Role of Cancer Associated Fibroblast Heterogeneity on Immunotherapeutic Potentials of Pancreatic Ductal Adenocarcinoma

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Acknowledgments

This experiment was conducted as a part of the iResearch Institute program beginning in July 2021.

Madhav Subramanian provided the foundational background surrounding the bioinformatics tools used as well as key concepts explored in the presented study. Additionally, he assisted in verifying that the conclusions of the study were valid and extrapolated correctly. Throughout the research process, Madhav was an incredible mentor and teacher.

Dr. McCalla and the iResearch Institute team taught general research procedures, statistical analysis techniques, and research paper and presentation expectations. Dr. McCalla helped lay the foundation for the presented paper.

Abstract:

Pancreatic Ductal Adenocarcinoma (PDAC) has the poorest prognosis of all cancers with a mere 9% 5-year survival rate. The aggressiveness of PDAC is attributed to an abundance of heterogeneous cancer associated fibroblasts (CAFs) which regulate tumor promotion and immunotherapeutic response. The purpose of the presented study was to delineate CAF functional heterogeneity in immune cell regulation to identify subsets of patients that could respond favorably to immunotherapies. Using bulk RNA-sequencing data, five population-level clusters of patients were identified based on differential presence of fibroblast subpopulations. Patients in the population level clusters had significantly different immune cell concentrations, cytolytic scores, and PD-L1 expression, demonstrating the possible differential responses to therapy. Further analysis was conducted on CAF subtypes to determine whether high concentrations correlated with an immunosuppressive environment. Of the four subtypes analyzed, high expressions of two CAF subtypes correlated with significantly poorer survival rates and lower expressions of CD4 T cells, indicating a pro-tumoral and immunosuppressive role. Interestingly, high expressions of the remaining two subtypes correlated with significantly higher PD-L1 expression and cytolytic score, illustrating a potentially better immunotherapeutic response. The results identify CAF subpopulations as immunotherapeutic targets that allow for more personalized, tumor-specific treatment which can significantly improve survival.

Introduction:

Pancreatic Ductal Adenocarcinoma (PDAC) remains one of the deadliest cancers worldwide [1]. By 2030, PDAC deaths are projected to increase rapidly, becoming the second leading cause of cancer death [2]. The low survival rates are primarily due to late detection and ineffective treatments. More than 80% of PDAC tumors are unresectable and only 20% of patients respond to chemotherapy [3, 4]. Synergistic therapies combining chemotherapy and immunotherapies have shown promise. One study found that PDAC patients receiving a combination of dendritic cell-based immunotherapy and gemcitabine chemotherapy had prolonged survival [5, 6]. Immunotherapies such as Programmed Death-ligand 1 (PD-L1) checkpoint blockade have had encouraging results in many malignant cancers. In a recent study, overall survival estimates doubled in Non-Small-Cell Lung Cancer patients treated with anti-PD-L1 therapy [7]. However, the overall efficacy of immunotherapy in PDAC has been limited with checkpoint inhibition receiving approval for only 1-2% of tumors [8]. To further improve the overall survival of PDAC patients, target biomarkers that allow for improved response to immunotherapies need to be identified.

The minimal response to immunotherapies in PDAC is primarily due to the desmoplastic stroma and fibrosis which compose 90% of tumor volume [8]. Stroma directly results from cancer associated fibroblasts (CAFs) which deposit an excessive amount of extracellular matrix (ECM) components in the tumor microenvironment (TME) [9]. The immense fibrosis creates a biophysical barrier that prevents chemotherapy drug penetration and inhibits cytotoxic T cell infiltration [9, 10]. Minimal cytotoxic T cell infiltration correlates with poor immunotherapeutic outcomes [9]. Targeting the highly fibrotic dense stroma could provide for more effective drug delivery and prolonged survival. A recent study found that the inhibition of a signaling component associated with CAFs and poor CD8+ T cell infiltration resulted in improved response to immune checkpoint therapy and better overall survival [11]. CAFs secrete immunosuppressive cytokines, growth factors, and immune checkpoint ligands which cause immune escape and inhibit therapeutic efficacy [9]. Understanding the underlying mechanisms behind CAFs is vital for the development of anti-tumoral drug therapies.

Recent studies have explored the effect of intratumoral heterogeneity of CAFs on tumor progression and immune infiltration. Four primary fibroblast-like CAF subpopulations have been identified. Inflammatory CAFs (C0 CAFs) regulate cytokine and chemokine secretion [4]. Myofibroblastic CAFs (C3 CAFs) are characterized by increased expression of smooth muscle actin, transforming growth factor signaling, and ECM [4]. Highly metabolic CAFs (C4 CAFs) activate glycolysis and mitochondrial translation [4]. Weakly antigen-presenting CAFs (C5 CAFs) express MHC Class II and CD74 antigens [4]. The heterogeneous functionality of CAF subpopulations could differentially affect immunotherapeutic response in PDAC patients [4]. The presented study utilized RNA-seq data to analyze the role of CAF functional heterogeneity in immune cell regulation and overall survival. The results support that targeting specific subpopulations of CAFs may allow for more effective, personalized immunotherapies leading to better overall survival.

Materials and Methods:

Dataset and Normalization

PDAC bulk RNA-seq dataset was obtained from The Cancer Genome Atlas (TCGA, available online: https://cancergenome.nih.gov/). The raw counts data was normalized using the BiocManager package, DESeq2 library, and SummarizedExperiment library of R in RStudio. Marker genes of CAF subpopulations were obtained from Supplementary Table S6 assembled by [4].

Survival Analysis

The median expression value for CAF expression served as the cutoff for patients with a high-expression versus low-expression of CAF subclusters. Log-rank tests were performed and Kaplan-Meier survival curves were plotted in Prism Graphpad in order to determine differential survival between high-expression and low-expression groups. Survival analysis was further performed on five population-level clusters of patients based on the differential presence of distinct fibroblast subsets.

Calculation of Relative Immune Cell Concentrations

Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) accurately estimates immune cell concentrations in tumors using expression profiles of purified leukocyte subsets and support vector regression [12]. CIBERSORT was used to determine the relative concentrations of 22 immune cell subtypes in the TME of PDAC patients.

Classification of Population-Level Clusters

Gene Set Variation Analysis (GSVA) is an unsupervised method of assessing gene set enrichment and estimating the variation of pathway activity [13]. The GSVA library in R was used to determine five population-level clusters of patients that contain the differential expression of multiple CAF subclusters. The pheatmap library in R was used to delineate and graphically interpret the population level clusters.

Cytolytic Score

Cytolytic score (CYT score) is an index for cancer immunity as it correlates with CD8+ T cell infiltration [14]. The CYT score was calculated by taking the geometric mean of mRNA expression levels of granzyme (GZMA) and perforin (PRF1).

$$\text{CYT Score} = \sqrt{GZMA \times PRF1}$$

PD-L1 Expression

PD-L1 Expression is a predictive biomarker to determine the efficacy of immune checkpoint blockade [15]. PD-L1 expression was determined and organized using Microsoft Excel.

Statistical Analysis and Modeling

Microsoft Excel was used to organize and classify data. Results of each test were analyzed and plotted in Prism Graphpad using unpaired t-tests and one-way ANOVA analysis. A p-value of less than 0.05 was considered to be significant.

Results

Considering that CAFs are functionally heterogeneous and highly linked to the TME of PDAC, the relationship between fibrosis and immune cells or the stromal-immune crosstalk could affect the overall survival of PDAC patients. Understanding the effect of different CAF subpopulations could allow for greater insights into favorable tumor microenvironments and responses to immunotherapies.

C0 CAF Subpopulation Analysis

Figure 1.

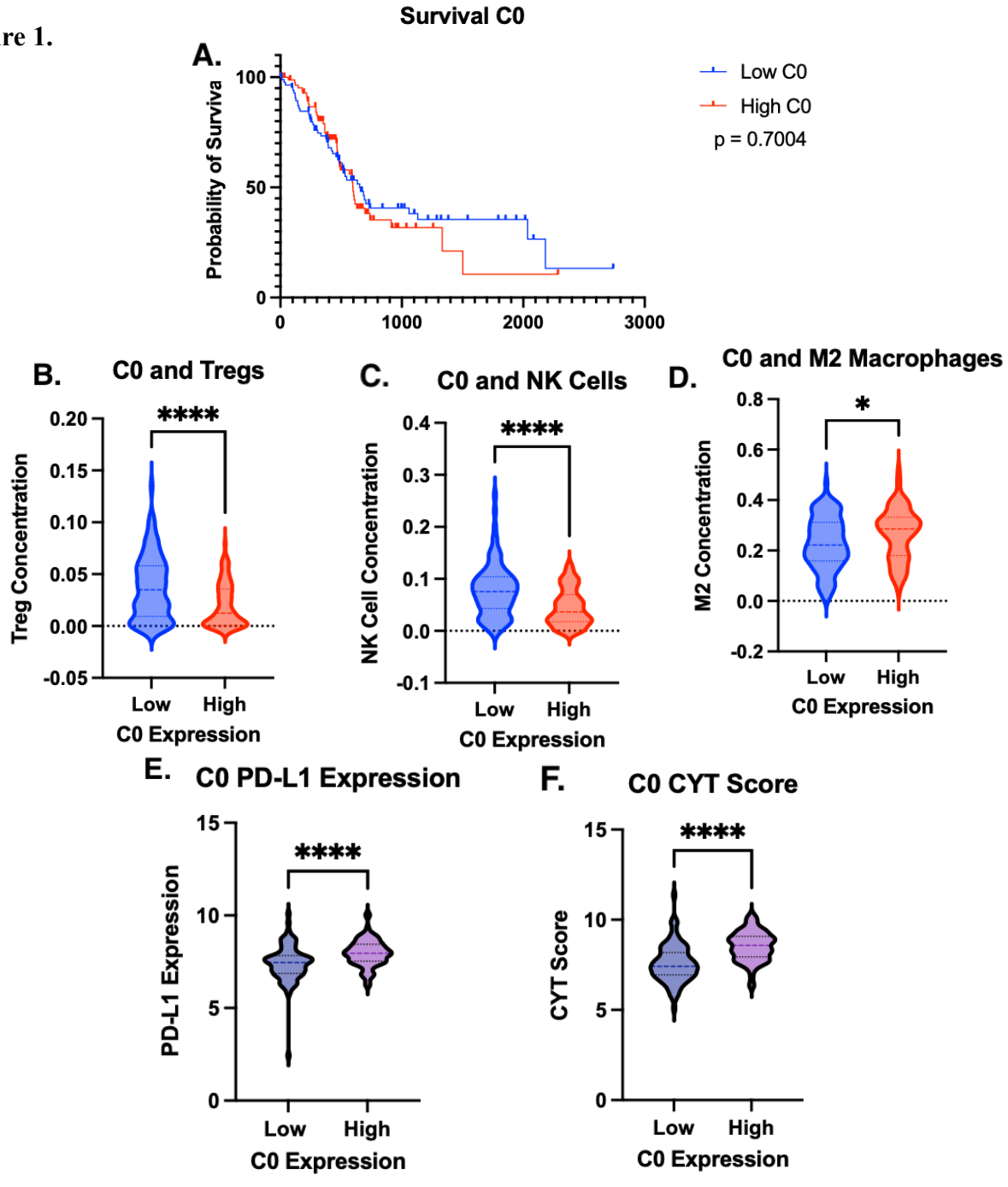


Figure 1. C0 CAF Subpopulation Analysis

A) Kaplan-Meier survival curve for overall survival of patients with low vs. high expression of C0 CAF subpopulation **B)** unpaired t-test violin plot of Treg concentration for patients with low vs. high expression of C0 CAF cluster **C)** unpaired t-test violin plot of NK cell concentration for patients with low vs. high expression of C0 CAF cluster **D)** unpaired t-test violin plot of M2 Macrophage concentration for patients with low vs. high expression of C0 CAF cluster. **F)** unpaired t-test violin plot of PD-L1 Expression for patients with low vs. high expression of C0 CAF cluster. **G)** unpaired t-test violin plot of CYT score for patients with low vs. high expression of C0 CAF cluster. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

To determine the correlation between CAF subpopulation expression and overall survival, patients were classified into high-expression and low-expression groups based on median score, and log-rank survival tests were performed. There was no statistically significant difference in survival between low vs. high C0 expression groups (Fig. 1A). Despite no survival difference, potential differences in tumor immune infiltration were determined by calculating immune cell concentrations using CIBERSORT. Of the 22 immune cell subtypes analyzed, higher expressions of C0 CAFs correlated with significantly lower regulatory T cell (Tregs) expression (mean values: 0.01971 vs. 0.03690, p-value < 0.0001, Fig. 1B). Additionally, patients with higher expressions of C0 CAFs correlated with a lower Natural Killer cell (NK cell) expression compared to low C0 CAF expression groups (mean values: 0.04500 vs. 0.07784, p-value < 0.0001, Fig. 1C). Higher expressions of C0 CAFs also correlated with higher expressions of M2 Macrophages (mean values: 0.2676 vs. 0.2288, p-value = 0.0114, Fig. 1E). To determine potential differences in immunotherapeutic response based on C0 CAF expression, PD-L1 expression, and CYT score was calculated. The high C0 CAF expression group correlated with a higher PD-L1 expression compared to the low C0 CAF expression group (mean values: 7.419 vs. 7.966, p-value < 0.0001, Fig. 1F). Similarly, higher expressions of C0 CAFs correlated with a higher CYT score (mean values: 7.579 vs. 8.530, p-value < 0.0001, Fig. 1G).

C3 CAF Subpopulation Analysis

Figure 2.

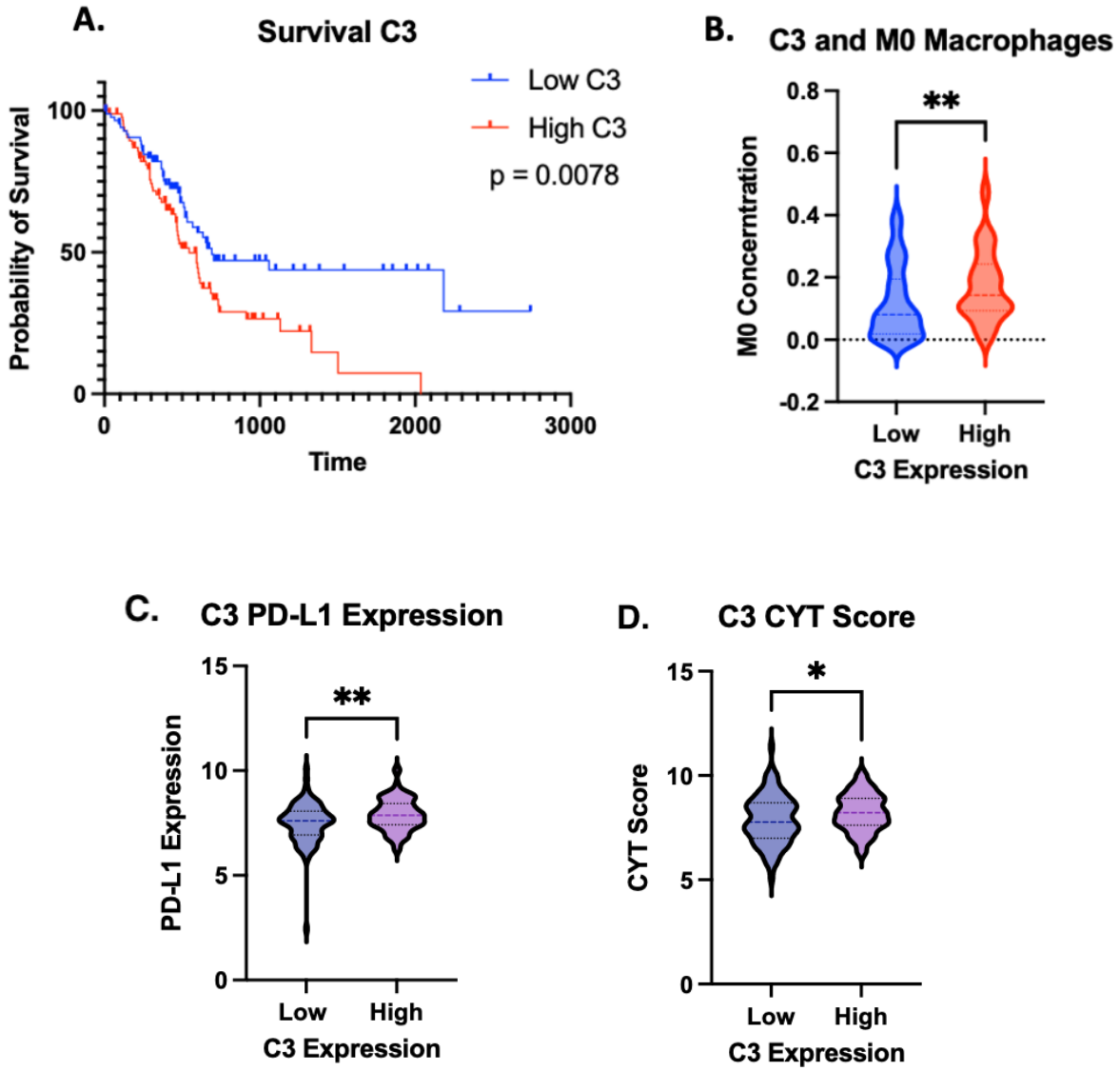


Figure 2. C3 CAF Subpopulation Analysis

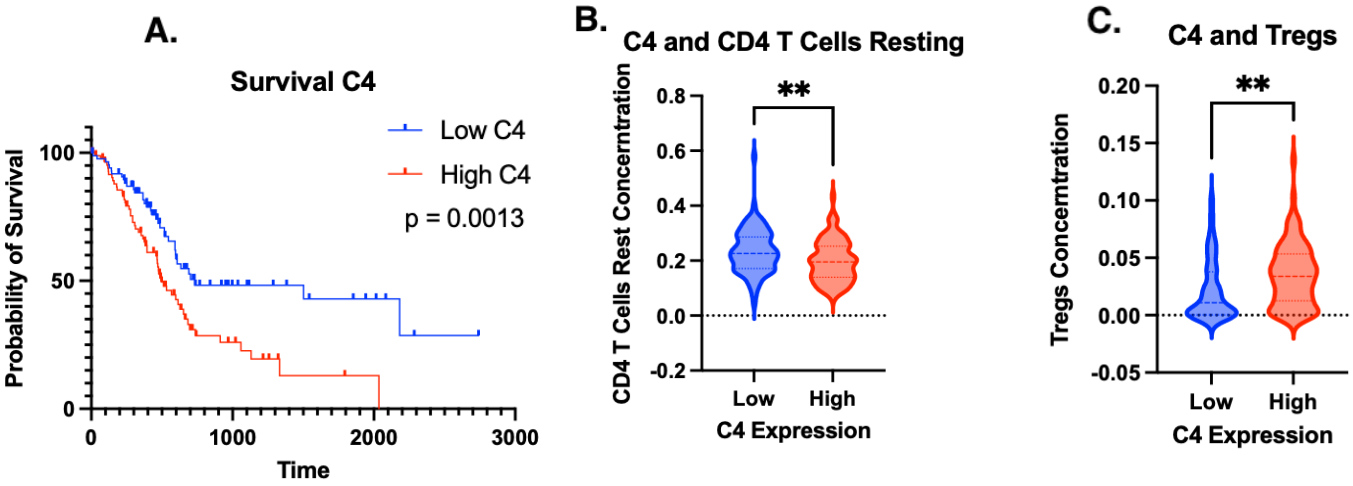
A) Kaplan-Meier survival curve for overall survival for patients with low vs. high expression of C3 CAF subpopulation **B)** unpaired t-test violin plot of M0 Macrophage concentration for patients with low vs. high expression of C3 CAF cluster **C)** unpaired t-test violin plot of PD-L1 expression for patients with low vs. high expression of C3 CAF cluster **E)** unpaired t-test violin plot of CYT score for patients with low vs. high expression of C3 CAF cluster. *$p \leq 0.05$;

**p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

Log-rank survival analyses were conducted between patients with a low expression vs. high expression of C3 CAFs to analyze differences in overall survival. The high C3 expression group showed a significantly higher frequency of poor overall survival as compared to the low C3 expression group (median survival: 545.0 days vs. 691.0 days, p-value = 0.0078, Fig. 2A). For further validation of overall survival and tumor immune infiltration, the relative proportions of immune cell subpopulations for high vs. low C3 expression groups were estimated by CIBERSORT. Patients with higher expressions of C3 CAFs showed a higher expression of M0 Macrophages (mean values: 0.1187 vs. 0.1720, p-value = 0.0022, Fig. 2B). Correlations between CAF subpopulation expression and PD-L1 expression were conducted to predict differential response to anti-PD-L1 immunotherapies. Higher expressions of C3 CAFs correlated with a higher PD-L1 expression as compared to the low C3 CAF expression group (mean values: 7.891 vs. 7.494, p-value = 0.0025, Fig. 2C). To determine overall cancer immunity and potentials for immunotherapeutic response, a differential CYT score was determined between high and low CAF expression groups. Patients with high expressions of C3 CAFs correlated with a higher CYT score compared to the low C3 CAF expression group (mean values: 8.226 vs. 7.883, p-value = 0.0261, Fig. 2D).

C4 CAF Subpopulation Analysis

Figure 3.



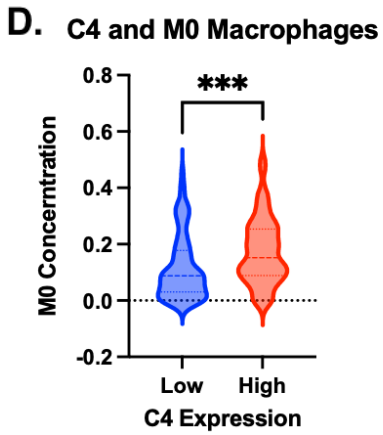


Figure 3. C4 CAF Subpopulation Analysis

A) Kaplan-Meier survival curve for overall survival for patients with low vs. high expression of C4 CAF subpopulation **B)** unpaired t-test violin plot of M0 Macrophage concentration for patients with low vs. high expression of C4 CAF cluster **C)** unpaired t-test violin plot of Treg concentration for patients with low vs. high expression of C4 CAF cluster **D)** unpaired t-test violin plot of resting CD4 T cell concentration for patients with low vs. high expression of C4 CAF cluster. **E)** unpaired t-test violin plot of Monocyte concentration for patients with low vs. high expression of C4 CAF cluster *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Similar to the C3 subset, patients with high expressions of C4 CAFs had significantly poorer overall survival than patients with low expressions of C4 CAFs (median survival: 498.0 days vs. 732.0 days, p-value = 0.0013, Fig. 3A). Relative immune cell concentrations were calculated based on C4 CAF expression to analyze tumor immune infiltration differences between low vs. high expression groups. Patients with higher expressions of C4 CAFs showed lower expressions of resting CD4 T Cells (mean values: 0.1981 vs. 0.2342, p-value = 0.0019, Fig. 3B). Higher expressions of C4 CAFs correlated with higher concentrations of Tregs (mean values: 0.03472 vs. 0.02189, p-value = 0.0015, Fig. 3C). Similarly, high C4 CAFs groups showed higher expressions of M0 Macrophages compared to low C4 CAF groups (mean values: 0.1740 vs. 0.1166, p-value = 0.0009, Fig. 3D). There was no significant difference in PD-L1 expression or CYT score based on the differential expression of C4 CAFs.

C5 CAF Subpopulation Analysis

Figure 4.

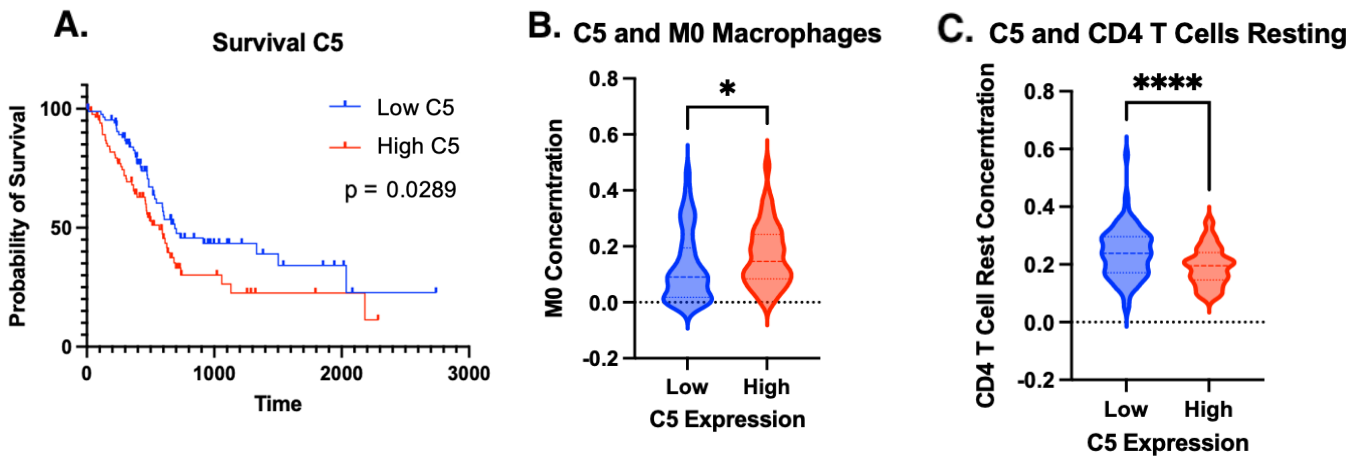


Figure 4. C5 CAF Subpopulation Analysis

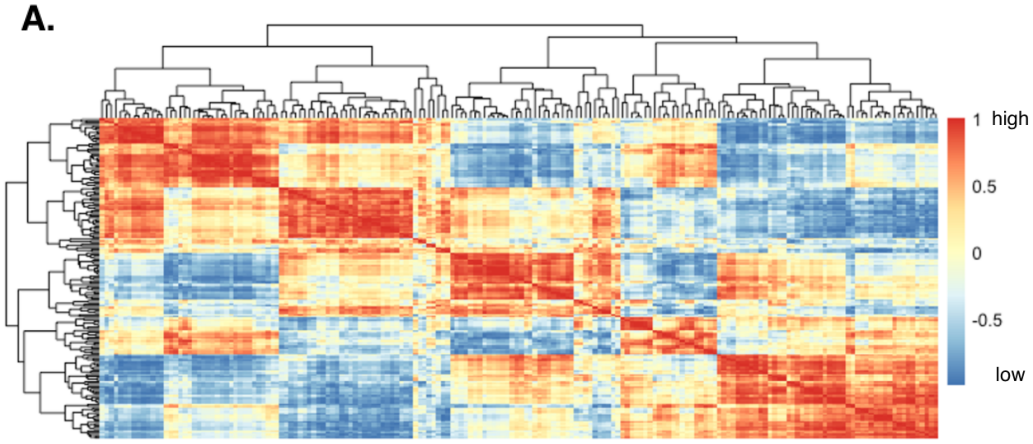
A) Kaplan-Meier survival curve for overall survival for patients with low vs. high expression of C5 CAF subpopulation **B)** unpaired t-test violin plot of M0 Macrophage concentration for patients with low vs. high expression of C5 CAF cluster **C)** unpaired t-test violin plot of resting CD4 T cells for patients with low vs. high expression of C5 CAF cluster *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Log-rank tests were performed on low vs. high C5 CAF expression groups to determine differences in overall survival. High C5 expression groups correlated with significantly poorer survival (median survival: 568.0 vs. 691.0, p-value: 0.0289, Fig. 4A). Immune cell concentrations were analyzed based on differential C5 CAF concentration. Patients with high expressions of C5 CAFs showed a higher expression of M0 Macrophages (mean values: 0.1659 vs. 0.1247, p-value = 0.0183, Fig. 4B). Higher expressions of C5 CAFs also correlated with lower expressions of resting CD4 T cells (mean values: 0.1935 vs. 0.2388, p-value < 0.0001, Fig. 4C). There was no statistically significant difference in PD-L1 expression or CYT score between low and high expression groups.

Population-Level Clusters

Figure 5.

A.



B.

Mean Values of CAF expression per Population Cluster				
	C0	**C3**	**C4**	**C5**
Population 1	0.2751	0.4015	-0.03635	-0.09259
Population 2	-0.1275	-0.3988	-0.1362	-0.1199
Population 3	-0.343	-0.2916	0.1231	0.1582
Population 4	0.2794	-0.2126	-0.3912	-0.2132
Population 5	-0.04527	0.4197	0.2259	0.06958

Fig. 5. CAF Subpopulation Distribution Across Population-Level Clusters

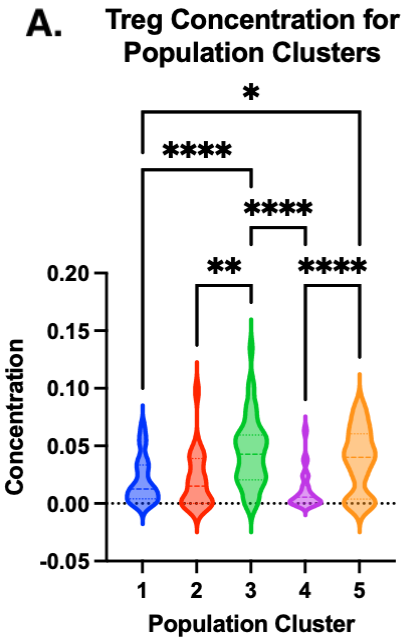
A) Heatmap depicting population-level clusters based on differential expression of CAF subpopulations. **B)** Enrichment values of CAF subpopulations for each population-level cluster

GSVA was used to compose a heatmap with five population-level clusters containing similar expressions of CAF subpopulations (Fig. 6A). Population 1 positively correlated with C0 and C3 CAFs and negatively correlated with C4 and C5 CAFs (Fig. 6B). In population 2 all of the CAF subtypes were negatively correlated (Fig. 6B). Patients in population 3 negatively correlated with C0 and C3 CAFs and positively correlated with C4 and C5 CAFs (Fig. 6B). Population 4 had a

positive correlation with C0 CAFs and a negative correlation with C3, C4, and C5 CAFs (Fig. 6B). Patients in population 5 expressed a negative correlation with C0 CAFs and a positive correlation with C3, C4, and C5 CAFs (Fig. 6B).

Figure 6.

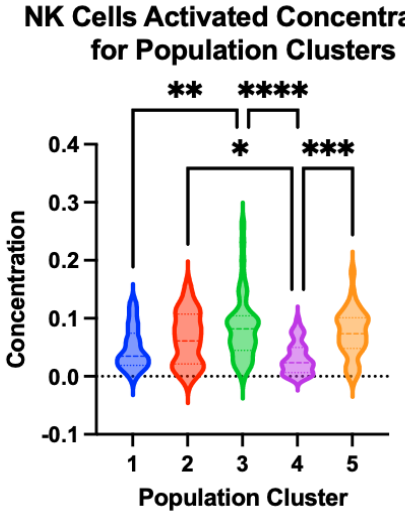
A.



B.

Treg Concentration Across Population Clusters					
	Mean 1	**Mean 2**	**Mean Diff.**	**Summary**	**P-Value**
1 vs. 2	0.01993	0.02055	-0.000618	ns	>0.9999
1 vs. 3	**0.01993**	**0.04462**	**-0.02469**	****	**<0.0001**
1 vs. 4	0.01993	0.01145	0.008476	ns	0.5554
1 vs. 5	**0.01993**	**0.03766**	**-0.01773**	*	**0.0175**
2 vs. 3	**0.02055**	**0.04462**	**-0.02407**	**	**0.0021**
2 vs. 4	0.02055	0.01145	0.009094	ns	0.6435
2 vs. 5	0.02055	0.03766	-0.01712	ns	0.0811
3 vs. 4	**0.04462**	**0.01145**	**0.03317**	****	**<0.0001**
3 vs. 5	0.04462	0.03766	0.006958	ns	0.6897
4 vs. 5	**0.01145**	**0.03766**	**-0.02621**	****	**<0.0001**

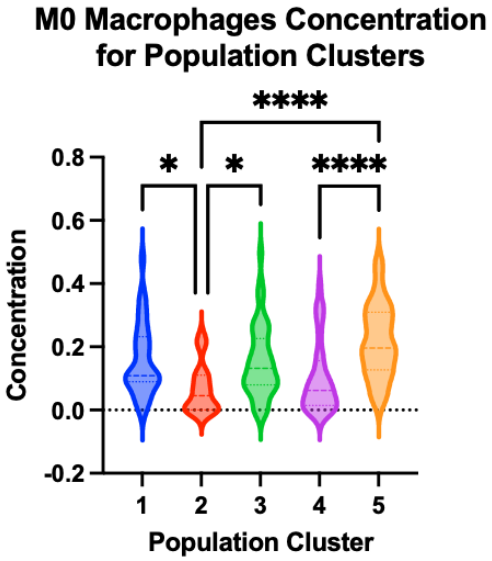
C.



D.

NK Cell Concentration Across Population Clusters					
	Mean 1	**Mean 2**	**Mean Diff.**	**Summary**	**P-Value**
1 vs. 2	0.04848	0.06548	-0.017	ns	0.5838
1 vs. 3	**0.04848**	**0.08422**	**-0.03575**	**	**0.0015**
1 vs. 4	0.04848	0.03129	0.01719	ns	0.3954
1 vs. 5	0.04848	0.07424	-0.02576	ns	0.0717
2 vs. 3	0.06548	0.08422	-0.01875	ns	0.4417
2 vs. 4	**0.06548**	**0.03129**	**0.03419**	*	**0.0267**
2 vs. 5	0.06548	0.07424	-0.008759	ns	0.9424
3 vs. 4	**0.08422**	**0.03129**	**0.05294**	****	**<0.0001**
3 vs. 5	0.08422	0.07424	0.009987	ns	0.8177
4 vs. 5	**0.03129**	**0.07424**	**-0.04295**	***	**0.0002**

E.



F.

M0 Macrophage Concentration Across Population Clusters					
	Mean 1	Mean 2	Mean Diff.	Summary	P-Value
1 vs. 2	**0.1558**	**0.06511**	**0.09069**	*	**0.0229**
1 vs. 3	0.1558	0.1537	0.002076	ns	>0.9999
1 vs. 4	0.1558	0.09944	0.05637	ns	0.1678
1 vs. 5	0.1558	0.2178	-0.06196	ns	0.1103
2 vs. 3	**0.06511**	**0.1537**	**-0.08862**	*	**0.019**
2 vs. 4	0.06511	0.09944	-0.03433	ns	0.7741
2 vs. 5	**0.06511**	**0.2178**	**-0.1527**	****	**<0.0001**
3 vs. 4	0.1537	0.09944	0.05429	ns	0.1487
3 vs. 5	0.1537	0.2178	-0.06404	ns	0.0614
4 vs. 5	**0.09944**	**0.2178**	**-0.1183**	****	**<0.0001**

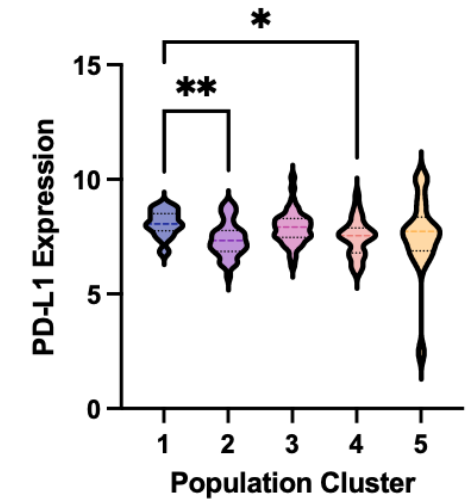
Figure 6. Immune Cell Concentration Population Cluster Analysis

A) one-way ANOVA violin plot of Treg concentration across population-level clusters **B)** Table of mean differences and significance for multiple comparisons of Treg concentration across population-level clusters **C)** one-way ANOVA violin plot of NK cell concentration across population-level clusters **D)** Table of mean differences and significance for multiple comparisons of NK cell concentration across population-level clusters **E)** one-way ANOVA violin plot of M0 Macrophage concentration across population-level clusters **F)** Table of mean differences and significance for multiple comparisons of M0 Macrophage concentration across population-level clusters. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Across the five population-level clusters, immune cell enrichment analysis was conducted using CIBERSORT to determine differential tumor immune microenvironments with potentially varying responses to immunotherapies. Treg cells were significantly enriched in populations 3 and 5 compared to the remaining populations (Fig. 6A, 6B). NK cells were significantly enriched in populations 2, 3, and 5 (Fig. 6C, 6D). M0 macrophages were significantly enriched in populations 1, 3, and 5 (Fig. 6E, 6F).

Figure 7.

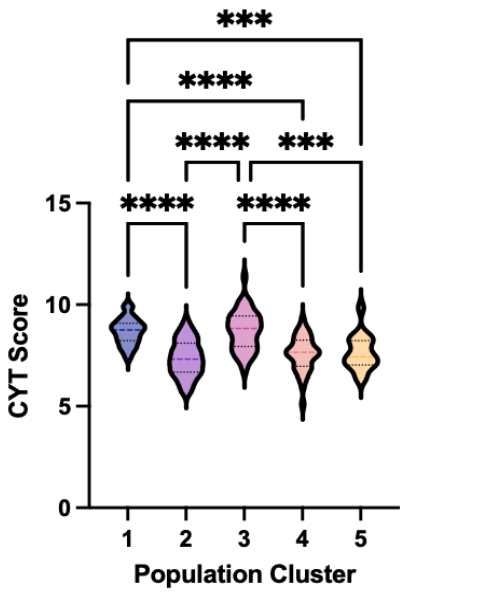
A.



B.

PD-L1 Expression Across Population Clusters					
	Mean 1	Mean 2	Mean Diff.	Summary	P-Value
1 vs. 2	**8.082**	**7.394**	**0.6882**	**	**0.0037**
1 vs. 3	8.082	7.881	0.2009	ns	0.8225
1 vs. 4	**8.082**	**7.47**	**0.6119**	*	**0.0165**
1 vs. 5	8.082	7.547	0.5352	ns	0.2068
2 vs. 3	7.394	7.881	-0.4873	ns	0.0671
2 vs. 4	7.394	7.47	-0.07626	ns	0.9946
2 vs. 5	7.394	7.547	-0.153	ns	0.9719
3 vs. 4	7.881	7.47	0.4111	ns	0.1897
3 vs. 5	7.881	7.547	0.3343	ns	0.6499
4 vs. 5	7.47	7.547	-0.07673	ns	0.998

C.



D.

CYT Score Across Population Clusters					
	Mean 1	Mean 2	Mean Diff.	Summary	P-Value
1 vs. 2	**8.684**	**7.36**	**1.324**	****	**<0.0001**
1 vs. 3	8.684	8.723	-0.03837	ns	0.9996
1 vs. 4	**8.684**	**7.582**	**1.102**	****	**<0.0001**
1 vs. 5	**8.684**	**7.643**	**1.041**	***	**0.0004**
2 vs. 3	**7.36**	**8.723**	**-1.362**	****	**<0.0001**
2 vs. 4	7.36	7.582	-0.2215	ns	0.7729
2 vs. 5	7.36	7.643	-0.2827	ns	0.781
3 vs. 4	**8.723**	**7.582**	**1.141**	****	**<0.0001**
3 vs. 5	**8.723**	**7.643**	**1.08**	***	**0.0002**
4 vs. 5	7.582	7.643	-0.06123	ns	0.9992

Figure 7. Immunotherapeutic Potentials for Population Clusters

A) one-way ANOVA violin plot of PD-L1 expression across population-level clusters **B)** Table of mean differences and significance for multiple comparisons of PD-L1 expression across population-level clusters **C)** one-way ANOVA violin plot of CYT score across population-level

clusters **D)** Table of mean differences and significance for multiple comparisons of CYT score across population-level clusters *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

PD-L1 expression and CYT score were calculated across the population clusters to determine differential response to immunotherapies. Population 1 had a significantly higher PD-L1 expression compared to populations 2 and 4 (Fig. 7A, 7B). Both population 1 and population 3 had significantly higher CYT scores compared to the remaining population clusters (Fig. 7C, 7D).

Discussion:

The presented study identified populations of CAFs that correspond to differential survival and potentially variable responses to immunotherapy. The results suggest that patients with lower expressions of C3 CAFs, C4 CAFs, or C5 CAFs will have better overall survival compared to patients with higher expressions of the aforementioned CAF subtypes, thus validating CAFs as a potential target against PDAC.

Furthermore, differing levels of fibroblast populations in the TME corresponded to distinct immune environments, providing insights into the stromal-immune crosstalk of PDAC. A higher expression of C3 CAFs, C4 CAFs, or C5 CAFs correlated with a higher expression of M0 macrophages and poorer prognosis. Recent studies have found that the accumulation of M0 macrophages contributes to lower overall survival [16]. The pattern of higher M0 macrophage expression across high CAF expression groups depicts the immunosuppressive effect of CAFs on the TME of PDAC. Additionally, low C4 and low C5 expression groups showed a higher expression of CD4 T cells. Recent studies have explored have found that CD4 T cells have anti-tumoral effects [17]. Depleting C4 and C5 CAF subtypes may allow for a more favorable tumor-microenvironment. Furthermore, a higher expression of C4 CAFs correlated with higher expressions of Tregs. Studies have found that Tregs hinder anticancer immunity and immunosurveillance [18]. The correlation of high C4 CAF expression and Tregs suggests a connection between C4 CAFs and immunosuppressive functions. Additionally, higher expressions C0 CAFs correlated with lower Treg cells, lower NK cells, and higher M2 macrophages. Analyzing differing levels of immune cells across low vs. high CAF expression

groups provides insights into which patients would respond preferentially to certain immunotherapies.

To further validate the effect of CAFs on specific immunotherapeutic potentials, PD-L1 expression and CYT score were determined. High expressions of C0 CAFs or C3 CAFs had higher PD-L1 expressions and CYT scores. Recent studies have shown that in many cancers higher PD-L1 expression and higher CYT score were associated with better outcomes for anti-PD-L1 immunotherapies such as pembrolizumab [19, 20]. Therefore, patients with higher expressions of C0 CAFs or C3 CAFs could respond preferentially to anti-PD-L1 therapy.

The results also identified 5 population-level clusters of patients based on the differential presence of fibroblast subpopulations. In population 1 only C0 and C3 CAFs were significantly enriched. None of the CAF subtypes were enriched in population 2. Patients in population 3 only correlated with C4 and C5 CAFs. In population 4 only C0 CAFs were enriched. Patients in population 5 only correlated with C3, C4, and C5 CAFs. Each of the 5 population-level clusters had distinct immune environments. Populations 3 and 5 had higher concentrations of Treg cells which correspond to an immunosuppressive microenvironment. Both of these populations contained higher expressions of C4 and C5 CAFs, illustrating the immunosuppressive effect of C4 and C5 CAFs in these patients. Populations 2, 3, and 5 had higher expressions of NK cells which are capable of killing cancerous cells [21]. Populations 1, 3, and 5 had higher concentrations of M0 macrophages which correlate to poorer prognosis [16]. Each of the population clusters with differential CAF expression corresponds to distinct immune environments that have both pro-tumoral and anti-tumoral effects.

Each population may respond differentially to various immunotherapies. Population 1 which contained higher expressions of C0 and C3 CAFs had a higher PD-L1 expression compared to populations 2 and 4. Both populations 1 and 3 had significantly higher CYT scores compared to the remaining population clusters. Since PD-L1 expression and higher CYT score is associated with better anti-PD-L1 therapy response, populations 1 and 3 may have more favorable responses to such immunotherapies [19] [20].

The results demonstrate the role of CAF subpopulations in immunotherapeutic response and identify subsets of patients that may respond better to certain forms of immunotherapies. The presented findings can inform treatment decisions for patients with PDAC, allowing for more

personalized medicine. Additionally, identifying targets to deplete pro-tumoral CAFs can skew the TME towards an anti-tumoral phenotype, thereby improving overall survival. Further in-vitro analyses need to be conducted in order to validate the results and determine differential immunotherapeutic responses based on CAF presentation.

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