Dissecting the stromal signaling and regulation of myeloid cells and memory T cells in pancreatic cancer

Alex B Blair[1, 2, 3], Victoria Kim[1, 2, 3], Stephen Muth[1, 2], May Tun Saung[1, 2, 4], Nathalie Lokker[5], Barbara Blouw[6], Todd D. Armstrong[1, 2], Elizabeth M Jaffee[1, 2, 4], Takahiro Tsujikawa[7, 8, 9], Lisa Coussens[8, 9], Jin He[1, 2, 3], Richard A Burkhart[1, 2, 3], Christopher L Wolfgang[1, 2, 3, 4], Lei Zheng[1, 2, 3, 4]

SUPPLEMENTAL MATERIALS:

METHODS:

Cell Medium:

KPC cells were maintained in Roswell Park Memorial Institute (RPMI) medium (LifeTechnologies), 10% fetal bovine serum (AtlasBiologicals), 1% L-glutamine (LifeTechnologies), 1% penicillin/streptomycin (LifeTechnologies), 1% sodium pyruvate (LifeTechnologies), and 1% nonessential amino acids (LifeTechnologies). B78H1-GM cells were maintained in RPMI (LifeTechnologies), 10% fetal bovine serum (AtlasBiologicals), 0.5% L-glutamine (LifeTechnologies) and 1% penicillin/streptomycin (LifeTechnologies). Immune analyses were performed using CTL medium consisting of RPMI (LifeTechnologies), 10% fetal bovine serum (AtlasBiologicals), 1% L-glutamine (LifeTechnologies), 0.5% penicillin/streptomycin (LifeTechnologies) and 0.1% 2-mercaptoethanol (LifeTechnologies).

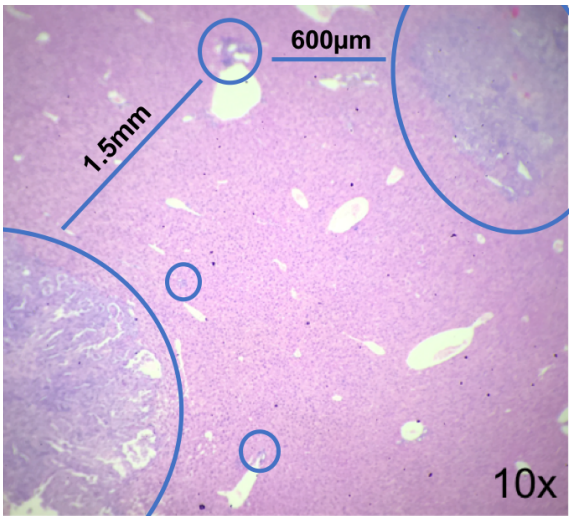
Quantitative real time reverse transcription polymerase chain reaction

TRIzol Reagent (ThermoFisher) was used to extract total RNA from isolated $CD8^+$, $CD11b^+$, and FAP^+ cells. The RNA was then converted to cDNA using the Superscript III First Strand Synthesis Supermix Kit (ThermoFisher). Quantitative real-time RT-PCR (qPCR) was performed on the StepOnePlus Real Time PCR System (ThermoFisher) and analyzed by the StepOne software V2.1. Expression of *Ifnγ*, *Cxcl12*, *Cxcr4*, and *Ccr7* were measured by SYBR

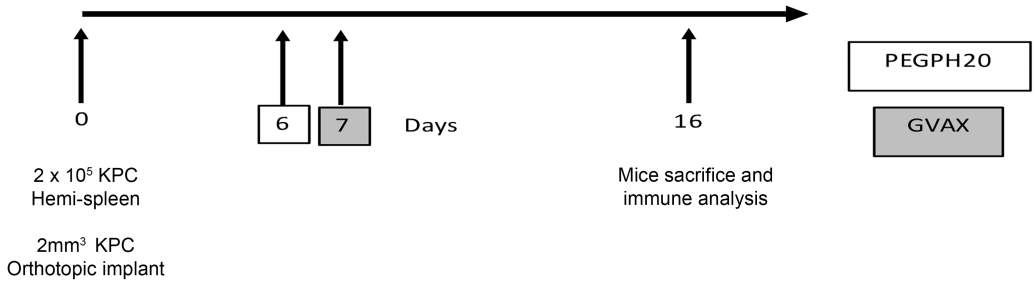
Green-based qPCR. Gene expression was normalized to the expression of *β-actin*. All PCR reactions were performed in triplicate.

Mouse IFNγ Enzyme-Linked Immunosorbent Assays

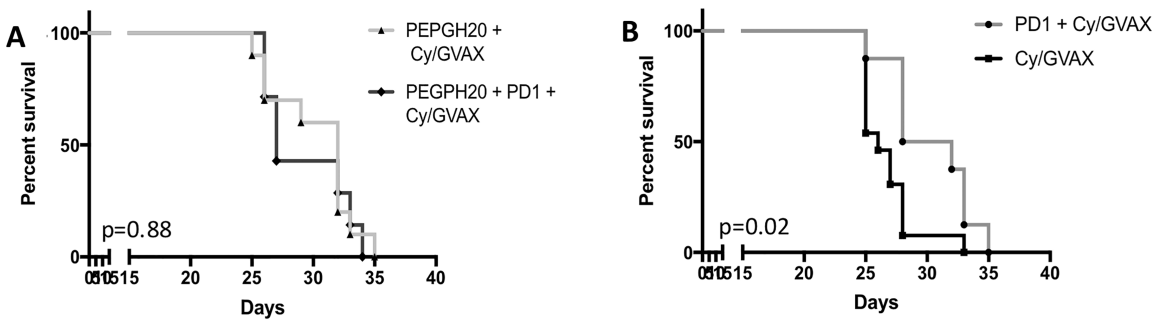
KPC cells were irradiated with 50 Gy, added to $CD8^+$ T-cells at a ratio of 5:1 ($2x10^5$ $CD8^+$ T-cells:$4x10^4$ irradiated KPC tumor cells) and incubated for 18 hours at 37° C. Mouse IFNγ Enzyme-Linked Immunosorbent Assay (ELISA) Ready-Set-Go assay was conducted per the manufacturer's protocol (eBioscience).



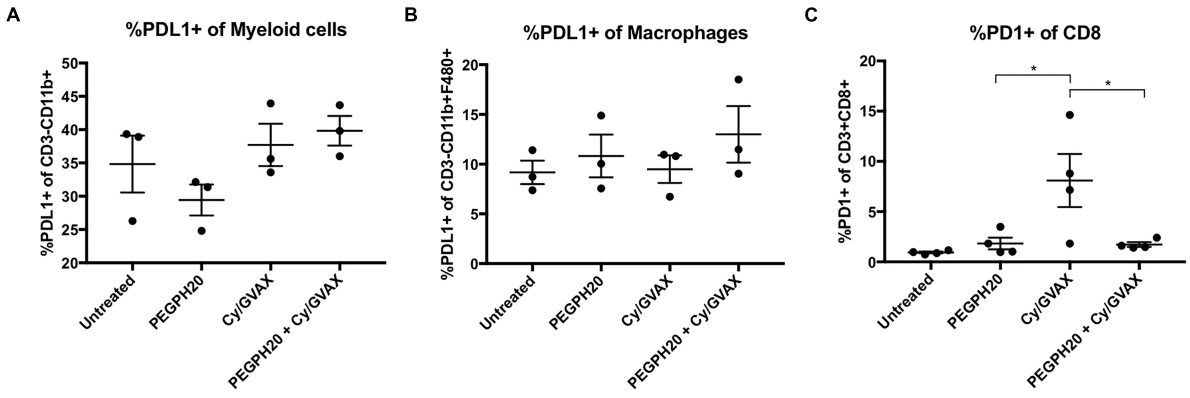
Supplemental Figure 1. Pathologic representation of diffuse liver metastases following the hemispleen procedure with KPC cells. Mice were inoculated with PDAC KPC tumor cells by the hemispleen procedure. Mice were sacrificed on Day 16 and liver harvested, sectioned and embedded in paraffin. Hematoxylin and Eosin staining was performed. 10x magnification shown.



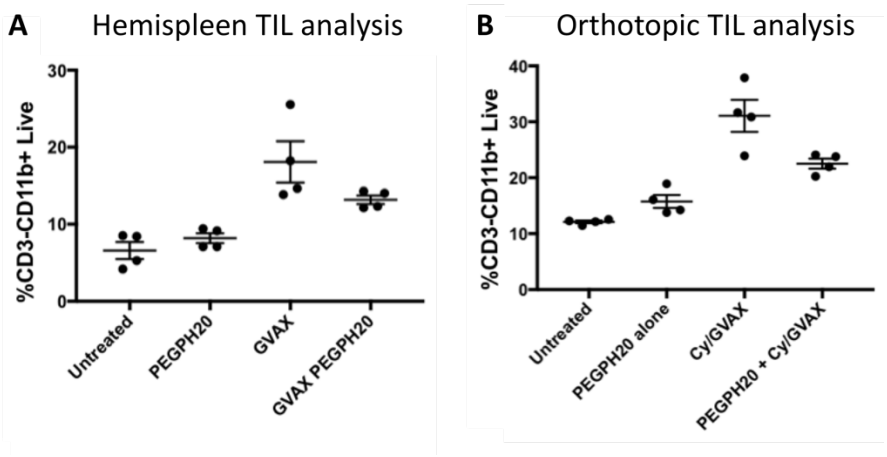
Supplemental Figure 2. Schematic representation of KPC tumor inoculation and treatment schema. Following orthotopic tumor implantation or the development of diffuse liver metastases by the hemispleen procedure, mice were treated with immunomodulatory Cyclophosphamide (100 mg/kg) on Day 6, PEGPH20 (40μg/kg) on Day 6 and GVAX on Day 7. Mice were sacrificed on day 16 for immune analysis. Survival experiments continued until death or survival endpoints were met.



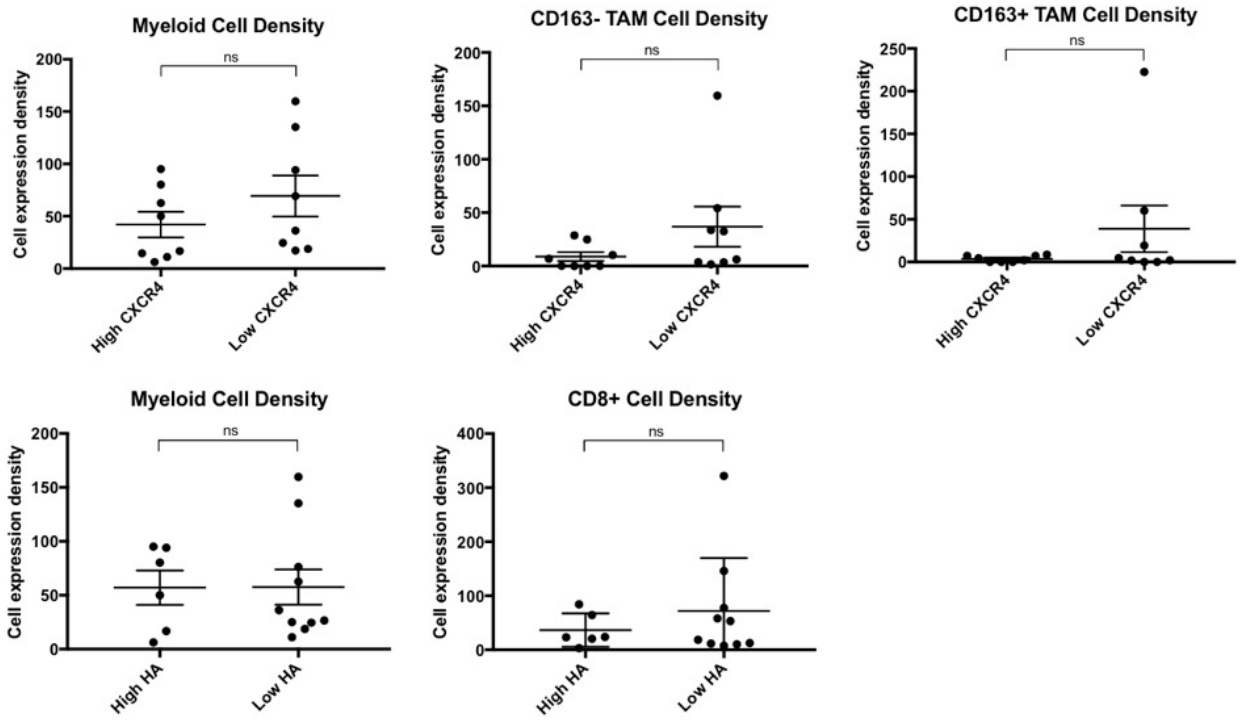
Supplemental Figure 3. Survival of tumor bearing mice following. (**A**) Kaplan Meier survival curves of mice implanted with PDAC cells and treated with the PEGPH20/GVAX combination treatment (n=8) or PEGPH20/GVAX and anti-PD1 antibody triple combination treatment (n=8), (**B**) GVAX alone (n=8) or GVAX and anti-PD1 antibodies (n=7).



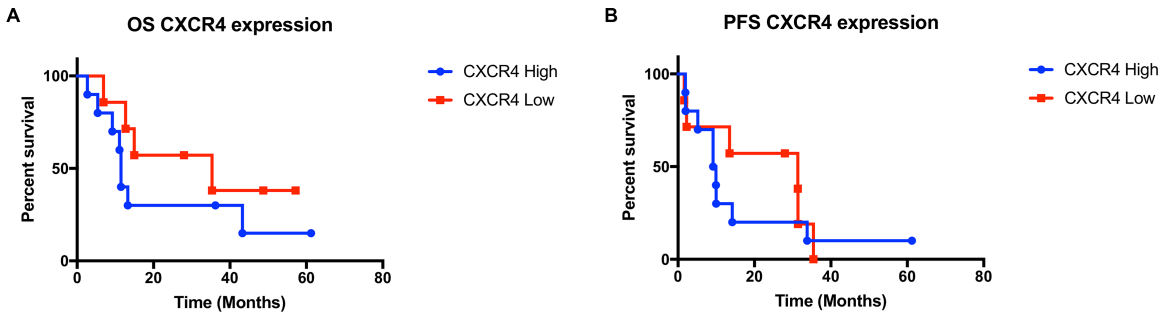
Supplemental Figure 4. Flow cytometry assessment of the expression of PD1/PD-L1 pathway in tumor infiltrating lymphocytes of KPC tumor-bearing mice. Following the inoculation of KPC tumor cells by orthotopic implantation, mice were treated with Cyclophosphamide (100 mg/kg) on Day 6, PEGPH20 (40μg/kg) on Day 6 and GVAX on Day 7. Flow cytometry was performed on tumor infiltrating lymphocytes (TIL) isolated from processed dissected orthotopic tumor. Each experimental group consisted of 3 or 4 mice analyzed individually. Relative percentage of (**A**) PD-L1 expression among total myeloid cells ($CD3^-$ $CD11b^+$) (**B**) PD-L1 expression among total macrophages ($CD3^-CD11b^+F480^+$) (**C**) PD1 expression among total $CD3^+CD8^+$ cells.



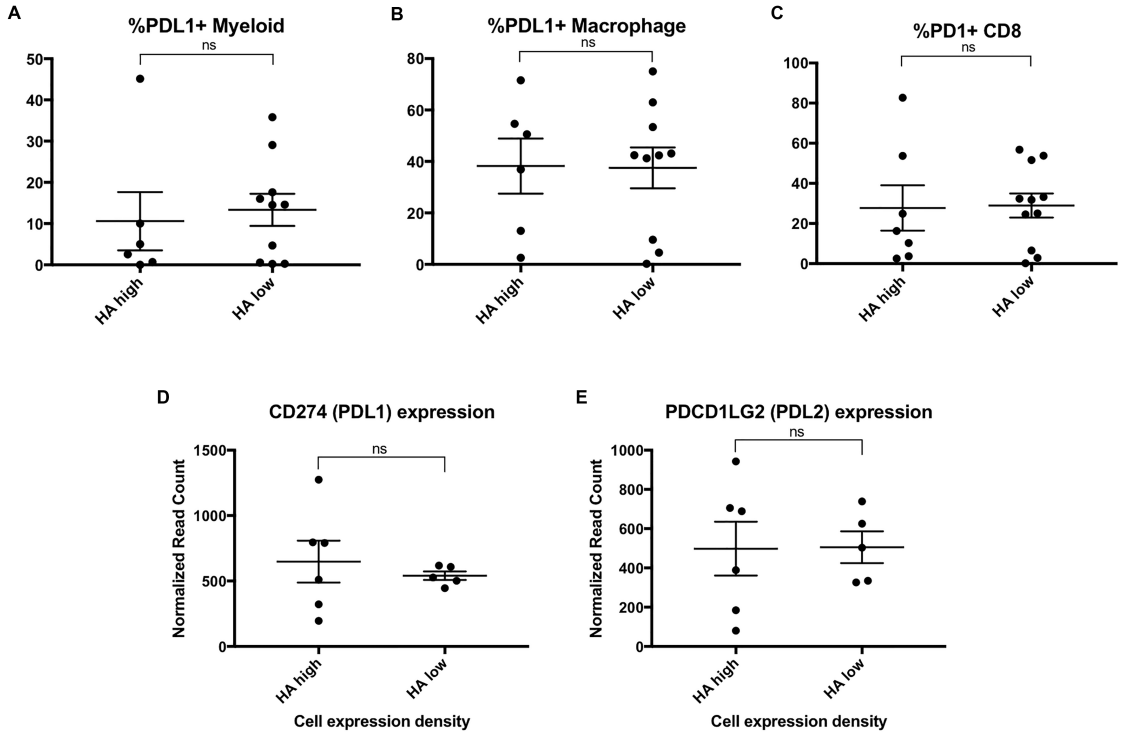
Supplemental Figure 5. Flow cytometry assessment of myeloid cells in KPC tumor-bearing mice. Flow cytometry was performed on tumor infiltrating lymphocytes (TIL) isolated from (**A**) processed murine liver of hemispleen mice and (**B**) dissected pancreatic tumor from the orthotopic model. Percentage of $CD3^{-}CD11b^{+}$ cells of total live cells as determined by immune analysis of 4 mice analyzed individually by flow cytometry.



Supplemental Figure 6. Multiplex immunohistochemistry assessment of myeloid cell density in GVAX treated human PDAC tissue. Immunohistochemistry (IHC) staining of CXCR4 and HA was performed. (**A**) Analysis of the density of Myeloid cells, (**B**) CD163- Tumor Associated Macrophage (TAM) cells, and (**C**) CD163+ TAM cells density as determined by multiplex IHC in GVAX treated PDAC patients with high stromal CXCR4 expression compared to patients with low CXCR4 expression. (**D**) Analysis of the density of Myeloid cells and (**E**) CD8+ cells density as determined by multiplex IHC in GVAX treated PDAC patients with high HA expression compared to patients with low HA expression. ns= not significant



Supplemental Figure 7. Survival comparison of GVAX treated resected human PDAC. Human PDAC tissue were collected from patients treated with one dose of neoadjuvant GVAX and subsequent surgical resection at our institution and immunohistochemistry staining of CXCR4 was performed (n=17). CXCR4 high and low groups were determined by IHC cell expression density. Kaplan Meier survival estimates for (**A**) Overall survival and (**B**) Progression free survival comparing the subgroups of CXCR4 high and CXCR4 low tumor expression. Not significant.



Supplemental Figure 8. Assessment of the expression of PD1/PD-L1 pathway in GVAX treated resected human PDAC. Human PDAC tissue were collected from patients treated with one dose of neoadjuvant GVAX and subsequent surgical resection at our institution and immunohistochemistry staining of HA was performed (n=15). HA high and low groups were determined by IHC cell expression density. The percentage of total cell subsets, as determined by multiplex IHC, were compared between high vs low HA expression groups, including: (**A**) the relative percentage PD-L1 expression among myeloid cells, (**B**) the relative percentage PD-L1 expression among macrophages and (**C**) the relative percentage PD1 expression among $CD8^+$ T cells. Whole exome RNA sequencing was performed on dissected pancreatic stroma of the same cohort of patients (n=17). Whole exome RNA sequencing was performed on dissected pancreatic stroma of the same cohort of patients (n=17). Normalized RNA expression of (**D**) *CD274* and (**E**) *PDCD1LG2* were compared between the high vs low HA expression groups. Data represent mean ± SEM. Not significant.