SUPPLEMENTARY SECTION

Loss of PTEN can promote corticosteroid resistant MMP-9 epithelial expression in the chronically inflamed lung microenvironment.

Amanda Vannitamby[1,2], Huei Jiunn Seow[1], Gary Anderson[2], Ross Vlahos[1,2], Michelle Thompson[3], Daniel Steinfort[3], Louis B. Irving[3], Steven Bozinovski[1,2]

Supplementary methods and materials

Patient samples and FFPE processing for QPCR. The studies were approved by the ethics committee of the participating institutions and informed written consent was obtained. Lung tissue from resection surgery for treatment of a solitary peripheral carcinoma was collected from subjects with COPD (n=10; 7 GOLD I stage: 3 GOLD II stage). This cohort included 7 males: 3 females; mean age (range) was 71 (63-80) with a mean (range) smoking history of 58 (25-140) pack years. Of the 10 resection samples collected, 6 were confirmed adenocarcinoma and 4 were confirmed squamous cell carcinoma. Tissue blocks from the tumour site and the adjacent subpleural parenchyma avoiding areas involved by tumor were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5-μm sections were prepared for H&E staining. FFPE sections were used for RNA in accordance with the ReliaPrep™ FFPE Total RNA Miniprep System protocol (Promega, Madison, WI, USA). Genomic DNA was removed using the DNA-free™ DNA Removal Kit (Invitrogen, Carlsbad, CA, USA) and purified RNA was converted to cDNA using the SuperScript IV First Strand Synthesis (Invitrogen, Carlsbad, CA, USA). TaqMan® PreAmp Master Mix (Applied Biosystems, Foster City, CA, USA) was used to pre-amplify gene-specific targets with pooled Taqman primers as per manufacturers' instructions. Q-PCR was performed on the pre-amplified cDNA for gene expression analysis of PTEN and MMP-9 normalised to the geometric mean of the two reference genes PUM1 and TBP validated for FFPE lung samples [1]. The comparative (2^{-ddCt}) method was used to present relative expression of target genes relative to single adjacent control sample.

Reagents. Culture medium (Minimum Essential media + Earle salts and Keratinocyte serum-free media including supplements except FBS), Ethidium bromide, high capacity RNA to cDNA kit, pre-optimised target primers and Taqman Fast Advanced PCR Master mix were purchased from Life technologies (Grand Island, NY), while Fetal Bovine Serum was purchased from Lonza (Basel, Switzerland). Lipopolysaccharide (LPS), Budesonide (BUD) and Acridine orange were purchased from Sigma-Aldrich (St Louis, MO).

Macrophage and neutrophil staining. To assess the presence of leukocytes, primary antibody to CD68 and MPO (Dako, Glostrup, Denmark) was used to stain for macrophages and neutrophils respectively. Primary antibody to MMP-9 (RnD systems MAB911) was used to detect MMP-9 immuno-reactivity. Whole lung sections were scanned using whole slide scanner (Olympus VS-120) and morphometric analysis was performed using CellSens Dimensions software from Olympus. Morphometric evaluation of adjacent control and tumour lung tissue sections was performed on the whole slide section where the percent positive stain area was determined using standardised threshold values.

Beas-2B cell culture. Beas- 2B human bronchial epithelial cells (ATCC: CRL-9609) isolated from normal bronchial epithelium were used as previously described [2]. Briefly, cells were cultured in complete media with a 1:1 mixture of Keratinocyte-Serum Free Media (supplemented with 5µg/ml epidermal growth factor and 50µg/ml bovine pituitary extract) and Minimum Essential Medium (MEM) + Earle Salts (supplemented with 10% FBS, 2mM L-glutamine, 1.0mM sodium pyruvate, 0.1mM non-essential amino acids, 1.5g/L sodium bicarbonate, 25µg/ml gentamicin, 100µg/ml penicillin and 100µg/ml streptomycin). For stimulation assays, Beas-2B cells were transferred in a 24

well-plate format at a density of $0.25x10^6$cel/well and incubated in low serum media (1%). Cells were treated with media alone (vehicle group), 100ng/ml LPS, 10^{-7}M BUD or a combination of both BUD/LPS. The treated cells were then collected at 2 time points; 3h and 48h and cell pellets were resuspended in RLT buffer (Qiagen, Valencia, CA) and stored at -20^0C in preparation for Q-PCR.

PTEN siRNA Reverse Transfection and Quantitative Polymerase Chain Reaction (Q-PCR). Beas-2B cells were resuspended in low serum optiMEM media (Life technologies, Grand Island, NY) and seeded at a density of $2.5\ x10^5$ cells per well in 6 well plate. siPort NeoFx transfection reagent (Life Technologies) was combined with Ambion silencer select PTEN or control siRNA (Life Technologies) at a final siRNA concentration of 12.5pmol. After a 6hour incubation at 37^0C this media was replaced with complete media (K-SFM and MEM+Earle salts with 10% FBS) and further incubated at 37^0C for a total of 72hours. RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was prepared using the High Capacity RNA-to-cDNA synthesis kit previously published [3]. Q-PCR was conducted using ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) with each assay containing validated Taqman probes/primer sets. Relative Expression above the vehicle was established using the ΔΔCT method, where values where normalised to the house keeping gene, GAPDH.

Western blot analysis. Cell pellets were resuspended in M2 lysis buffer (50mM Tris-HCL, 150mM NaCl, 1mM EDTA, 1% Triton® X-100) with 1:100 protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), as previously prepared [4]. Protein concentration was determined using the Pierce ® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and 40µg were loaded onto 10% gel slabs, transferred onto a Hybond-P PVDF membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA), on a semi-dry apparatus, (Bio-Rad Laboratories, Hercules, CA) as previously published [3]. Membranes were

blocked in Odyssey blocking buffer (Li-Cor biosciences, Lincoln, NE), followed by an overnight incubation with primary antibodies Actin (Santa Cruz biotechnology, Santa Cruz, CA) and PTEN (Cell Signalling technology, Danvers, MA) in blocking buffer + 0.1% Tween-20 in the cold room. Membranes were washed and then incubated with secondary IRDye® fluorescence antibodies (1, Li-Cor biosciences, Lincoln, NE); 800CW donkey anti-rabbit and 680 LT conjugated donkey (polyclonal) anti-goat IgG. Following extensive washes, membranes were scanned using the Li-Cor Odyssey Infrared Imaging System and detected bands were quantified using the Li-Cor software.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism and SPSS. Results were presented as Tukey box plots where the box indicates the interquartile range with median. For direct comparison of results in tumour vs. adjacent regions, a Wilcoxon's signed rank test was used as the data was not normally distributed. For comparison of tumour subtypes, a Mann-Whitney t test was used. For comparison of control and PTEN KD cells, a two way ANOVA with bonferroni multiple comparison correction was used. The Spearman correlation coefficient was calculated between the percent of positively stained lung tissue section and the expression of MMP-9. Statistical inferences used a $p = 0.05$ two-sided significance level.

References:

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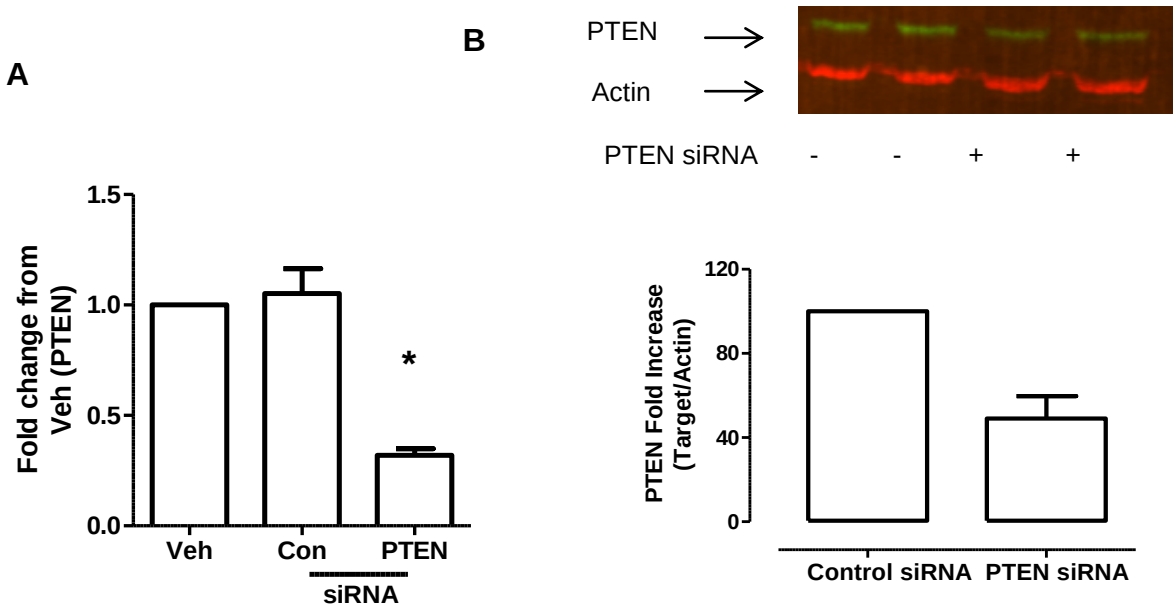
4. Bozinovski S, Jones JE, Vlahos R, et al. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFkappa B and AP-1 in vivo. J Biol Chem 2002;**277**(45):42808-14.

Supplementary Results



Efficacy of functional PTEN knockdown. Using an siRNA transfection knockdown approach, replacement of low serum optiMEM media with complete media 6 hours after transfection improved cell viability across all groups in comparison to media change at 24 hours, therefore this time point was adapted. This approach resulted in a 70% reduction in PTEN transcript expression in comparison to the control siRNA group (Figure A n=6, $p<0.001$). Western blot analysis was conducted to determine whether the reduction in PTEN protein levels would be similar to the reduction observed in mRNA levels. A representative fluorescence Western Blot image is presented (Figure B, top panel)

demonstrating multiplexing for Actin and PTEN expression within the same blot. (Figure B) Densitometric analysis of this data demonstrated a 51% reduction in PTEN protein compared to the control siRNA.