was significantly upregulated in the prostates of *Pten*^{fl/fl} *Erk5*^{fl/fl} mice, along with a trend for increased *Ccl5* expression (Figure 3C). Furthermore, in cell lines derived from *Pten*^{fl/fl} (P1) and *Pten*^{fl/fl} *Erk5*^{fl/fl} (PE) prostate tumours, which were validated to lack expression of *Pten* and both *Pten* and *Erk5* respectively (Figure S2A and S2B), we observed a dramatic increase in *Ccl5* and *Cxcl10* mRNA expression in PE compared to P1 cells (Figures 3D).

CCL5 (rantes) and CXCL10 are members of the CC and CXC cytokine families respectively. They are produced by a wide range of cell types, including endothelial cells, epithelial cells and fibroblasts, in response to stimulation by CD40L, IL-15 (for CCL5) and IFN γ (for CXCL10). CCL5 and CXCL10 are potent chemo-attractants for T lymphocytes upon binding to the appropriate cell surface receptors on T cells: CCR5 for CCL5 and CXCR3 for CXCL10 (36,37). *In situ* hybridisation revealed upregulated expression of *Ccl5* and *Cxcl10* mRNA in end point *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} tumours (Figure 3E), with near-significant increase and significant increase in the number of probe copies per cell within the malignant epithelium respectively (Figure 3F), which is in keeping with QPCR data (Figure 3C). IHC staining was also performed to examine the pattern of expression of CCL5, CXCL10 and their cognate receptors, CCR5 and CXCR3 respectively (Figure S2C). CCL5 and CXCL10 immunoreactivity was elevated in *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours, and in infiltrating immune cells in the stroma. While CCR5 expression was largely negative in *Pten*^{fl/fl} prostate tumours, patchy CCR5 immunoreactivity was detected in *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate epithelium and immune cells in the stroma. CXCR3 expression was also

identified in tumour epithelium and immune cells present in the stroma in *Pten^{fl/fl} Erk5^{fl/fl}* prostates.

CD3 positive cells within the epithelium and stromal aggregates were elevated in *Pten^{fl/fl} Erk5^{fl/fl}* compared to *Pten^{fl/fl}* prostate tumours

To investigate the functional correlates of increased CCL5 and CXCL10 expression in the prostate, we studied T lymphocytes in *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours by IHC for CD45 (marker for general lymphocyte) and CD3 (a component of the T cell receptor complex). CD45 staining revealed areas of intense immunoreactivity in the stroma of both *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours at end point, indicating significant lymphoid infiltration (Figure S3A). Similar to CD45, areas of CD3 positive T cells were observed in the stroma (referred to herein as stromal lymphoid aggregates) as well as within the malignant epithelium at 9-10 month time point (Figure S1B) and at end point (Figure 4A) in both *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours. At end point, there were significantly more CD3 positive cells in the prostate tumour epithelium of *Pten^{fl/fl} Erk5^{fl/fl}* than in *Pten^{fl/fl}* samples (Figure 4B). Furthermore, CD3 positive T cells were also statistically more abundant in the tumour stroma of *Pten^{fl/fl} Erk5^{fl/fl}* than in *Pten^{fl/fl}* prostate tumour samples at study end point (Figure 4C). In keeping with these data, we observed a trend for increased CD3 immunoreactivity in both tumour epithelium and stroma at the earlier 9-10 month time point in *Pten^{fl/fl} Erk5^{fl/fl}* prostates when compared to the control *Pten^{fl/fl}* prostates (Figure 4B, 4C). Interestingly, we did not detect substantial CD3 positive cell infiltration in *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostates taken at 3 months of age (Figure S1A), suggesting that lymphoid infiltration may take place as the tumour progresses.

CD4 positive T cells were abundant in *Pten^{fl/fl} Erk5^{fl/fl}* tumours

We next studied the subtypes of T lymphocytes in the *Pten^{fl/fl} Erk5^{fl/fl}* tumours, focusing on those that may have an anti-tumour immunity function, namely CD8 positive cytotoxic T lymphocytes (T_C) and CD4 positive T lymphocytes, which include helper T lymphocytes (T_H) and Natural Killer (NK) T Cells (T_{NK}). ISH analysis using dual RNA scope for *Cd4* and *Cd8* mRNA was performed. While ISH signals for both *Cd4* (fast red) and *Cd8* (DAB) were detected (Figure 4D), *Cd4* positive T cells were more abundant, particularly within the lymphoid aggregates of *Pten^{fl/fl} Erk5^{fl/fl}* tumours. As an additional validation to the ISH experiment, dual IHC staining for CD3 and CD4 protein was also done. In keeping with the *Cd4* ISH signal which we observed in the lymphoid aggregates in *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours, we observed strong immunoreactivity for CD4 positive cells (Figure S3B). At higher magnification, there were cells which were positive for both CD3 and CD4 as well as cells expressing either CD3 or CD4.

Discussion

The current standard of care treatment for patients with advanced PCa remains unsatisfactory, with significant risk of premature death among these patients. For the first time, we demonstrated that *Erk5* deletion in the prostates of PB-Cre4:*Pten^{fl/fl}* mice suppressed prostate tumorigenesis, resulting in smaller tumours and prolonged mouse survival (Figures 1, 2A and B). Interestingly, we also observed increased *Erk5* expression in

Pten^{fl/fl} compared to WT prostate at end point by both QPCR and ISH analysis (Figures 2C and 2D), which has not previously been reported.

Our RNA sequencing analysis to compare *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate epithelial tumours revealed pathways relating to inflammatory responses, immune responses and regulation of cytokine and chemokine expression to be significantly altered. There are several reports in the literature indicating a role for ERK5 in inflammation, with both anti-inflammatory (38–40) and pro-inflammatory effects (41–43) being documented. ERK5 signalling has also been implicated in inflammation-driven skin cancer (17).

The cytokine encoding genes *Ccl5* and *Cxcl10* were highly upregulated in their levels of expression as well as being members of significantly enriched pathways in *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours when compared to *Pten*^{fl/fl} control prostate tumours (Figure 3, Tables S5 and S6). There are emerging roles of the CCL5/CCR5 and CXCL10/CXCR3 axes in cancer [reviewed in (36,37)]. Importantly, CCL5 and CXCL10 can direct the innate and adaptive immune response to mediate an anti-tumour effect via their ability to attract leukocytes to inflammatory sites, including cytotoxic (CD8⁺), helper (CD4⁺) and NK T lymphocytes. Recent reports have found that local secretion of CCL5 and CXCL10 correlates with CD8⁺ T-cell infiltration in colorectal cancer (44) and esophageal squamous cell carcinoma (45). In metastatic melanoma, overexpression of CXCL10 and CCL5 was associated with responsiveness to adoptive therapy with tumour-infiltrating lymphocytes (46). Similarly, epigenetic silencing of CXCL9 and CXCL10 appeared to promote tumour immune-evasion in ovarian cancer (47). Restoring the expression of these cytokines enhanced effector T cell infiltration, reduced tumour progression and increased tumour response to immune-based therapy *in vivo*. Overexpression of CXCL10 has previously been found to inhibit the proliferation of LNCaP prostate cancer cells, an effect that was accompanied by induced CXCR3 expression (48). A strong positive correlation was observed between CXCL10 and local CD8 expression in human prostate tumour explants (49). Our data demonstrating an increase in T cell infiltrate to the tumour microenvironment of *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate tumours which have enhanced local bio-availability of CCL5 and CXCL10 (Figure 4) is in keeping with these reports in the literature.

While we observed some *Cd8* mRNA ISH signal in the stromal aggregates of *Pten*^{fl/fl} *Erk5*^{fl/fl} tumours, the levels of *Cd4* mRNA ISH signal were striking (Figure 4D). Moreover, from CD3/CD4 dual IHC protein analysis, we also saw high levels of CD4 immunoreactivity (Figure S3B), indicating a predominantly CD4 positive cell population. Interestingly, it can be seen from the dual IHC staining that there are CD3/CD4 dual positive cells, which could incorporate T_H and NK T cells. There are also cells that are positive for just CD3 [these could include CD8 positive cells and NK T cells] or CD4 [these could comprise regulatory T cells (T_{reg})].

In conclusion, our data support ERK5 as a target for therapy in PCa with the loss of *Erk5* in a *Pten* null background impairing prostate tumorigenesis and extending the survival of the study animals. Shown in Figure 5 is a schematic summary of our other key findings that *Erk5* deletion in combination with *Pten* deletion resulted in upregulation of the T cell recruiting cytokine genes, *Ccl5* and *Cxcl10*, and that this was associated with enhanced recruitment of

CD4 (predominantly) and CD8 positive T lymphocytes to the tumour epithelium itself and distinct sites within the surrounding stroma. The role of the ERK5 pathway in the host immune response warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Pten^{fl/fl} Erk5^{fl/fl}* mice survived longer and had smaller prostate tumours compared to *Pten^{fl/fl}* mice

(A) Oncoprint of genetic alteration of ERK5 (MAPK7) and MEK5 (MAP2K5) in a human prostate tumour TCGA dataset available on cBioportal (Cancer Genome Atlas Research Network, 2015). ‘Deep deletion’ refers to homozygous deletion. (B) Kaplan-Meier survival curve demonstrating increased survival in *Pten^{fl/fl} Erk5^{fl/fl}* (n=11) compared to *Pten^{fl/fl}* (n=13) mice (**p=0.0006; Log-rank (Mantel-Cox) Test) (see Table S1 also). (C) Representative images of isolated prostates from WT, *Erk5^{fl/fl}*, *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}*

mice taken at end point. Highlighted in the WT image are Anterior Prostate (AP) (indicated by red arrows), Dorsal-Lateral Prostate (DLP), Ventral Prostate (VP) and Seminal Vesicles (SV). **(D-F)** Scatter plots comparing differences between *Pten*^{fl/fl} and *Pten*^{fl/fl} *Erk5*^{fl/fl} prostate weights at 3 months (**D**), 9-10 months (**E**) and end point (11-15 months for *Pten*^{fl/fl}; 15 months for *Pten*^{fl/fl} *Erk5*^{fl/fl}) (**F**). Also included in (**F**) are weights of WT and *Erk5*^{fl/fl} prostates (age 15 months). In (**E**) and (**F**), because of the cystic nature of the tumours, isolated prostates were weighed prior to removal of the cystic fluid [termed Wet Weight (WW)], then re-weighed to assess the solid tumour mass with the cysts drained [termed dry weight (DW)]. Cystic component was also calculated $[(WW-DW)/WW]*100$. Shown in **(D-F)** are individual data points; long horizontal line indicates the MEAN; error bars represent SEM; t test (unpaired, 2 tailed) was used to calculate p values; key significant changes ($p<0.05$) are shown (see Tables S2-S4 also).

Figure 2. Histopathological analysis revealed that *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours were less proliferative compared to *Pten^{fl/fl}* prostate tumours and that *Pten^{fl/fl}* prostate tumours expressed more *Erk5* compared to WT prostate

(A) H+E staining was performed to examine the tissue histology and Ki67 IHC was employed to investigate the level of cellular proliferation in prostates from WT, *Erk5^{fl/fl}*, *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* mice (n=minimum of 3 for each genotype). Representative micrographs of H+E and Ki67 staining for each genotype are shown (main images 10x; insets are higher magnification (40x) view of prostate epithelium). (B) % Ki67 positive cells in each sample were scored using an automated method (Leica software). 12 representative

fields of view per sample were scored for n=6 samples for each genotype at end point. (WT all 15 months; *Erk5^{fl/fl}* all 15 months; *Pten^{fl/fl}* age range 11-15 months; *Pten^{fl/fl} Erk5^{fl/fl}* all 15 months) (C) QPCR analysis of RNA isolated from mouse prostate epithelium by LCM in WT, *Erk5^{fl/fl}*, *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* mice (n=3 for each genotype) for *Erk5* Exon 4 (the floxed allele) and *Pten*. (D) RNA ISH analysis by RNA Scope was utilised to examine the level of *Erk5* expression in WT prostate and *Pten^{fl/fl}* prostate tumours (n=3 for each genotype). Representative micrographs (100x) of RNA Scope staining for *Erk5*, *Ppib* (positive control for RNA integrity) and *Dapb* (negative control for probe specificity) of WT prostate and *Pten^{fl/fl}* tumours at end point are presented. Shown in (B) and (C) are the means; error bars represent SEM; t test (unpaired, 2 tailed) was used to calculate p values; key significant changes (p<0.05) are shown.

Figure 3. Validation of RNA-Seq data confirmed an increase in the expression of T-cell recruiting cytokines, *Ccl5* and *Cxcl10*, in *Pten^{fl/fl} Erk5^{fl/fl}* compared to *Pten^{fl/fl}* prostate tumours

(A) Heatmap of RNA-Seq data. Presented are the 67 genes that were identified from analysis of RNA-Seq data as having significantly altered expression ($P < 0.05$; > 1.5 fold change) between *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours ($n = 3$ for each genotype). In the heatmap, blue represents down-regulation of gene expression (Row Z-Score < 0) and red represents up-regulation of gene expression (Row Z-Score > 0). (B,C) QPCR analysis of LCM prostate epithelial RNA from *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* tumours for *Ccl5* and

Cxcl10 at **(B)** 9-10 month time point (n=3 for each genotype) and **(C)** at end point (n=3 for each genotype). **(D)** QPCR analysis of RNA from tumour-derived cell lines, P1 (*Pten*^{fl/fl}) and PE (*Pten*^{fl/fl} *Erk5*^{fl/fl}), for *Ccl5* and *Cxcl10*. **(E)** RNA ISH by RNA Scope was used to examine *Ccl5* and *Cxcl10* expression in *Pten*^{fl/fl} (n=4) and *Pten*^{fl/fl} *Erk5*^{fl/fl} (n=5) prostate tumours at end point. Representative micrographs (main images 20x; inset (malignant epithelium) 100x) of RNA Scope staining for *Ccl5*, *Cxcl10*, *Ppib* and *Dapb* are shown. **(F)** Average number of *Ccl5* and *Cxcl10* probe copies per cell was calculated using an automated method (HALO software). A minimum of 12 representative areas of prostate epithelium per slide were analysed for each sample [*Pten*^{fl/fl} (n=4) and *Pten*^{fl/fl} *Erk5*^{fl/fl} (n=5)]. Shown in **(B-D and F)** are means; error bars represent SEM; t test (unpaired, 2 tailed) was used to calculate p values and those with significance (p<0.05) are specified.

Figure 4. Infiltration of CD3 positive cells was increased in *Pten^{fl/fl} Erk5^{fl/fl}* compared to *Pten^{fl/fl}* prostate tumours and T-cell infiltrates were predominantly CD4 positive

(A) IHC for the T cell marker, CD3, was used to investigate the presence and localisation of T cells in *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours (n=3 for each genotype) and representative micrographs (top panel – 4x; bottom panel – 40x) of the staining are shown. In the top panel, red arrows indicate CD3-stained stromal lymphoid aggregate areas in the stroma; in the bottom panel, yellow arrows highlight the presence of CD3 positive cells within the prostate tumour epithelium. (B) % CD3 positive cells within the tumour

epithelium at 9-10 month time point and end point in *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours was determined by automated scoring of total number of cells (Leica Software) followed by manual counting of the number of CD3 positive cells per field studied; 12 fields of view per sample for n=3 mice of each genotype at each time point were analysed. **(C)** The proportion of CD3 stromal lymphoid aggregate area per total area of prostate on slide in *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* tumours was calculated by using Leica software to measure the area of lymphoid stromal aggregates and total area of prostate per slide (n=3 samples for each genotype at each time point). Shown in **(B and C)** are means; error bars represent SEM; t test (unpaired, 2 tailed) was used to calculate p values; key significant changes (p<0.05) are shown. **(D)** Dual RNA ISH was used to investigate the levels of T cell markers, *Cd4* (Fast Red) and *Cd8* (DAB), in *Pten^{fl/fl}* and *Pten^{fl/fl} Erk5^{fl/fl}* prostate tumours (n=3 for each genotype) and representative micrographs of the staining (40x) are shown. Blue arrows highlight areas of *Cd8* staining.

Figure 5. Schematic representation summarising the compartmentalisation and potential role of the cytokines, CCL5 and CXCL10, in the context of *Pten* and *Erk5* loss in prostate epithelium

The cytokines, CCL5 and CXCL10 are produced by a variety of cell types, including epithelial cells, endothelial cells, fibroblasts, in response to external stimuli such as IFN- γ , CD40L and IL-15. CCL5 and CXCL10 can exert autocrine and paracrine effects on the cells from which they were produced. However, the major function of CCL5 and CXCL10 is to recruit T lymphocytes to their site of production via recognition of the cytokines by their corresponding receptors on the surface of the T lymphocytes (CCR5 for CCL5 and CXCR3 for CXCL10). T cells can also produce CCL5 and CXCL10. We propose that loss of *Erk5* in combination with loss of *Pten* leads to increased CCL5 and CXCL10 expression within the malignant tumour epithelium. This in turn associates with recruitment of T cells (predominantly CD4⁺) to the tumour epithelium and distinct sites within the stroma (lymphoid aggregates).