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RESEARCH ARTICLE

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Characterization of natural killer and cytotoxic T-cell immune infiltrates in pancreatic ductal adenocarcinoma

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Abstract

Background and Objectives: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with poor response to systemic therapies, including immunotherapy. Given the immunotherapeutic potential of natural killer (NK) cells, we evaluated intratumoral NK cell infiltrates along with cytotoxic T cells in PDAC to determine their association with patient outcomes.

Methods: We analyzed tumors from 93 PDAC patients treated from 2012 to 2020. Predictor variables included tumor-infiltrating lymphocytes (TILs), T-cell markers (CD3, CD8, CD45RO), NK marker (NKp46), and NK inhibitory marker (major histocompatibility complex class I [MHC-I]) by immunohistochemistry. Primary outcome variables were recurrence-free survival (RFS) and overall survival (OS).

Results: Mean TILs, CD3, and NKp46 scores were 1.3 ± 0.63, 20.6 ± 17.5, and 3.1 ± 3.9, respectively. Higher expression of CD3 and CD8 was associated with higher OS, whereas NK cell infiltration was not associated with either RFS or OS. There was a tight positive correlation between MHC-I expression and all T-cell markers, but not with NKp46.

Conclusions: Overall NK cell infiltrates were low in PDAC and did not predict clinical outcomes, whereas T-cell infiltrates did. Further characterization of the immune infiltrate in PDAC, including inhibitory signals and suppressive cell types, may yield better biomarkers of prognosis and immune targeting in this refractory disease.

KEYWORDS

adenocarcinoma, immune infiltrates, pancreatic cancer

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy which is resistant to most systemic therapies, including immunotherapy. Pancreatic cancer is the fourth most common cause of cancer related death in the United States and is projected to be number two by 2030.[1,2] According to the American Cancer Society, approximately 60 430 new cases in the United States were diagnosed in 2021 with estimated 48 220 deaths.[1] While surgical resection is the mainstay of treatment for PDAC,

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approximately 80%–85% of patients present at an advanced stage with unresectable or metastatic disease.[1] Moreover, the prognosis remains poor even in the setting of surgical resection with a 5-year survival rate of only 20% for patients with localized, resectable disease.[3] The dismal prognosis associated with PDAC is multifactorial in nature, but includes advanced stage at presentation, aggressive tumor biology, and hostile tumor microenvironment (TME) which contributes to its resistance to chemotherapy, targeted therapy, and immunotherapy. In fact, even successful chemotherapy regimens such as FOLFIRINOX have only modestly improved survival rates,[4] thus highlighting the importance of examining alternative therapeutic approaches to improve outcomes.

The immune system plays a key role in the surveillance, elimination, and progression of malignancies in the host.[5] Over the last decade, immunotherapy has revolutionized clinical oncology with breakthrough advances in multiple cancers, including melanoma, non-small cell lung cancer, and renal cell carcinoma.[6–8] Strategies targeting immune checkpoints, such as cytotoxic T-lymphocyte-associated antigen 4 and programmed death 1, have demonstrated impressive antitumor responses in solid tumors and have become a standard pillar in cancer treatment.[6,9] However, not all patients respond, and some cancers show minimal, if any, response to immunotherapy. PDAC is a prime example of an immunotherapy resistant cancer with response rates less than 5%.[10,11] Barriers include low T-cell infiltration in tumors and an immunosuppressive TME with adverse metabolic conditions which reduce T-cell recruitment and inhibit those which are present leading to exhaustion.[12] Additionally, immunosuppressive PDAC environments contain abundant desmoplastic stroma which is a mechanical barrier to immune cell infiltration.[13–15]

As a result, PDAC is characterized as a stereotypical "cold" tumor with low immune infiltration, particularly devoid of $CD8^+$ T cells.[16] However, although PDAC has been linked to low T-cell infiltrate, the contribution of natural killer (NK) cells has received less attention. NK cells are a vital component of the innate immune system and can identify and eliminate virally infected and malignant cells without prior sensitization.[17] They are cytotoxic due to their ability to release toxic molecules such as perforin and granzymes, forming pores in the membranes of targeted cells. NK cells also express a broad array of receptors, allowing them respond to diverse stimuli.[17] While NK cell therapies have shown efficacy in hematologic malignancies and are under intense investigation to extend the promise of immunotherapy, they have not been proven effective in solid tumors to date as adoptive therapy.[18] However, tumor-infiltrating NK cells have been associated with improved responses to neoadjuvant treatments and overall prognosis in solid tumors such as breast cancer.[19,20] Given the need for novel immunotherapy approaches in PDAC, our objective was to evaluate immune parameters in PDAC, particularly markers of NK cells, to determine if NK cell infiltrates were associated with patient outcomes.

2 | MATERIALS AND METHODS

We performed a retrospective analysis of patients diagnosed with resectable PDAC from 2012 to 2020. Our research protocol was approved by the Institutional Review Board at the University of California, Davis (Protocol Number 1727704-1).

2.1 | Patient clinical characteristics

We identified 93 patients with stages I to III PDAC based on the American Joint Committee on Cancer Cancer Staging Manual, 8th edition.[21] Patient demographic, clinicopathologic, and treatment data were abstracted from the medical record, including patient age, sex, race, tumor grade, tumor size, lymph node involvement, and receipt of chemotherapy and radiotherapy. Primary outcome variables included recurrence-free survival (RFS) and overall survival (OS), analyzed by the Kaplan–Meier method. RFS and OS were defined as described previously.[22] OS was calculated as the number of months between the date of initial diagnostic biopsy to the date of death. RFS was calculated from the date of initial diagnostic biopsy to the date of recurrence or the date of last follow up reported in the electronic medical records. Date of death was established using electronic medical records and confirmed with the California Cancer Registry.

2.2 | Immunohistochemical (IHC) analysis

Predictor variables included tumor-infiltrating lymphocytes (TILs), T-cell markers (CD3, CD8, CD45RO), NK marker (NKp46) and NK inhibitory marker (major histocompatibility complex class I [MHC-I]). Tissue microarrays (TMA) containing approximately three patient samples per slide were constructed with 1.5 mm cores from archived specimens, which were reviewed before TMA construction to select areas of viable tumor.[23] IHC staining was performed using mouse anti-human CD3 (Abcam; clone SP7), mouse anti-human CD8 (Dako; clone C8/144B), mouse anti-human CD45RO (Cell Signaling; clone UCHL1), and rabbit anti-human NKp46/NCR1 (AbCam; clone ERP22403-57). Slides were scored by a blinded pathologist (M. A. D.) using a scale of 0–3 for TILs and scale of 0–300 for immune markers by IHC as described previously.[23] Staining was performed in triplicate, and these values were averaged for statistical analysis. Individual patient mean values of the triplicate scores were then classified as high or low with reference to the median score for the entire cohort for each marker, as described previously.[24]

2.3 | Statistical analysis

Survival outcomes were compared using Kaplan–Meier curves and log-rank test. Correlations between variables were performed using Spearmen correlation test. Additionally, multivariate proportional

hazard models were fitted for OS and RFS. Age, sex, and stage were adjusted with separate models, and biomarkers were inputted into a combined model designed to assess collinearity and the impact of confounding factors.

3 | RESULTS

3.1 | Patient clinicopathologic characteristics

The clinicopathologic characteristics of our study cohort are reported in Table 1. Mean age was 70, 55% were female, and the mean tumor size was 3.1 ± 1.1 cm. Eighty-nine percent of tumors involved the pancreatic head, and 63% were lymph node positive. In our cohort, 10% of patients received neoadjuvant therapy, and 63% received adjuvant therapy. However, few patients tolerated complete adjuvant therapy following surgery. With a median follow up of 27 months, the median OS for the cohort was 25 months, and the median RFS was 23 months (Table 2).

TABLE 1 Characteristics of retrospective PDAC cohort, 2012–2020.

Characteristic	Number (n = 93)	%
Gender		
Female	51	55
Male	42	45
Age at diagnosis (mean ± SD)	69.6 ± 10.2	
Race/ethnicity		
Caucasian	74	80
Asian	4	4
Hispanic	7	8
Black	3	3
Other	4	4
Tumor site		
Head/uncinate	90	84
Body/tail	10	9
Histology		
Adenocarcinoma	93	100
Maximal tumor size, median (range)	3.0 cm (0.4–7.25)	
Procedure		
Pancreaticduodenectomy	87	94
Partial pancreatectomy	6	6
Lymph node positive	59	63
AJCC stage at surgery		
I	16	17
II	63	68
III	14	15

Abbreviations: AJCC, American Joint Committee on Cancer; PDAC, pancreatic ductal adenocarcinoma.

3.2 | NK and T-cell infiltrates

Analyzing the surgical specimens following resection, we observed mean TIL scores of 1.3 ± 0.63, mean CD3 scores of 20.7 ± 17.5, and mean NKp46 scores of 3.1 ± 3.9 (Table 3). Figure 1 shows representative staining of TIL scores 0–3 (Figure 1A), including high and low expression of immune markers of interest (Figure 1B).

3.3 | Association of immune infiltrates with outcomes

We then analyzed the correlation between immune infiltrates and clinical outcomes. A higher expression of both CD3 (p = 0.009) and CD8 (p = 0.001) was associated with higher OS (Figure 2A,B), while neither TIL scores nor NK cell infiltrates were associated with OS (Figure 2C,D). However, we observed that lower expression of MHC-I was associated with greater OS (p = 0.047) (Figure 2E). Memory T-cell marker, CD45RO, was not associated with OS (Figure 2F).

TABLE 2 Patient survival.

Characteristic	Number (n = 93)	%
Disease status		
No evidence of disease	8	9
Alive with disease	16	17
Dead of disease	69	74
Median follow up in months (range)	27 (1–85)	
Median RFS in months	23	
Median OS in months	25	

Abbreviations: OS, overall survival; RFS, recurrence-free survival.

TABLE 3 Immune marker expression scores.

Marker	Mean expression score (range)
TILs	1.3 ± 0.63 (0–3)
CD3	20.7 ± 17.5 (1.66–93.3)
CD8	13.0 ± 12.5 (0–71.7)
NKp46	3.15 ± 3.97 (0–20.0)
MHC-I	84.3 ± 47.9 (6.67–206)
CD45RO	12.4 ± 10.7 (0–58.3)

Abbreviations: MHC-I, major histocompatibility complex class I; TIL, tumor-infiltrating lymphocytes.

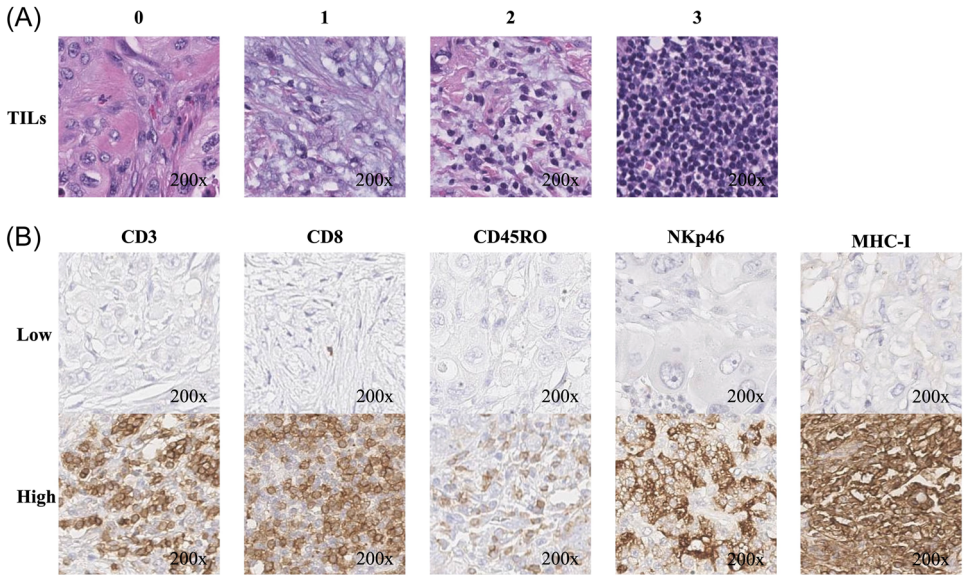

FIGURE 1 Representative staining of tumor-infiltrating lymphocytes (TILs) and immunohistochemical (IHC) immune markers. (A) Hematoxylin and eosin photomicrographs of TILs, scoring 0–3. (B) IHC photomicrographs of high and low staining for CD3, CD8, CD45RO, NKp46, and MHC-I. MHC-I, major histocompatibility complex class I.

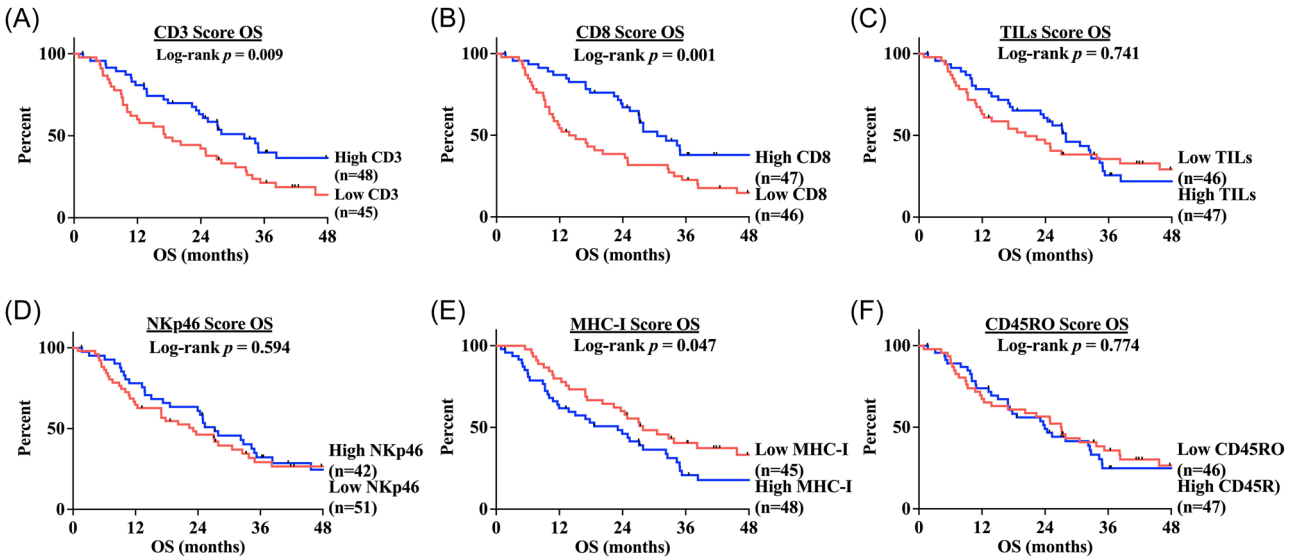

FIGURE 2 T-cell infiltrates are linked with favorable overall survival (OS) in PDAC patients. Kaplan–Meier analysis of OS stratified by median (A) CD3 score, (B) CD8 score, (C) TILs score, (D) NKp46, (E) MHC-I, and (F) CD45RO. Significance of Kaplan–Meier analysis was determined by log-rank test. High- and low-expression groups were determined by median scores. MHC-I, major histocompatibility complex class I; PDAC, pancreatic ductal adenocarcinoma; TIL, tumor-infiltrating lymphocytes.

Additionally, none of the single color IHC immune markers nor TILs were significantly associated with RFS (Figure 3). We did observe a positive correlation between MHC-I expression and all T-cell markers, CD3/MHC-I ($r = 0.33$, $p = 0.001$), CD8/MHC-I ($r = 0.34$, $p = 0009$), and CD45RO/MHC-I ($r = 0.49$, $p < 0.0001$) (Figures 4A–C, respectively). Additionally, as shown in Figure 4E, there was a significant association between MHC-I expression and TILs ($r = 0.43$, $p < 0.0001$), while NKp46 infiltrates were not associated with MHC-I expression (Figure 4E). Additionally, there was a tight correlation between TIL scores and CD8 expression ($r = 0.65$, $p < 0.0001$) noted in Table 4.

3.4 | Multivariable adjusted analysis of predictors of outcome

We performed multivariable adjusted analysis to determine if immune infiltrates were independently associated with survival

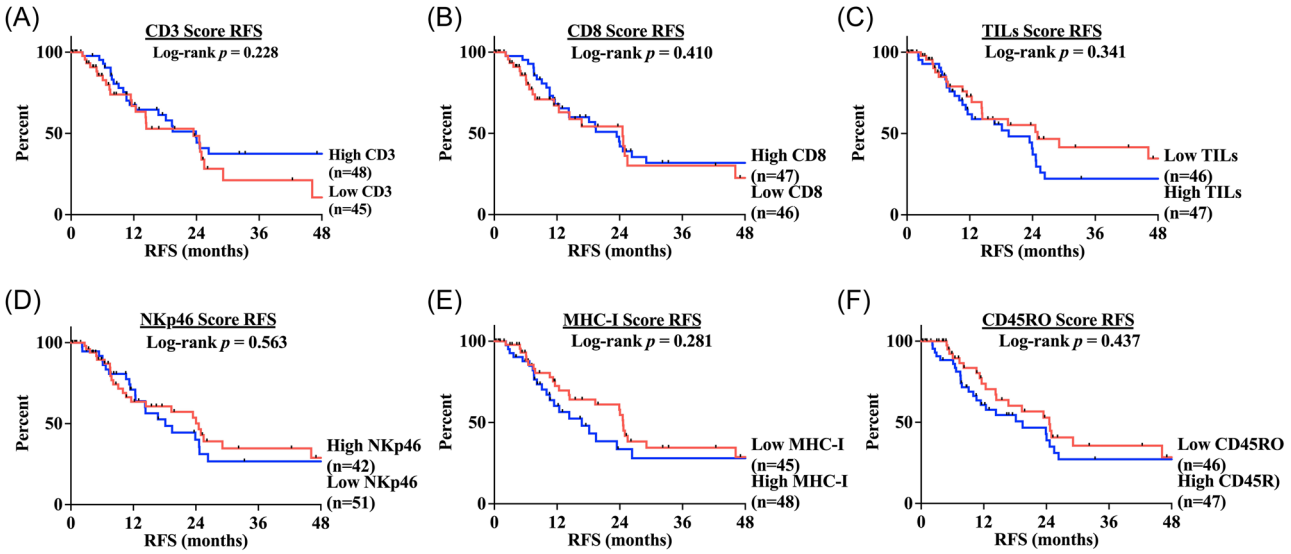

FIGURE 3 Intratumoral immune cell markers are not associated with differences in recurrence-free survival (RFS). Kaplan–Meier analysis of RFS stratified by median (A) CD3 score, (B) CD8 score, (C) TILs score, (D) NKp46, (E) MHC-I, and (F) CD45RO. Significance of Kaplan–Meier analysis was determined by log-rank test. High- and low-expression groups were determined by median scores. MHC-I, major histocompatibility complex class I; TIL, tumor-infiltrating lymphocytes.

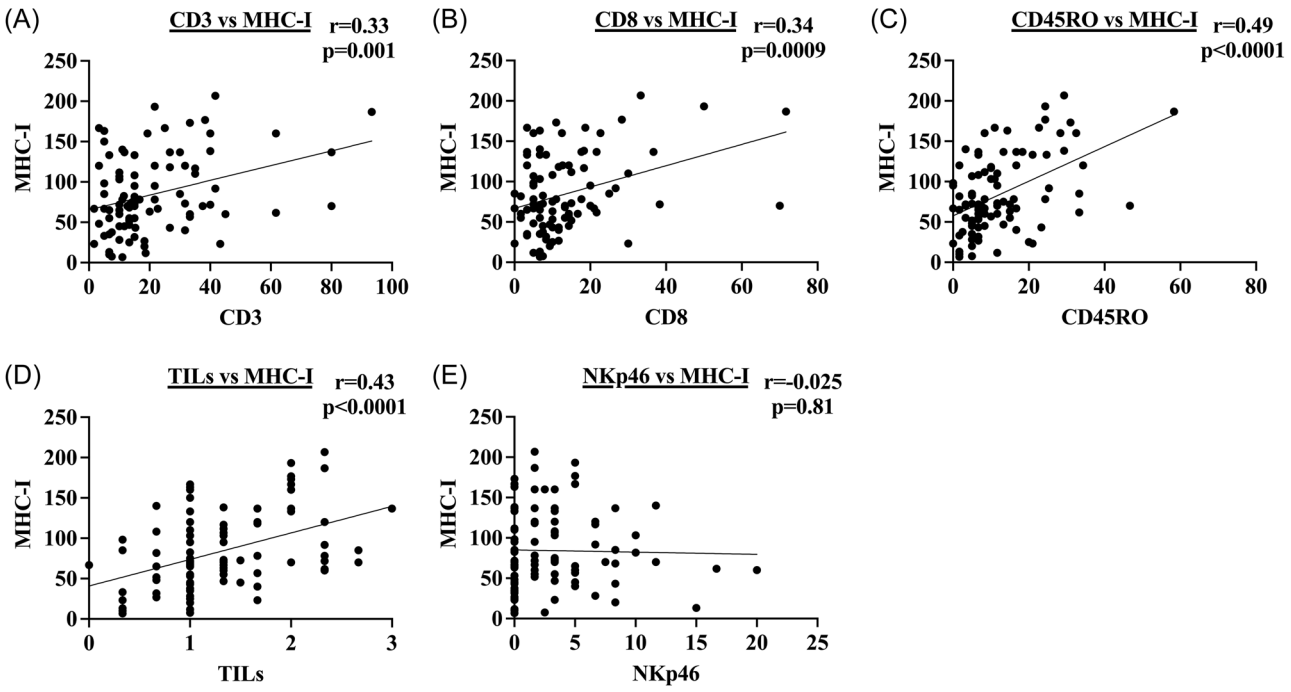

FIGURE 4 MHC-I expression correlates with T-cell markers in PDAC. Positive correlation of MHC-I with (A) CD3 ($r = 0.33$, $p = 0.001$), (B) CD8 ($r = 0.34$, $p = 0009$), (C) CD45RO ($r = 0.49$, $p < 0.0001$), and (D) TILs ($r = 0.43$, $p < 0.001$). (E) MHC-I was not associated with NKp46 expression ($r = 0.025$, $p = 0.81$). Correlations determined by two-tailed Spearman coefficient. MHC-I, major histocompatibility complex class I; PDAC, pancreatic ductal adenocarcinoma; TIL, tumor-infiltrating lymphocytes; vs, versus.

outcomes. The results of our multivariable analysis are depicted in Tables 5 and 6. Age was the only significant predictor for OS ($p = 0.008$), with a hazard ration and 95% confidence interval of 1.04 (1.01–1.07). There were no significant predictor variables for RFS.

4 | DISCUSSION

In this analysis, we observed overall low infiltration of TILs, T cells, and NK cells in a consecutive series of PDAC patients undergoing surgery at a single institution over an 8-year period. Our findings

TABLE 4 Spearman correlation coefficients between CD8, TILs, and NKp46.

	CD8	TILs	NKp46
CD8	1	0.65	0.28
		<0.0001	0.0066
TILs	0.65	1	0.39
	<0.0001		0.0001
NKp46	0.28	0.39	1
	0.0066	0.0001	

Abbreviation: TILs, tumor-infiltrating lymphocytes.

TABLE 5 Multivariable adjusted analysis for overall survival.

Variable	Adjusted hazard ratio	(95% CI)	*p* Value
Gender			
Female	1.73	(1.04–2.87)	0.034
Male	1		
Age at diagnosis	1.03	(1.01–1.06)	0.018
AJCC stage at surgery			
I	1		
II	1.43	(0.73–2.82)	0.30
III	1.73	(0.73–4.13)	0.22
Mean TIL score	0.93	(0.56–1.55)	0.79
Mean NKp46	0.98	(0.93–1.04)	0.53
Mean CD8	0.99	(0.96–1.02)	0.59

Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; TIL, tumor-infiltrating lymphocyte.

are consistent with those of other studies assessing immune infiltrates in clinical PDAC specimens.[16,25] Historically, PDAC has been characterized as an immune desert due to low CD8 T-cell infiltration; however, NK cell infiltration has only recently been analyzed.[26] Few studies have explored the association of NK cell infiltration and survival in PDAC, and the results have generally been inconclusive.[27,28] However, there are select preclinical studies exploring the antitumor effects of NK cells for PDAC in mouse models, showing that adoptive transfer of NK cells exhibits antitumor activity leading to a significant delay in tumor growth in mice.[11,29,30] Although it is possible that our study and others like it are underpowered to show an association between NK infiltrates and outcomes in PDAC surgical specimens, the discrepancy in results between preclinical studies showing an association of NK cells with antitumor responses in PDAC in mouse models and retrospective analyses of human samples suggests that key species differences as well as idiosyncrasies of mouse modeling may be impacting the generalizability of those preclinical results.

TABLE 6 Multivariable adjusted analysis for recurrence-free survival.

Variable	Adjusted hazard ratio	(95% CI)	*p* Value
Gender			
Female	1.75	(0.91–3.36)	0.095
Male	1		
Age at diagnosis	0.99	(0.96–1.02)	0.47
AJCC stage at surgery			
I	1		
II	1.15	(0.51–2.62)	0.73
III	2.14	(0.78–5.90)	0.14
Mean TIL score	0.99	(0.52–1.89)	0.97
Mean NKp46	1.00	(0.92–1.07)	0.93
Mean CD8	0.99	(0.95–1.04)	0.81

Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; TIL, tumor-infiltrating lymphocyte.

Our observation that NK cell infiltrates were not significantly associated with oncologic outcomes may also be related to several other factors. It is possible there is a required threshold of tumor-infiltrating NK cells needed to observe significant differences in survival, and the low NK infiltrates in the tumors we analyzed may not have met this threshold. Alternatively, inhibitory signals suppressing NK cell function in the PDAC TME could be contributing to the observed results. There are potential immunosuppressive factors including intratumoral hypoxia, which is common in solid tumors such as PDAC, as well as elaboration of immunosuppressive cytokines like transforming growth factor-β.[31] NK cells have been shown to be less cytotoxic in hypoxic environments due to reduced expression of NKG2D, CD107a, and granzyme B.[32] Additionally, the use of NKp46 in our study as a marker for NK cells may contribute to discrepancies with previous studies, most of which show NK cell infiltration to be associated with improved survival using CD56 as the NK marker.[33] Although CD56 is a reliable NK cell marker by flow cytometry, by single color IHC, it can be nonspecific and stain neural cells and other lymphoid cell types. On the other hand, although NKp46 is highly specific for NK cells, it may not capture all NK cells present since it is frequently expressed at low levels in resting NK cells.[34,35]

In contrast, our data showed that T-cell markers, including CD3 and CD8, were associated with improved survival outcomes, suggesting that targeting intratumoral CD3 and CD8 infiltrates in PDAC may be a viable strategy for eliciting an immune response, if appropriate ways to increase intratumoral T cells and prevent their suppression can be unlocked. Notably, we did not observe an association between TIL infiltration, as assessed by hematoxylin and eosin staining, and survival outcomes in our study. However, TIL scoring includes multiple immune cells, all of which have various

inhibitory and activating receptors. While our IHC was performed by a blinded pathologist, immune scoring is subjective which may impact data obtained and therefore results. Additionally, TMAs capture a small portion of a viable tumor. Areas with elevated inflammation are avoided as it would significantly obscure visualization of the tumor. This may have contributed to sampling bias.

Currently, few papers have investigated MHC-I expression in PDAC.[36] We analyzed MHC-I because it is a both stimulatory molecule for T cells and an inhibitory molecule for NK cells.[37] T cells recognize antigens in the context of MHC presentation, with CD8 T cells able to respond to tumor cells expressing MHC-I presenting cognate antigen.[38] We observed a strong positive correlation between MHC-I expression and all T-cell markers (CD3, CD8, and CD45RO) which was statistically significant. Therefore, strategies to increase MHC-I expression may increase T cells and ideally reverse the PDAC immune desert. Conversely, we did not observe a correlation of MHC-I expression with NK infiltration. MHC-I negatively regulates NK cell activation via cytoplasmic tyrosine immunoreceptors, such as killer-immunoglobulin like receptors, therefore loss of MHC-I can lower the threshold for NK cell activation.[37,39] Given the inhibitory effects of MHC-I on NK cell function, further studies should assess the spatial localization of NK and CD8 T cells in relation to MHC-I expressing cells and how this impacts outcomes.

Although we were able to analyze a consecutive series of surgically treated patients with translationally relevant tumor tissue, our cohort size of 93 patients may have resulted in an underpowered study, potentially limiting our ability to evaluate our hypothesis regarding NK infiltrates in the PDAC TME. Studies by Ino et al. and Carsten et al. which observed an association of TIL infiltrates with survival in PDAC included sample sizes of 132 and 241, respectively, both of which are greater than ours.[40,41] In addition, there is a risk of missing or incomplete data in population-based datasets, and this can skew the data especially for potentially ambiguous endpoints such as RFS. The lack of significant results regarding RFS and discrepancy with OS is likely due to the retrospective nature of this analysis including patients lost to follow up. Although survival dates were obtained from a highly validated source, the California Cancer Registry, and were likely definitive, documentation of recurrence was much less rigorous and impacted for patients lost to follow up. This likely biases to the null hypothesis our analysis of RFS as an outcome variable.

5 | CONCLUSION

In summary, we observed that NK cells as assessed by IHC expression of NKp46 do not significantly correlate with survival outcomes in PDAC, while CD3 and CD8 expression do. This may be the result of immunosuppressive features of the PDAC TME, such as hypoxia and MHC-I expression, which inhibit NK cells. Further characterization of the immune infiltrate in PDAC is needed for improved prognostication and immune targeting.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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