Inhibition of PAK1 suppresses pancreatic cancer by stimulation of anti-tumour immunity through down-regulation of PD-L1

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Abstract

Immunotherapies have not yielded significant clinical benefits for pancreatic ductal adenocarcinoma (PDA) mainly because of the existence of an immunosuppressive tumour microenvironment (TME) characterised by a desmoplastic stroma containing infiltrated immune cells and activated pancreatic stellate cells (PSCs). This study aimed to investigate the involvement of PAK1 in anti-tumour immunity. In PDA patients low PAK1 expression, low activation of PSC and high $CD8^+$ T cell/PAK1 ratios correlated with longer overall survival. In a murine PDA model PAK1 knockout increased intra-tumoral $CD4^+$ and $CD8^+$ T cells, inhibited PSCs activation and extended survival. Inhibition of PAK1 reduced PSC-stimulated PDA cell proliferation and migration, blocked PSC-mediated protection of PDA cells from killing by cytotoxic lymphocytes and decreased intrinsic and PSC-stimulated PD-L1 expression in PDA cells. The latter decrease further sensitized PDA cells to cytotoxic lymphocytes. Inhibition of PAK1 stimulated anti-tumour immunity by increasing intra-tumoral $CD4^+$ and $CD8^+$ T cells, and by sensitizing PDA cells to killing by cytotoxic lymphocytes via down-regulation of intrinsic and PSC-stimulated PD-L1 expression. The use of PAK1 inhibitors, especially in combination with immune checkpoint inhibitors, may result in improved efficacy of immunotherapy of PDA.

Key words: p21-activated kinase 1; tumour microenvironment; pancreatic stellate cells; programmed death-ligand 1.

Abbreviations: α-SMA, alpha-smooth muscle actin; ECM, extracellular matrix; IHC, immunohistochemistry; KPC, *LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *Pdx-1-Cre*; PAK1, p21-activated kinase; PD-1, programmed death 1; PDA, pancreatic adenocarcinoma; PD-L1, programmed death-ligand 1; PSC, pancreatic stellate cell; TMA, tissue microarray; TME, tumour

microenvironment; TIL, tumour infiltrating lymphocytes; Treg, regulatory T cell; KD, knockdown; KO, knockout; BMI, body mass index

1. Introduction

Pancreatic adenocarcinoma (PDA) is a highly lethal malignancy with a dismal prognosis because of a lack of early detection, aggressive tumour biology and a limited number of effective therapeutics. In contrast to the improvement in long-term survival for some other cancer types, little progress has been made in pancreatic cancer, and the overall five-year survival rate is only 9% [1]. Chemotherapy currently still plays an important role in the clinical management of pancreatic cancer. Although extensive clinical trials have been performed with different combinations of chemotherapies, to discover effective and safe regimens that could extend survival, only modest improvements in survival have been observed, and high toxicity and early occurrence of resistance led to low tolerance and poor health of patients.

The newly developed immunotherapy has delivered promising results in several tumour types by activation of anti-tumour immunity. The immune checkpoint programmed death 1 (PD-1) receptor is expressed in immune cells, including activated T cells, B cells and myeloid cells, [2] while its ligand PD-L1 is expressed in many human cancers including PDA [3]. Binding of PD-L1 to PD-1 induces T cell apoptosis and suppresses the subsequent anti-tumour immune response. Thus, PD-1/PD-L1 blockade has emerged as a potent anti-tumour immunotherapy [4, 5]. Increasing numbers of clinical trials with an emphasis on the combination of PD-1/PD-L1 blockade either with conventional chemo- or radio-therapies, or with targeted small molecular inhibitors, have aimed to enhance therapeutic efficacy and improve survival [6].

In PDA, however, immunotherapy has not produced many clinically significant results largely because of the existence of an immunosuppressive tumour microenvironment (TME)[7]. Pancreatic stellate cells (PSCs) play important roles in the development of the unique desmoplastic reaction in PDA, by contributing to stromal fibrosis, deregulation of the extracellular matrix (ECM)

and deficient vascularization [8, 9]. In pancreatic cancer, normally quiescent PSCs become activated to a phenotype characterized by fibroblast-like morphology, absence of vitamin A droplets, expression of α-smooth muscle actin (α-SMA) and increased secretion of ECM proteins [10]. Activated PSCs modulate the interaction between tumour cells and infiltrated immune cells in the TME and cause inhibition of anti-tumour immunity and immune evasion by inhibiting T cell infiltration, suppressing cytotoxic T cell activation and stimulating immunosuppressive cells [11, 12].

The p21-activated kinases (PAKs) are a family of serine/threonine kinases, that are activated by the Kras p21 protein and its relatives. PAKs are divided into 2 groups (PAKs 1, 2 and 3; and PAKs 4, 5 and 6) based on sequence homologies. PAK1 and PAK4 are the most important members in gastrointestinal carcinogenesis, and several studies have shown that both are up-regulated in PDA[13]. Inhibition of PAK1 increased the sensitivity of PDA to gemcitabine *in vitro* and *in vivo*[14-16], decreased proliferation of PSCs isolated from the dense stroma of human PDA samples, and increased apoptosis of these PSCs by blocking their activation [17]. Inhibition or depletion of PAK1 decreased pancreatic cancer growth which seemed to be associated with stimulation of T cell infiltration of tumours[18].

The present study aimed to investigate the role of PAK1 in anti-tumour immunity in PDA. The correlations between PAK1 expression, lymphocyte numbers, and survival in human PDA were evaluated. The effects of PAK1 knockout in a murine genetically engineered PDA model that spontaneously develops autochthonous tumours (the KPC mouse [19] (*LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre*)) were also examined. The mechanisms involved were investigated by determining the effects of PAK1 knockout on PSC activation and infiltration of intra-tumoral immune cells. In addition, the effect of PAK1 inhibition on basal and PSC-stimulated PD-L1

expression by tumour cells, and on the killing of tumour cells by active cytotoxic lymphocytes, were assessed.

2. Materials and Methods

2.1. Patient information and composition of tissue microarray

All patients (n=91) undergoing pancreatic resection from 2008 to 2015 with a diagnosis of PDA confirmed by two independent pathologists at Austin Health, Melbourne, Australia, were included in this study. Patients who died from severe post-surgery complications and early-stage lethal recurrence or from metastasis within 6 months (n=14) after surgery were excluded, to ensure that patients who probably did not benefit from surgery were not assessed. Patients dying from the metastatic disease within 6 months of surgery likely had metastatic disease present at the time of surgery, were therefore incorrectly staged, and thus should be excluded. Written consent was acquired from all patients before surgery. All samples obtained were fixed in 10% formalin and embedded in paraffin. Three core samples (1 mm in diameter) were taken from the tumour centre and assembled into a tissue microarray (TMA). The use of human tissue samples was approved by the Human Research Ethics Committee at Austin Health (H2013-04953).

2.2. TMA staining and evaluation

The TMA samples were cut into 4-μm sections. For immunohistochemistry (IHC), antigens were retrieved by boiling the samples in citrate buffer (10 mM citric acid, pH6, Sigma-Aldrich, Castle Hill NSW, Australia) for 30 min. Samples were then incubated with hydrogen peroxidase blocker for 15 min followed by 5% normal goat serum for 30 min at room temperature for endogenous peroxidase quenching and protein blocking, respectively. After incubation with

primary antibodies against PAK1 (1:300, Cell Signalling Technology, Danvers, MA), α-SMA (1:300, Abcam, Melbourne, Australia), CD4 (1:100, eBiosciences, San Diego, CA), CD8 (1:100, eBiosciences), PD-L1 (1:100, Cell Signalling Technology), or IgG (1:300, Cell Signalling Technology) overnight at 4 °C, sections were stained with an EnVision kit (Dako, Botany, Australia) and counterstained with haematoxylin (Sigma-Aldrich). The images of all slides were captured with an Aperio Digital Slide Scanning System (Leica Microsystem, Melbourne, Australia).

IHC staining of PAK1 and α-SMA was analysed using Aperio Image Scope (v12.3). Mean values of the intensity of positively stained areas of all samples were calculated and used to divide the samples into high (> mean value) and low (< mean value) expression of PAK1 and α-SMA. For evaluation of the IHC staining of CD8 and CD4, mean values of the numbers of positively stained cells from all samples were calculated and used to divide the samples into high (> mean value) and low (< mean value). The overall survival (OS) of patients in relation to various indexes as described in the results was assessed by Kaplan-Meier and Log rank analysis.

2.3. KPC Mouse study

All mouse experiments were approved by the Austin Health Animal Ethics Committee (A2016/05317) and adhered to the standards articulated in the Animal Research: Reporting of In Vivo Experiments (ARRIVE). Experimental mice were housed in the BioResource Facility at Austin Health and monitored for 12 months for disease progression according to strict health criteria. KPC (*LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre*) mice were crossed with PAK1$^{-/-}$ C57BL/6 mice to generate PAK1 wildtype (KPC PAK1 WT, n=10, 5 female, 5 male aged from 5-22 weeks old), PAK1 heterozygous (KPC PAK1 Het, n=17, 10 female, 8 males, aged from 8-28

weeks) or PAK1 knockout (KPC PAK1 KO, n=9, 5 female, 4 male, aged from 14-34 weeks) mice on a KPC background. Briefly, the triple mutant (*LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre*) C57BL/6 mice were crossed with PAK1 knockout (PAK1$^{-/-}$) C57BL/6 mice to generate dual (*LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre*) or triple (*LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre*) mutant mice with PAK1$^{+/-}$. The female *LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre; PAK1*$^{+/-}$ mice were then bred with male *LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre; PAK1*$^{+/-}$ mice to get KPC mice in PAK WT, het and KO background. At the experimental endpoint, mice were euthanized, and pancreatic tumour mass and spleen were collected. Mice survival was assessed by Kaplan-Meier analysis and compared between PAK1 genotypes.

For IHC staining, pancreatic tumours were fixed in 10% formalin, embedded and cut into 4μm sections. After antigen retrieval by incubation in citrate buffer, followed by blocking with 5% BSA in 1% TBST (20 mM Tris, 0.8% NaCl, 0.05% Tween 20, pH 7.6), the sample slides were incubated with antibodies against PAK1, α-SMA, desmin, CD4, CD8 or rabbit IgG (Santa Cruz, Dallas, TX) overnight at 4 °C. After washing with TBST, slides were incubated with horseradish peroxidase-labelled goat anti-rabbit IgG for 1 h, followed by diaminobenzidine and haematoxylin-counter staining. Images were taken with a Leica microscope at 20X magnification and analysed using the Image Pro-Plus 6.0 image analysis program (Media Cybernetics Inc., Silver Spring, MD).

2.4. Mouse PDA tail model and FACS analysis

Pancreatic tail tumours were induced in 8-10 weeks old, 6 male PAK1 WT and 6 male PAK1 KO C57BL/6 mice by injecting pancreatic cancer cell TB33117 (isolated from the pancreatic tumour of a KPC mouse [20]) into the tail of the pancreas as previously described [21]. After 4 weeks, the mice were culled, and tumours were isolated and weighed. Tumour cell suspensions were made

by digesting tumour tissues with 7mg/ml collagenase (Type III, Worthington Biochemicals, Freehold, NJ). The tumour cells were incubated for 1 h on ice with antibodies against CD45 (1/1000, BV510), B220 (1/200, FITC), CD3 (1/200, PE), CD8 (1/1000, PECy7), CD4 (1/1000, ApcCy7) (BD Biosciences, North Ryde, Australia) or PD-1 (1/200, APC) (Miltenyi Biotec, Macquarie Park, Australia), after blocking of Fc receptors using CD16/CD32 (1/1000) (Miltenyi Biotec). Cells were washed 3 times in 0.5% BSA in PBS before analysis by FACS Canto II (BD Biosciences). Data were analysed using Weasel software (Cytometry Laboratory, Walter and Eliza Hall Institute, Parkville, Australia).

2.5. In vitro co-culture study

A co-culture model was used to evaluate the interaction between tumour cells, PSCs and lymphocytes. Murine tumour-associated pancreatic stellate cells (PSC) were established from tumour fragments from KPC PAK1 WT and KPC PAK1 KO mice by the outgrowth method [17]. Splenic lymphocytes were isolated from C57BL/6 mice as described previously [18] and activated in RPMI1640 medium with 1 μg/ml CD3, 1 μg/ml CD28 (eBioscience) and 30 units/ml IL-2 (Miltenyi Biotec) for 24h.

To determine the effects of PSCs on the killing of cancer cells by lymphocytes, PSC cells (2000 cells/well) were seeded in 48-well plates and incubated overnight. The luciferase-labelled TB33117 cell (2000 cells/well) were seeded next morning, activated splenic lymphocytes (10^6 cells/well) were added that afternoon, and the mixed cells were continuously cultured for another 24 h. Cancer cell survival was measured by the luciferase activity as assessed by the Dual-Luciferase® Reporter Assay (Promega, Sydney, Australia) following the manufacturer's

instructions. Luciferase intensity was measured with a FLUOstar OPTIMA microplate reader (BMG Labtech, Melbourne, Australia).

To determine the effect of PAK1 inhibition on the killing of cancer cells by lymphocytes, luciferase-labelled TB33117 cells were pre-treated with PF-3758309 (Active Biochemical Co. Maplewood, NJ) for 24 h followed by co-culture with activated lymphocytes for another 24 h. Cancer cell survival was determined as described above.

2.6. Western Blot

Cancer cells isolated from KPC PAK1 WT or KPC PAK1 KO mice, and PAK1 WT cancer cells treated with PF-3758309 or with PSC-conditioned medium, were lysed in SDS sample buffer and the resultant cell lysates were electrophoresed on 10% SDS gels followed by blotting with antibodies against PD-L1, phosphorylated PAK1 (pPAK1), total PAK1 and GAPDH (Cell Signalling Technology). The cell lysates from PSCs were also blotted with antibodies against α-SMA and CK19.

2.7. Migration/invasion assay

Cell migration/invasion was measured using a Boyden Chamber assay. On day 1, PSC cells (PAK1 WT and KO; 3×10^4 cells/well) were seeded in a 24-well plate and allowed to adhere overnight. On day 2, the original medium was replaced by fresh medium with different treatment. For PAK1 WT PSC, cells were divided into 2 treatment groups: control, PF-3758309 (10 nM). For PAK1 KO PSC, cells were incubated in a normal medium as control. After 24-hour incubation, all the treatments were replaced by a fresh serum-free medium containing 0.1% BSA. The inserts (8 μm pore size, Corning Incorporated, Corning, NY) were coated with 3μg of human fibronectin on the

lower surface. TB33117 cells were seeded into the upper chamber at a density of 5×10^4/100µl and allowed to migrate for 24 hours. After 24-hour migration, non-migrated cells were removed by a cotton swab from the upper surface. The inserts were fixed and stained with Quick-Dip (Fronine, Sydney, Australia). Then the membranes were cut, mounted in DPX (VWR International Ltd; QLD, Australia). The cells migrated to the lower surface of the membranes were counted. Images were captured in at least 10 fields at 10X magnification using a Leica microscope (Leica Microsystems Pty Ltd, Australia).

2.8. Statistical analysis

Quantitative variables are presented as mean ± SEM or median with range. Categorical variables were expressed as numbers and percentages. Student's *t* test or Mann-Whitney *U* test was used to compare quantitative variables and the χ^2 test was used to compare categorical variables. Pearson's correlation and scatter plots were used for correlation analysis. The Kaplan-Meier method was used for survival analysis, with the log-rank test for statistical difference. Variables with a *p* value < 0.1 in univariable analysis were further included in the Cox regression model for multivariable survival analysis. Two-sided P values were calculated and considered to be statistically significant when $p< 0.05$. All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. High PAK1 or α-SMA correlated with shorter PDA patient survival

To construct a tissue microarray (TMA), 77 samples were selected from tumour tissue (which included both tumour cells and stromal cells) of 91 patients with PDA, after exclusion of individuals who had died within 6 months after surgical resection. The clinical and pathological characteristics of the 77 patients are described in Table 1. The expression of PAK1 and α-SMA proteins was determined by immunohistochemical (IHC) staining of the TMA; representative images are shown in Fig.1A. Significantly longer overall survival was observed in patients with low PAK1 expression across all cohorts (Fig. 1B), in the cohort with tumours in T3 and T4 stages (Fig. 1C), or in the cohort with N1 lymph node metastasis (Fig. 1D). Significantly increased overall survival was also observed in patients with low α-SMA expression in the cohort with tumours in T3 and T4 stages (Fig. 1F) or in the cohort with N1 lymph node metastasis (Fig.1G), but not in the overall cohort (Fig. 1E). PAK1 expression was positively correlated with α-SMA expression in the overall cohort (Fig. 1H). The results indicate that high PAK1 and α-SMA expression significantly reduced the survival of patients with PDA.

3.2. High ratios of CD8$^+$ T cells to PAK1 or to CD4$^+$ T cells were associated with longer patient survival

The numbers of CD4$^+$ and CD8$^+$ T cells were also determined by IHC staining of the TMA, and representative images are shown in Fig. 2A. Consistent with previous reports [22, 23], a higher ratio of CD8$^+$ to CD4$^+$ T cells correlated with longer overall survival (Fig.2B). The correlation of CD8$^+$ T cell numbers with overall survival did not reach statistical significance (Fig. 2C). However, patients with a higher ratio of CD8$^+$ T cells to PAK1 expression survived significantly longer than those with a lower ratio (Fig. 2D). Neither CD4$^+$ T cell number alone nor its ratio to PAK1 expression was significantly correlated with patients' survival (data not shown). The correlation of PAK1 expression to CD8$^+$ or CD4$^+$ T cell number was not significant (data not shown), perhaps because of the limited number of samples. Other factors involved in the regulation of tumour infiltration of T cells will be discussed further in the Discussion section.

PAK1 intensity and tumour histological grade were significant factors in the univariable analysis for overall survival. By using a backward conditional selection procedure with a cutoff value of $p < 0.1$, PAK1 intensity and tumour histological grade still had a significant impact on patients' overall survival from multivariable analysis (Table 2). The resection margin and α-SMA intensity were also included in the Cox regression model for multivariable analysis (data not shown) though both of them did not reach statistically significant in the univariable analysis (Table 2).

3.3. In a murine PDA model PAK1 deletion extended survival, increased the numbers of intra-tumoral CD4$^+$ and CD8$^+$ T cells, and decreased the activation of PSCs

To validate the results obtained from the human TMA and to test the hypothesis that inhibition of PAK1 suppresses PDA by stimulation of anti-tumour immunity via modulation of the TME, the effects of PAK1 on PDA survival, tumour immune response and PSC activation

were determined using PAK1 wildtype (KPC PAK1 WT), PAK1 heterozygous (KPC PAK1 Het) or PAK1 knockout (KPC PAK1 KO) mice on the KPC background. Both KPC PAK1 KO ($p = 0.008$) and KPC PAK1 Het mice ($p = 0.048$) survived significantly longer than KPC PAK1 WT mice (Fig. 3A). The median survival times were 15, 20 and 24 weeks for KPC PAK1 WT, KPC PAK1 Het and KPC PAK1 KO mice, respectively, suggesting that the mice survival was increased in a PAK1 dose-dependent manner, the less PAK1 the longer survival.

Furthermore, the numbers of intra-tumoral $CD4^+$ and $CD8^+$ T cells were significantly increased in KPC PAK1 KO mice (Fig. 3B&C). In contrast, the expression markers for the activation of PSCs (α-SMA and desmin) were decreased significantly in the tumour tissues of KPC PAK1 KO mice compared to the KPC PAK1 WT mice (Fig. 3D-F). Together these results suggest that the deletion of PAK1 suppressed PDA and increased the survival of mice with PDA by stimulating anti-tumour immunity possibly via modulation of PSCs.

Consistent with the above data, in a murine model of cancer of the pancreatic tail, where syngeneic pancreatic cancer cells were injected into the tail of the pancreas to induce tumour formation in the pancreatic tail, the numbers of intra-tumoral $CD3^+$, $CD4^+$ and $CD8^+$ T cells were also increased in PAK1 KO mice compared to PAK1 WT mice (Fig.4B&C), while tumour growth was significantly decreased in PAK1 KO mice compared to PAK1 WT mice (Fig.4A). Tumours were induced in the pancreatic tail in all 6 PAK1 WT mice, but in only 4 out of 6 PAK1 KO mice. The differences in PD-1 positive T cells between PAK1 WT and KO mice did not reach statistical significance although lower levels of PD-1-positive CD4 and CD8 cells were observed in one PAK1 KO mouse (Fig.4C).

3.4. Depletion of PAK1 reduced PD-L1 expression by PDA cells and sensitized them to killing by cytotoxic lymphocytes

Tumour cells cause exhaustion and apoptosis of tumour-infiltrating lymphocytes by producing PD-L1 (programmed cell death protein ligand 1)[24], which has become a key target in cancer immunotherapy, and an important marker for prognosis. To investigate the mechanism by which PAK1 affects anti-tumour immunity, the effect of PAK1 on PD-L1 expression by tumour cells and its impact on tumour immune response were investigated. Expression of the PD-L1 protein was lower in three isolates of tumour cells from KPC PAK1 KO mice, compared with three isolates from KPC PAK1 WT mice (Fig.5A&B). Expression of the PD-L1 protein was also decreased in two PAK1 knockdown (KD) clones of the human pancreatic cancer line PANC-1 (Fig. 5C). Furthermore, treatment of the murine PDA cell line TB33117 with the PAK1/4 inhibitor PF-3758309 (PF) reduced PD-L1 protein expression while suppressing the activity of PAK1 (Fig. 5D).

In addition, pre-treatment with PF enhanced killing of TB33117 cancer cells by activated cytotoxic lymphocytes in co-culture (Fig. 5E&F). Without PF pre-treatment, cytotoxic lymphocytes killed 15$\pm$2% of TB33117 cells and thus reduced cell survival to 85$\pm$2% of the value for cells incubated without lymphocytes (Fig. 5F). PF pre-treatment alone decreased the cell survival to 73$\pm$4% of the value for cells incubated without lymphocytes. PF pre-treatment increased the cell death induced by cytotoxic lymphocytes with a further reduction in cell survival to 57$\pm$3% of the value for cells incubated without PF and without lymphocytes (Fig. 5F). Together these data indicate that depletion or inhibition of PAK1 suppressed the expression of PD-L1 by PDA cells and enhanced the induction of cell death by cytotoxic lymphocytes.

3.5. Depletion of PAK1 decreased PSC-stimulated cancer cell proliferation and blocked PSC-induced protection of PDA cells from killing by cytotoxic lymphocytes

The unsuccessful outcome of immunotherapy in PDA is mainly caused by the existence of the immunosuppressive TME which is dominated by PSCs. Modulation of PSCs should contribute to the reprogramming of the TME and improve the anti-tumour immune response. To investigate this hypothesis, tumour-associated PSCs were isolated from the pancreatic tumours of KPC PAK1 WT and KPC PAK1 KO mice as described in Materials and Methods. The isolated PSCs expressed the activation marker α-SMA, and were free from epithelial cell contamination, as assessed by the absence of CK19 on Western blotting (Fig. 6A&B). When the PSCs were co-cultured with luciferase-labelled TB33117 PDA cells at different ratios, PDA cell proliferation was stimulated in a dose-dependent manner. However, the stimulation by PSCs from KPC PAK1 KO mice was significantly less than that by PSCs from KPC PAK1 WT mice (Fig. 6C). Additionally, PAK1 WT PSCs significantly increased cell migration of TB33117 cells compared to negative control with no PSC (Fig.6D&E). This stimulation on cell migration by PAK1 WT PSCs was blocked by PF-3758309. PAK1 KO PSCs did not increase TB33117 cell migration when compared to negative control with no PSC (Fig. 6D&E).

Furthermore, when the PAK1 WT PDA cell lines WT942 and TB33117 were cultured with the conditioned medium collected from PSCs isolated from KPC PAK1 WT or KPC PAK1 KO mice, PD-L1 expression in the two PDA cell lines was increased by KPC PAK1 WT PSC-conditioned medium but not by KPC PAK1 KO PSC-conditioned medium (Fig. 6F). To investigate the effect of PSCs on cancer cell killing by lymphocytes, the luciferase-labelled TB33117 cells were cultured with or without PSCs from either KPC PAK1 WT or KPC PAK1 KO mice at a 1:1 ratio, before culture with activated splenic lymphocytes for a further 24 hours (Fig. 6G). In the

absence of PSCs, activated lymphocytes killed some tumour cells and cell survival was reduced to <70% of the survival of tumour cells without lymphocyte treatment (Fig. 6H). PSCs from KPC PAK1 WT mice increased tumour cell survival to a level comparable to that of the tumour cells without lymphocyte treatment. In contrast, no such protective effect was observed with PSCs from KPC PAK1 KO mice (Fig. 6H). These results indicate that the deletion of PAK1 enhanced anti-tumour immunity by blocking PSC-induced protection of tumour cells from killing by cytotoxic lymphocytes through suppression of PSC-stimulated expression of PD-L1 in PDA cells.

4. Discussion

The role of PAK1 in the development of pancreatic cancer has been controversial. Overexpression of PAK1 has been identified in both PDA tissues and cell lines, and the deregulation of PAK1 contributes to increased PDA cell survival, proliferation and invasion and decreased gemcitabine sensitivity [25, 26]. However, although high PAK1 expression is related to poor histopathological differentiation and prognosis in ovarian and colorectal tumours [27, 28], in pancreatic tumours PAK1 overexpression has been reported to correlate with better survival and higher differentiation [29, 30]. In contrast, in this study we found that patients with low PAK1 expression had longer overall survival compared to patients with high PAK1 expression, not only

in the total cohort analysed, but also in the cohorts with advanced (T3-T4 stages) and metastatic (N1) tumours (Fig. 1B-D). Similarly, KPC PAK1 KO mice survived significantly longer than either KPC PAK1 Het or KPC PAK1 WT mice, and even KPC PAK1 Het mice survived longer than KPC PAK1 WT mice (Fig. 3A). In a murine orthotopic model for pancreatic cancer, tumour growth was significantly reduced in PAK1 KO mice compared to PAK1 WT mice (Fig.4A). These results are consistent with the previous observations that PAK1 acts down-stream of Kras, the oncogene that is mutated in 95% of PDA and plays a key role in the progression from PanIN to PDA [31]. Our findings indicate that deletion of PAK1 reduced the initiation and progression of PDA, and that PAK1 expression acts as a predictive factor for survival.

PSCs, which dominate the desmoplastic reaction in PDA, are modulated by PAK1. α-SMA, a marker for PSC activation, contributes to cell migration/invasion and tumour metastasis in solid tumours [9, 32, 33]. In PDA, high expression of α-SMA was associated with a dismal prognosis in a prospective randomized phase III clinical study [34]. The results presented here demonstrate firstly, that patients with low α-SMA expression had a better survival than patients with high α-SMA expression in the cohorts with advanced (T3-T4) and metastatic (N1) tumours ($p < 0.05$) (Fig. 1F&G), secondly, that PAK1 expression was positively correlated to α-SMA expression in human PDA tissues (Fig.1H), and thirdly, that expression of α-SMA and desmin was decreased in KPC PAK1 KO tumours compared to KPC PAK1 WT tumours (Fig. 3D-F). Inhibition of PAK1 by FRAX597 (a group I PAK inhibitor) reduced the expression of α-SMA and desmin in PSC [17]. Previous studies have shown that PAK1 stimulates Wnt/β-catenin signalling by increasing β-catenin phosphorylation, nuclear translocation and transcriptional activity [35], and ATRA down-regulates α-SMA in PSCs by affecting Wnt/β-catenin signalling [36]. Cdc42, a small GTPase protein acting up-stream of PAK1, also regulates the activation of the α-SMA promoter through

PAK1 [37]. Furthermore activated PSCs reduced the infiltration of cytotoxic $CD8^+$ T cells to the stroma immediately adjacent to the tumour epithelial cells of PDA by secretion of IL-6 and IL-8 [7]. This evidence together with the findings presented here implies that PAK1 could affect tumour infiltrating lymphocytes (TILs) by modulation of PSCs.

Cytotoxic $CD8^+$ T cells recognize and kill tumour cells. PDA patients without TILs had more advanced tumours and a poorer prognosis [38], and an increased number of $CD8^+$ T cells was associated with improved survival [39]. In this study, patients with a high ratio of $CD8^+$ T to $CD4^+$ T cells or a high ratio of $CD8^+$ T cells to PAK1 had significantly better survival (Fig. 2B&D). Moreover, in a murine PDA model, the numbers of intra-tumoral $CD8^+$ and $CD4^+$ T cells were increased in KPC PAK1 KO tumours compared to KPC PAK1 WT tumours (Fig. 3B&C), and PAK1 KO significantly extended survival. In the orthotopic pancreatic cancer tail model, the numbers of intra-tumoral infiltrating $CD3^+$, $CD4^+$ or $CD8^+$ T cells were also significantly increased in PAK1 KO mice compared to PAK1 WT mice (Fig.4B&C). These findings indicate that the deletion of PAK1 suppressed PDA and extended survival by stimulating anti-tumour immunity via modulation of PSCs and intra-tumoral T cells. However, no significant correlation between PAK1 expression and the level of $CD8^+$ infiltration was observed in human TMA. Possible explanations for the failure to observe a correlation are the limited number of samples analysed, and the variation in sampling time as tumour samples were taken at various stages of tumour development. Clark *et al.* have reported that there is a dynamic immune response to PDA from tumour initiation to advanced malignancy [11], and that $CD8^+$ T cells were scarce in early-disease stage, and only presented in a subset of advanced stages. Moreover, immunosuppressive tumour-associated macrophages, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) cells are also found in preinvasive lesions. A strong inverse correlation between MDSC and $CD8^+$

T cell infiltration was observed [40]. In addition, chemotherapeutic agents re-modulate the immune response within TME. Gemcitabine attenuated the immunosuppressive response by reducing the numbers of Tregs and MDSCs in PDA patients [41].

Overexpression of PD-L1 in the tumour microenvironment of pancreatic cancer is associated with an advanced tumour stage and a poor prognosis [3, 42]. Inhibition or depletion of PAK1 decreased expression of PD-L1 in both murine and human PDA cells (Fig. 5A-D), which in turn sensitised pancreatic cancer cells to killing by cytotoxic lymphocytes (Fig.5F). The depletion of PAK1 also inhibited the stimulation by tumour-associated PSCs of PDA cell proliferation, migration/invasion and PD-L1 expression (Fig.6C-F). More importantly, depletion of PAK1 in tumour-associated PSCs blocked PSC-induced protection of PDA cells from killing by cytotoxic lymphocytes (Fig.6H). Nomi and colleagues have reported that PD-L1 expression was inversely correlated with the numbers of TIL, especially $CD8^+$ T cells [3]. This observation, taken together with the findings here, implies that inhibition of PAK1 up-regulates intra-tumoral lymphocytes and stimulates anti-tumour immunity *via* down-regulation of PD-L1. PD-L1 expression can be up-regulated by PI3K/AKT activation and interferon-γ [43, 44], both of which interact closely with PAK1 [25]. Further study will be needed to explore the underlying mechanisms involved in PAK1 regulation of PD-L1 expression.

PSCs promote proliferation and migration/invasion of PDA cells *in vitro* and *in vivo* [8, 45, 46]. Overexpression of Galectin-1 in PSCs significantly induced $CD4^+$ and $CD8^+$ T cell apoptosis and Th2 cytokine (IL-4 and IL-5) secretion, which in turn contributed to the immunosuppressive microenvironment of PDA [47]. The expression of PD-L1 in PSCs may also contribute to inhibition of anti-tumour immunity [48]. This study demonstrates for the first time that tumour-associated PSCs stimulated the expression of PD-L1 by PDA cells (Fig. 6F) and protected cancer

cells from killing by cytotoxic lymphocytes (Fig. 6H), indicating that PSCs promote tumour progression by up-regulation of PD-L1 in cancer cells, which in turn causes apoptosis of cytotoxic lymphocytes. The fact that inhibition of PAK1 decreased/blocked both intrinsic as well as PSC-stimulated PD-L1 expression by PDA cells, and thereby increased the induction of cancer cell death by cytotoxic lymphocytes, implies that PAK1 promotes PDA progression by inhibiting the anti-tumour immune reaction via modulation of PSCs and expression of PD-L1 in cancer cells. PSCs can regulate PDA cell functions by producing various growth factors and cytokines [49]. PSCs produce TGF-β to stimulate PDA cell proliferation [49]. TGF-β stimulates PD-L1 expression in both lung and breast cancer via PI3-K/AKT- and MEK/ERK- dependent signalling pathways [50-52]. This evidence suggests that PSCs could stimulate PD-L1 expression by PDA cells through secretion of factors including TGF-β.

The low positive staining of PD-L1 in the samples from both patients and KPC mice made it difficult to analyse the correlation of PD-L1 expression to PAK1 expression and to overall pancreatic cancer survival. Pancreatic cancer samples are often selected with varying disease stages, sampling times and fixation procedures [53, 54]. The fact that samples from both patients and KPC mice were collected at different time points with different disease stages could contribute to the low rate of positive staining of PD-L1 in this study.

In summary, as indicated in Fig.7, we have demonstrated that inhibition of PAK1 decreased PSC-stimulated proliferation and migration/invasion of tumour cells. More importantly, we have shown that inhibition of PAK1 suppressed the intrinsic and PSC-stimulated PD-L1 expression of tumour cells, which would, in turn, have decreased the amount of PD-L1 bound to PD-1, a receptor expressed on the surface of active T cells. The reduction in receptor-bound PD-L1 would reduce

or block the ability of tumour cells to escape anti-tumour immune surveillance mediated by PD-L1.

Conclusion

This study has revealed a crucial role for PAK1 in regulating stromal desmoplasia and the anti-tumour response in PDA. Inhibition of PAK1 not only decreased PSC activation and increased TIL, but also enhanced lymphocyte-induced tumour cell death and blocked the protective effect of PSCs via down-regulation of PD-L1. The data presented here provide important insights into the potential role of PAK1 inhibition in re-programming the TME to enhance anti-PD-1/PD-L1 immunotherapy and lay a solid basis for the development of combination therapies using PAK1 inhibitors together with immunotherapy in the management of PDA.

Ethics approval and consent to participate

The use of human tissue samples in this study was approved by the Human Research Ethics Committee at Austin Health (H2013-04953). All mouse experiments were approved by the Austin Health Animal Ethics Committee (A2016/05317).

Author Contributions

Mehrdad Nikfarjam, Yifan Zhan and Hong He conceived and designed the study; Kai Wang, Yifan Zhan, Nhi Huynh, Chelsea Dumesny, Xiao Wang, Yang Yang, Mehrdad Nikfarjam and Hong He

conducted experiments and collected data; Kai Wang, Yifan Zhan and Hong He analysed and interpreted data; Kai Wang and Hong He drafted the manuscript; Khashayer Asadi, Paul Timpson, David Herrmann and Katrina Walsh provided technical or material support; Graham Baldwin, David Herrmann and Paul Timpson provided valuable comments and suggestions to improve the manuscript and data presentation; Graham Baldwin, Mehrdad Nikfarjam and Hong He supervised the whole study; All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no potential conflicts of interest.

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Figure Legend

Fig.1. Low expression of PAK1 and α-SMA correlated with better patients' survival. The tissue microarray samples obtained from the patients described in Table 1 were stained immunohistochemically for the expression of PAK1 and α-SMA proteins. The intensity of positively stained areas was determined using computer software as described in Materials and Methods. The mean values calculated from all samples analysed were used to divide the samples into high or low expression of PAK1 or α-SMA, and representative images taken at 20x magnification, and zoomed in at 40x magnification are shown in A. Cytoplasmic expression of PAK1 is higher in cancer cells (A, arrow), and lower in surrounding stroma (A, arrowhead). Cytoplasmic and membrane expression of α-SMA is low in cancer cells (A, arrow), and higher in surrounding stroma (A, arrowhead). Low PAK1 protein expression was correlated to better survival of patients in overall (B), T3-T4 (C) and N1 (D) cohorts while low α-SMA was related to better survival of patients in T3-T4 (F) and N1 (G) cohorts, but not in the overall (E) cohort. The expression of PAK1 and α-SMA proteins was significantly correlated in the overall cohort (H).

Fig.2. High ratios of $CD8^+$ cells to $CD4^+$ cells and to PAK1 expression correlated to better patients' survival. The tissue microarray samples obtained from the patients described in Table 1 were stained immunohistochemically for $CD4^+$ and $CD8^+$ cells. The numbers of total positively stained cells were counted. The means calculated from all samples analysed were used to divide the samples into high or low $CD4^+$ and $CD8^+$, and representative images taken at 20x magnification, and zoomed in at 40x magnification, are shown in A. High ratios of $CD8^+$ to $CD4^+$ (B) and of $CD8^+$ to PAK1 (D) were correlated to better survival of patients in the overall cohort. The correlation between high $CD8^+$ and overall survival of patients was not significant difference (C).

Fig.3. PAK1 depletion extended mouse survival of pancreatic cancer and increased tumour infiltrating $CD4^+$ and $CD8^+$ T cells and reduced the expression of α-SMA and desmin proteins. PAK1 wild type (WT), heterozygous (het) and knockout (KO) mice were established on a KPC background and monitored according to health score as approved by the Austin Health Animal Ethics Committee. Mice survival (A) was calculated as described in Materials and Methods. Both KPC PAK1 KO and KPC PAK1 Het mice survived significantly longer than KPC PAK1 WT mice. Pancreatic tumour tissues collected from KPC PAK1 WT and KPC PAK1 KO mice were stained for $CD4^+$ and $CD8^+$ cells (B&C), and PAK1, α-SMA and desmin proteins (D-F). The images in B and D were taken at 20x magnification and zoomed in at 40x magnification. *, $p < 0.05$, **, $p < 0.01$, compared with the values obtained from KPC PAK1 WT mice.

Fig.4. Tumour growth was decreased and intra-tumoral T cells were increased in pancreatic tail tumours in PAK1 knockout mice. Pancreatic tail tumours were induced in 6 PAK1 wild type (WT) and 6 PAK1 knockout (KO) mice. After 4 weeks the mice were culled, and tumours isolated and weighed. Single-cell suspensions were prepared from tumour tissues and intra-tumoral lymphocytes were analysed as described in Materials and Methods. PAK1 KO reduced tumour growth (A) and increased the tumour infiltration of $CD3^+$, $CD4^+$ and $CD8^+$ T cells (B, C). *, $p<0.05$; **, $p<0.01$; compared to the values obtained from PAK1 WT mice. The differences in PD-1 expression between PAK1 WT and KO mice did not reach statistical significance although much lower levels of PD-1 were observed in CD4 and CD8 T cells in one PAK1 KO mouse (C).

Fig.5. Inhibition of PAK1 sensitized cancer cells to cytotoxic lymphocytes by down-regulation of PD-L1 expression in the cancer cells. PAK1 wild type (WT, 3 clones) and knockout (KO, 3 clones) PDA cells were isolated from the pancreatic tumours of KPC mice, and PD-L1, PAK1 and GAPDH proteins were measured by Western blot (A&B). PD-L1expression was significantly lower in PAK1 KO clones; **, $p < 0.01$, compared to the values obtained in PAK1 WT clones. PAK1 knockdown (KD) clones of the human pancreatic cancer cell line PANC-1 (C) were generated as previously described[16]. The PAK1 WT pancreatic cancer cell line TB33117 (TB) was treated with the PAK1/4 inhibitor PF-3758309 (PF) and the expression of active phosphorylated PAK1 (pPAK1) and PD-L1 was determined by Western blot (D). *, $p<0.05$, ***, $p<0.001$ compared to values obtained from no PF treated cells. Luciferase-labelled TB33117 cells were pre-treated with PF for 24h and then co-cultured with activated lymphocytes for another 24h as indicated in E. TB33117 cell survival was measured by luciferase activity and the value obtained from cancer cells treated with neither PF nor lymphocytes was taken as 100% (F). **, $p < 0.01$, compared to the values obtained in cells without PF-treatment and without lymphocyte co-culture. #, $p < 0.05$, ##, $p < 0.01$, compared to the values obtained from TB33117 cells pre-treated with PF before co-culture with activated lymphocytes (F).

Fig.6. PAK1 deletion inhibited PSC-stimulated PDA cell proliferation, migration/invasion and blocked PSC protection of PDA cells from killing by cytotoxic lymphocytes by inhibiting PSC-stimulated PD-L1 expression in cancer cells. PSCs (A) isolated from the pancreatic tumours (labelled with asteroid mark) of KPC PAK1 WT and KPC PAK1 KO mice were characterized by Western blot (B) and incubated with luciferase-labelled TB33117 (TB) cells at different ratios (C). Cancer cell proliferation was assayed by luciferase activity, and the values

obtained in the absence of PSCs were taken as 1 (C). ^, $p < 0.05$ compared to the values obtained from cancer cells incubated with PSCs isolated from KPC PAK1 WT mice. PSC-stimulated PDA cell migration/invasion were evaluated using a Boyden chamber assay as described in Materials and Methods (D&E). *, $p<0.05$, **, $P<0.01$ comparison as shown. PDA cells isolated from KPC PAK1 WT mice (WT942), or TB33117 cells, were incubated either with untreated media (CT) or with media conditioned by PSCs isolated from KPC PAK1 WT (WT) or KPC PAK1 KO (KO) mice, and PD-L1 protein expression was measured by Western blot (F). *, $p<0.05$, ***, $P<0.001$ compared to untreated media (CT). The luciferase-labelled TB cells were incubated with PSCs isolated from KPC PAK1 WT or KPC PAK1 KO mice for 24 h before co-culture with activated lymphocytes for another 24 h (G). Cell survival was measured by relative luciferase activity and the value obtained from the cancer cells treated with neither PSCs nor lymphocytes was taken as 100% (H). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the values obtained from the cells cultured without lymphocytes. ##, $p < 0.01$, ###, $p < 0.001$, compared to WT PSC as shown.

Fig. 7 Regulation by PAK1 of tumour and stroma of pancreatic cancer in the anti-tumour immune response. Inhibition of PAK1 increased apoptosis and decreased the proliferation of pancreatic cancer-associated stellate cells (PSC) [17]. Inhibition of PAK1 also suppressed PSC-stimulated proliferation and migration/invasion of tumour cells. Inhibition of PAK1 suppressed both the intrinsic and PSC-stimulated (presumably via some soluble factors) PD-L1 expression of tumour cells, and thus reduced the amount of PD-L1 bound to PD-1, a receptor on the surface of active T cells. As a result of the reduction in receptor-bound PD-L1, the ability of a tumour cell to escape the anti-tumour immune surveillance by a PD-1-mediated mechanism would be inhibited.

Fig.1

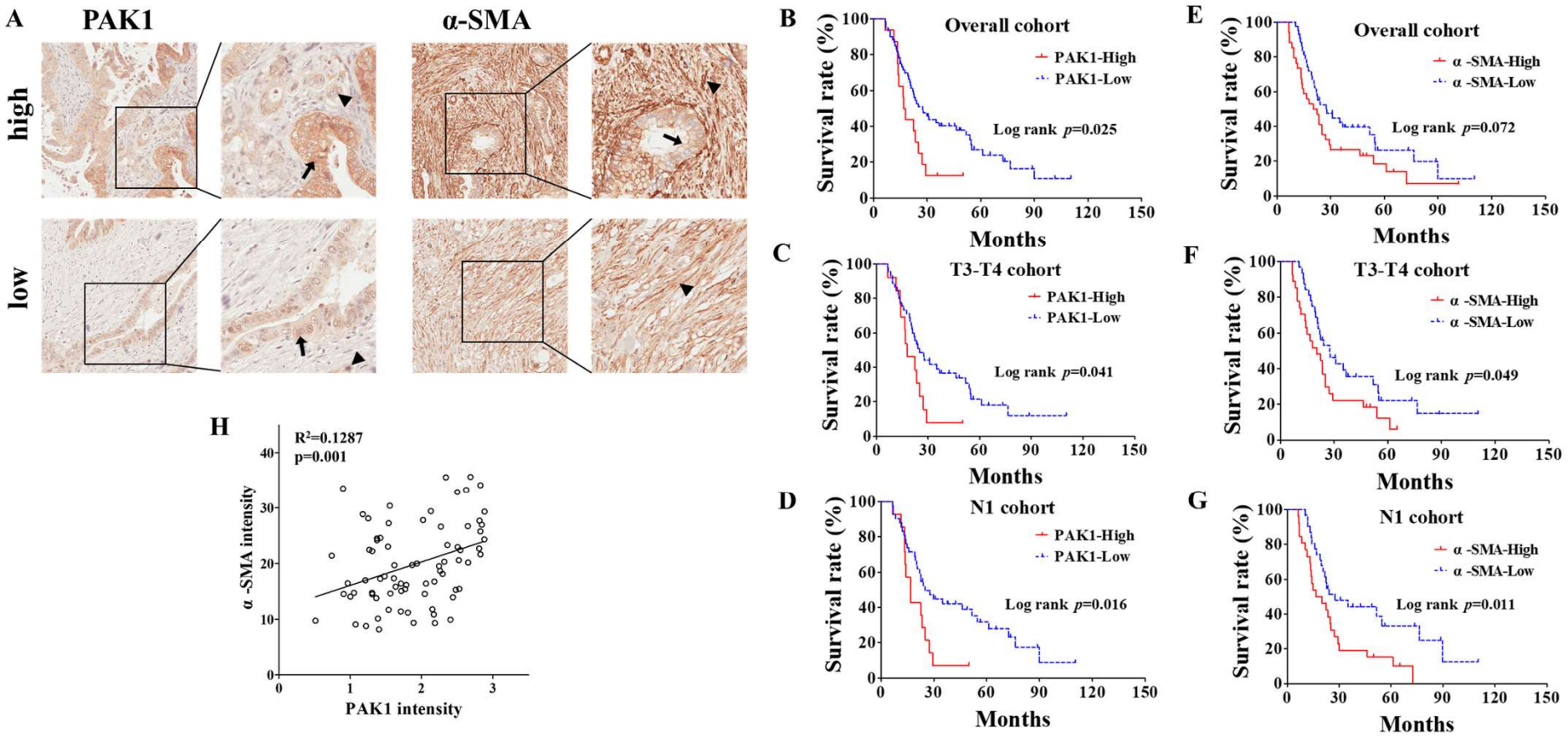

A
PAK1
α-SMA
high
low
B
Overall cohort
PAK1-High
PAK1-Low
Log rank p=0.025
Survival rate (%)
Months
C
T3-T4 cohort
PAK1-High
PAK1-Low
Log rank p=0.041
Survival rate (%)
Months
D
N1 cohort
PAK1-High
PAK1-Low
Log rank p=0.016
Survival rate (%)
Months
E
Overall cohort
α -SMA-High
α -SMA-Low
Log rank p=0.072
Survival rate (%)
Months
F
T3-T4 cohort
α -SMA-High
α -SMA-Low
Log rank p=0.049
Survival rate (%)
Months
G
N1 cohort
α -SMA-High
α -SMA-Low
Log rank p=0.011
Survival rate (%)
Months
H
R²=0.1287
p=0.001
α -SMA intensity
PAK1 intensity

Fig.2

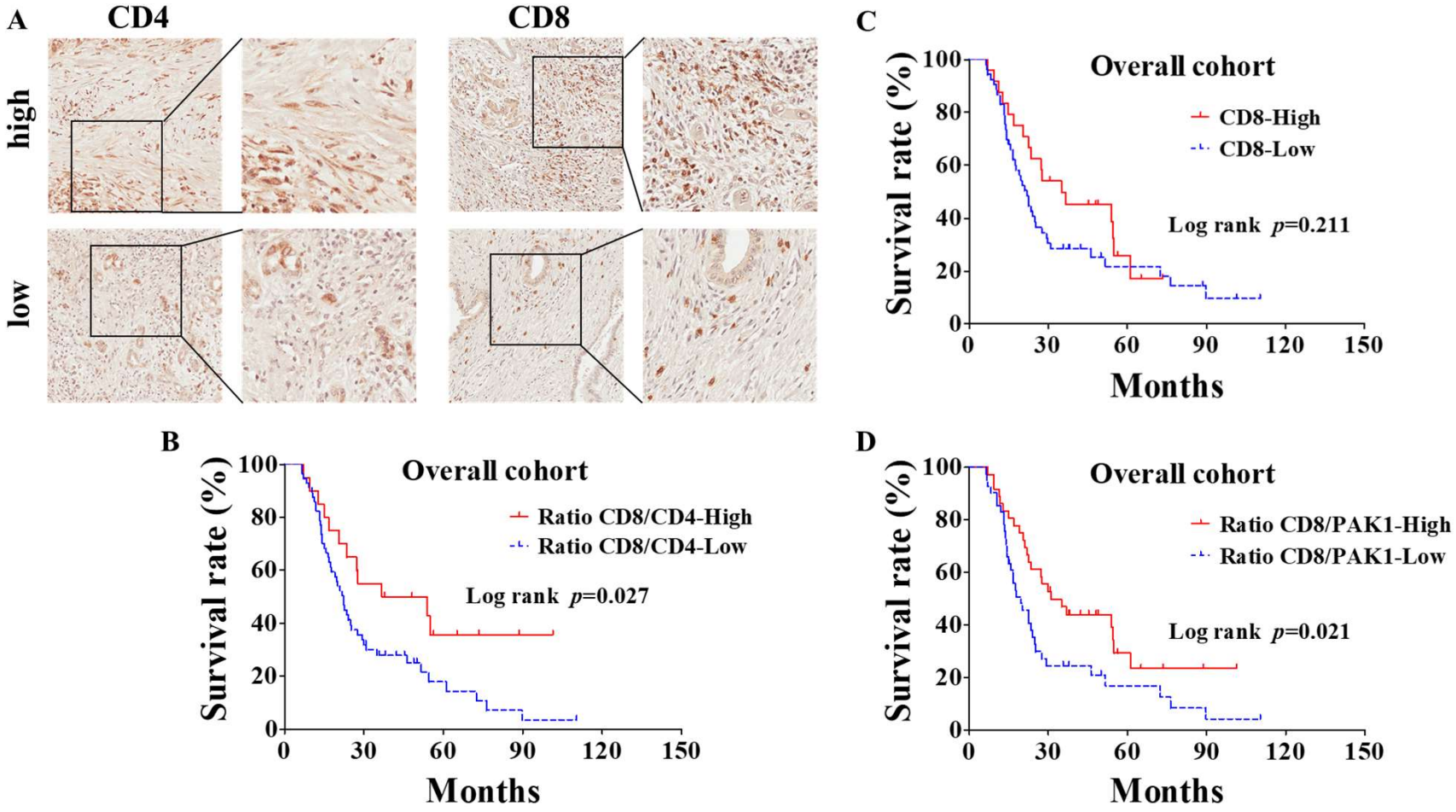

A
CD4
CD8
high
low
B
Overall cohort
Ratio CD8/CD4-High
Ratio CD8/CD4-Low
Log rank p=0.027
Survival rate (%)
Months
C
Overall cohort
CD8-High
CD8-Low
Log rank p=0.211
Survival rate (%)
Months
D
Overall cohort
Ratio CD8/PAK1-High
Ratio CD8/PAK1-Low
Log rank p=0.021
Survival rate (%)
Months

Fig.3

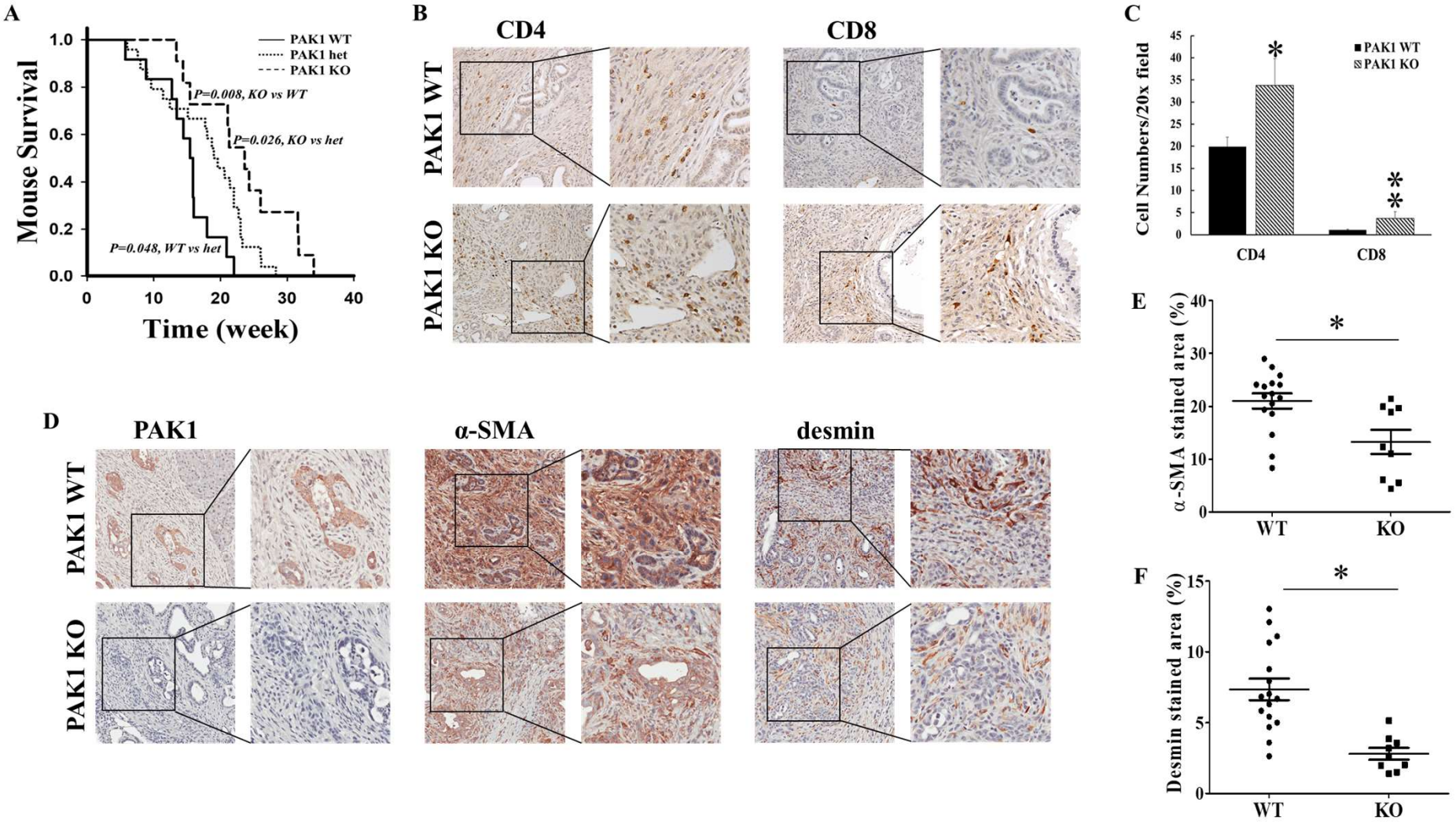

A
Mouse Survival
Time (week)
PAK1 WT
PAK1 het
PAK1 KO
P=0.008, KO vs WT
P=0.026, KO vs het
P=0.048, WT vs het
B
CD4
CD8
PAK1 WT
PAK1 KO
C
Cell Numbers/20x field
PAK1 WT
PAK1 KO
CD4
CD8
D
PAK1
α-SMA
desmin
PAK1 WT
PAK1 KO
E
α-SMA stained area (%)
WT
KO
F
Desmin stained area (%)
WT
KO

Fig.4

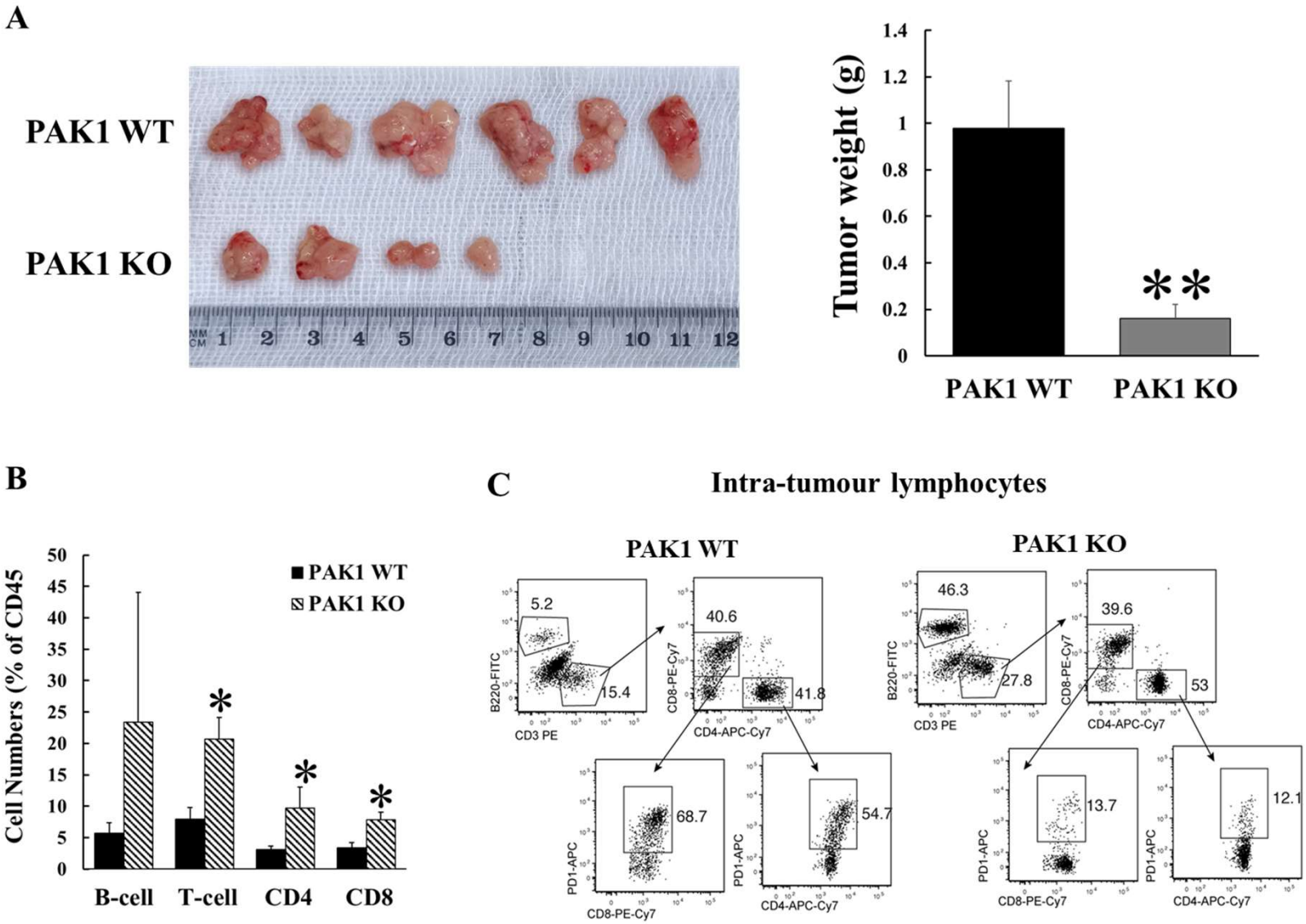

A
PAK1 WT
PAK1 KO
MM CM 1 2 3 4 5 6 7 8 9 10 11 12
Tumor weight (g)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
PAK1 WT
PAK1 KO
B
Cell Numbers (% of CD45
50
45
40
35
30
25
20
15
10
5
0
PAK1 WT
PAK1 KO
*
*
*
B-cell
T-cell
CD4
CD8
C
Intra-tumour lymphocytes
PAK1 WT
PAK1 KO
5.2
15.4
40.6
41.8
68.7
54.7
46.3
27.8
39.6
53
13.7
12.1
B220-FITC
CD3 PE
CD8-PE-Cy7
CD4-APC-Cy7
PD1-APC

Fig.5

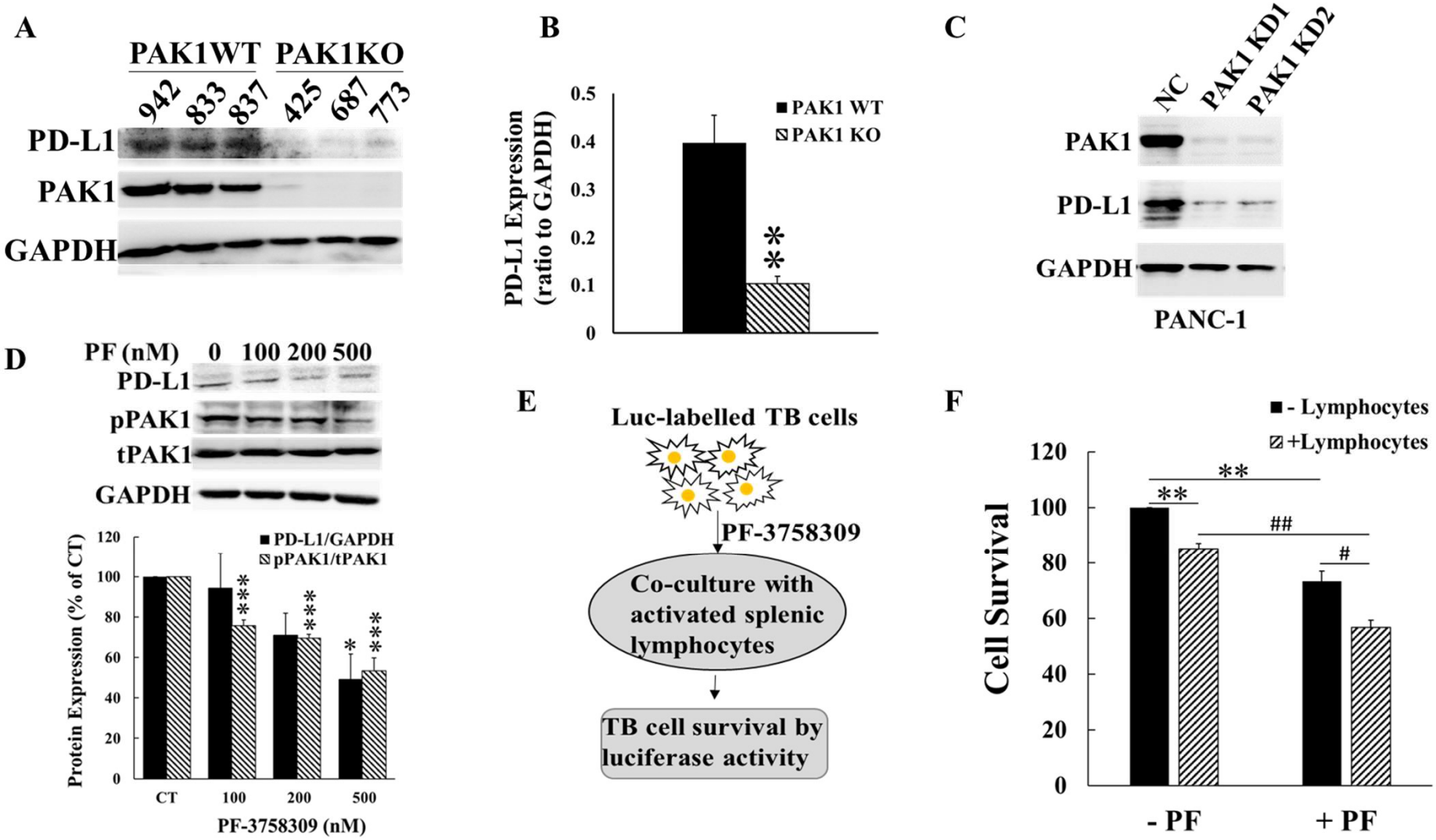

A
PAK1WT
PAK1KO
942
833
837
425
687
773
PD-L1
PAK1
GAPDH
B
PD-L1 Expression (ratio to GAPDH)
PAK1 WT
PAK1 KO
C
NC
PAK1 KD1
PAK1 KD2
PAK1
PD-L1
GAPDH
PANC-1
D
PF (nM) 0 100 200 500
PD-L1
pPAK1
tPAK1
GAPDH
PD-L1/GAPDH
pPAK1/tPAK1
Protein Expression (% of CT)
CT
100
200
500
PF-3758309 (nM)
E
Luc-labelled TB cells
PF-3758309
Co-culture with activated splenic lymphocytes
TB cell survival by luciferase activity
F
- Lymphocytes
+Lymphocytes
Cell Survival
- PF
+ PF

Fig.6

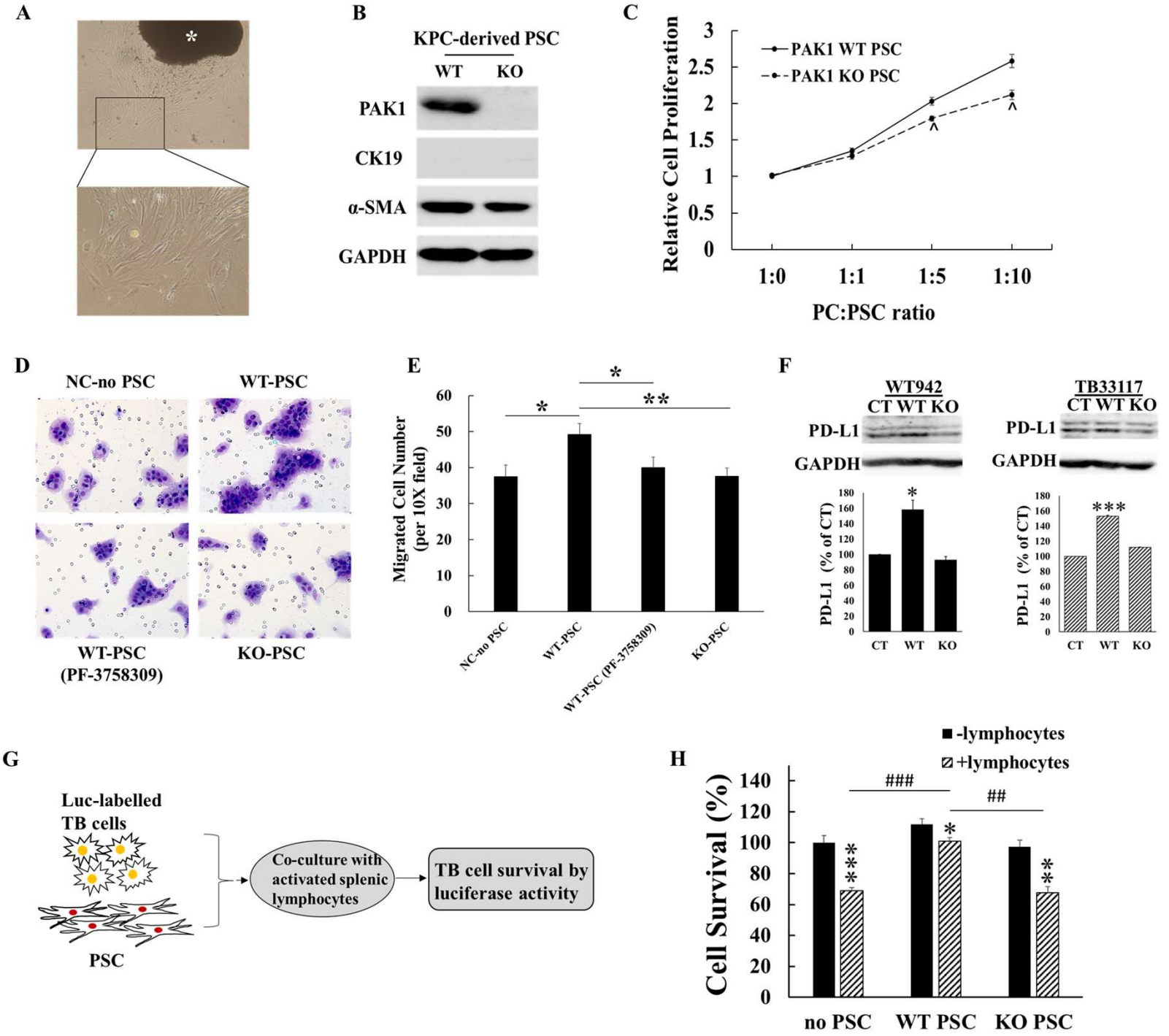
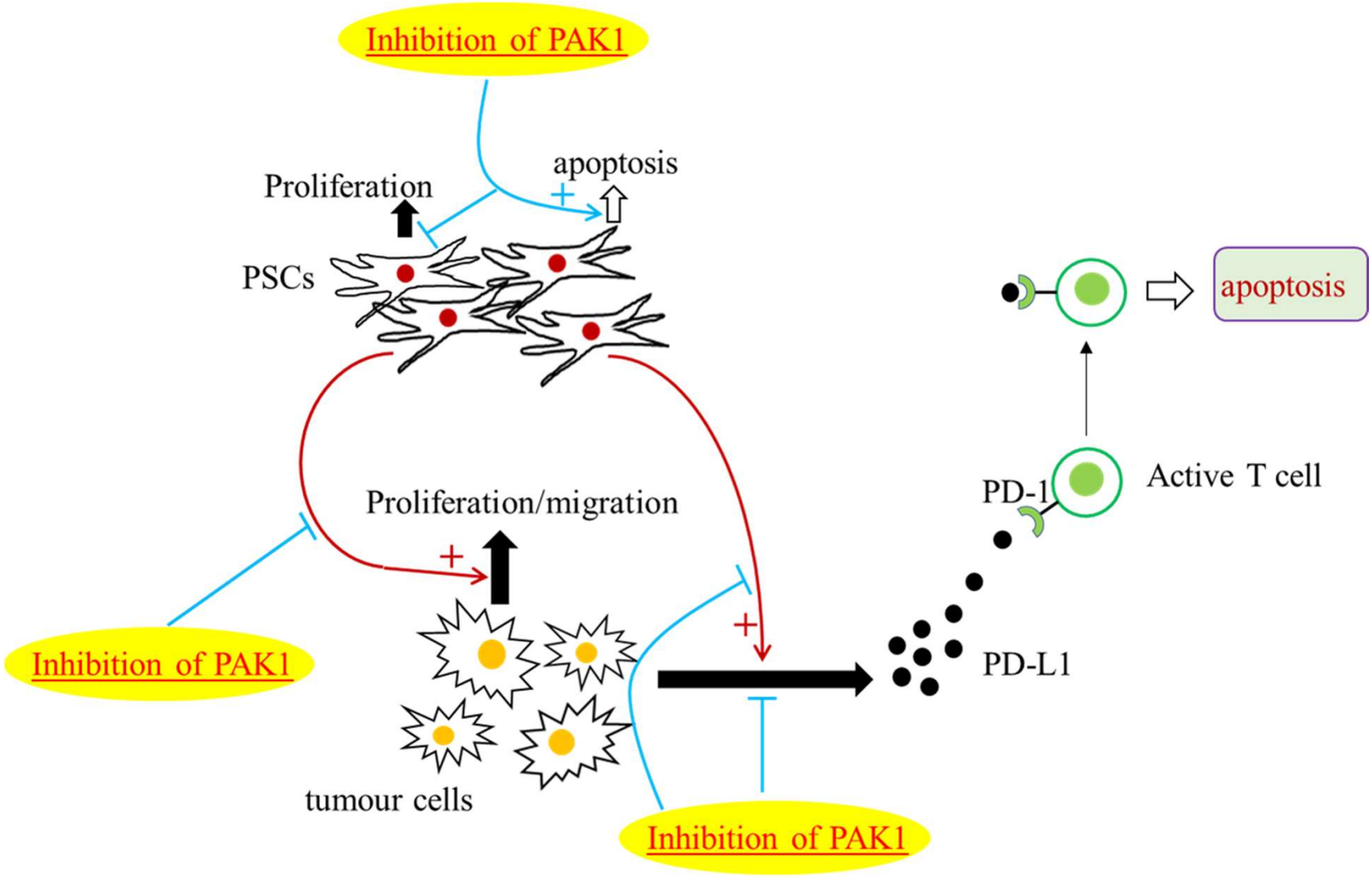

A
B
KPC-derived PSC
WT
KO
PAK1
CK19
α-SMA
GAPDH
C
Relative Cell Proliferation
PAK1 WT PSC
PAK1 KO PSC
PC:PSC ratio
1:0
1:1
1:5
1:10
D
NC-no PSC
WT-PSC
WT-PSC (PF-3758309)
KO-PSC
E
Migrated Cell Number (per 10X field)
NC-no PSC
WT-PSC
WT-PSC (PF-3758309)
KO-PSC
F
WT942
TB33117
CT WT KO
PD-L1
GAPDH
PD-L1 (% of CT)
G
Luc-labelled TB cells
PSC
Co-culture with activated splenic lymphocytes
TB cell survival by luciferase activity
H
-lymphocytes
+lymphocytes
Cell Survival (%)
no PSC
WT PSC
KO PSC

Fig.7


Inhibition of PAK1
Proliferation
apoptosis
PSCs
apoptosis
Proliferation/migration
PD-1
Active T cell
Inhibition of PAK1
PD-L1
tumour cells
Inhibition of PAK1