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ORIGINAL ARTICLE

Low intratumoral regulatory T cells and high peritumoral CD8$^+$ T cells relate to long-term survival in patients with pancreatic ductal adenocarcinoma after pancreatectomy

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Abstract The prognosis for pancreatic ductal adenocarcinoma (PDAC) remains extremely poor. Recent studies have focused on the role of lymphocytes in the PDAC microenvironment. Using immunohistochemistry, our study explored the clinical significance of intratumoral or peritumoral CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) and CD8$^+$ T cells in the tumor microenvironment and analyzed their relation to the prognosis of PDAC in a consecutive series of 92 patients after resection. CD8$^+$ T cells were more frequently seen within peritumoral sites, while CD4$^+$Foxp3$^+$ Tregs were more frequent within intratumoral areas. Neither exhibited any relationship with other clinicopathologic factors. Patients with low levels of intratumoral Tregs had longer disease-free survival than those with higher levels (DFS 22.2 vs. 11.2 months, $p < 0.001$), and patients with higher levels of peritumoral CD8$^+$ T cells had longer overall survival than those with lower levels (OS 31.0 vs. 14.2 months, $p < 0.001$). Multivariate analysis demonstrated that intratumoral Tregs (hazard ratio, HR 3.39, $p = 0.010$) and peritumoral CD8$^+$ T cells (HR 0.10, $p < 0.001$) are related to DFS and OS, respectively. These results indicate that intratumoral Tregs are a negative predictor of DFS, while peritumoral CD8$^+$ T cells are a positive predictor of OS for PDAC patients with pancreatectomy.

Keywords Pancreatic ductal adenocarcinoma · Pancreatectomy · Tumor microenvironment · Tregs · CD8$^+$ T cells · Prognosis

Abbreviations

CT	Computed tomography
CTLs	Cytotoxic T cells
DFS	Disease-free survival
DP	Distal pancreatectomy
FasL	Fas ligand
GrB	Granzyme B
HPF	High-power fields
HR	Hazard ratio
IHC	Immunohistochemistry
MRI	Magnetic resonance imaging
OS	Overall survival
PD	Pancreaticoduodenectomy
PDAC	Pancreatic ductal adenocarcinoma
PPPD	Pylorus-preserving pancreaticoduodenectomy
Th1	T helper type 1
Th2	T helper type 2
TILs	Tumor-infiltrating lymphocytes
Tregs	Regulatory T cells
UICC	Union for International Cancer Control

Introduction

The prognosis of patients with pancreatic cancer remains extremely poor, with a 5-year survival rate of only 7 % [1].

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For patients with resectable lesions, the 5-year survival rate following pancreatectomy is <20 %, despite improved diagnostic and treatment strategies [2–7]. In recent years, the microenvironment of pancreatic cancer that contributes to tumor initiation, progression, and metastasis has been investigated, and special focus has been on the role of tumor-infiltrating lymphocytes (TILs) in tumor progression [8, 9]. TILs are regarded as reflections of host antitumor immunity and have been studied in various tumors including pancreatic cancer; however, the results have been inconsistent. For example, high levels of $CD4^+$ T cells are an independent prognosticator for patients undergoing resection for pancreatic cancer [8], which is somewhat different from another study that indicated that the ratio of tumor-infiltrating T helper type 2 (Th2)/T helper type 1 (Th1) cells is an independent predictor of survival [9]. Recent data showed that regulatory T cells (Tregs) could offer a new explanation of the pro- and antitumor implications of TILs. Tregs, a subpopulation of T lymphocytes with an immunophenotype of $CD4^+CD25^+$, are thought to hamper T cell immunity against cancer and participate in immune regulation. On the other hand, Foxp3, a transcription factor of the forkhead/winged-helix family, fulfills the criteria of being a Treg-specific marker [10]. In actuality, $CD4^+CD25^+Foxp3^-$ T cells do not show Tregs' function [11] and nonregulatory T cells do not express Foxp3 [12]; therefore, Foxp3 is used as a more specific marker to identify functional Tregs, which prompted our investigation of the level of Tregs in pancreatic cancer. Nevertheless, the results were contradictory because some reports showed a beneficial role of $Foxp3^+$ Tregs, while others suggested their adverse prognostic action. Some studies claim that the accumulation of $Foxp3^+$ Tregs in pancreatic, liver, and ovarian cancer is generally associated with a poor prognosis because of their capacity to suppress antitumor immunity [13, 14], while other studies claim that high tumor infiltration of $Foxp3^+$ Tregs in colorectal carcinoma and Hodgkin's lymphoma shows a favorable prognosis [14–16].

In contrast to Tregs, $CD8^+$ T cells can directly kill target cells, including cancer cells, through the perforin/granzymes cell death pathway or the Fas/Fas ligand (FasL) acquired immune response pathway, both of which are expected to take a high responsibility for antitumor immunity [17]. It was reported that a high level of infiltrating $CD8^+$ T cells in cancer tissue is an indicator for favorable prognostic in ovarian cancer [18], colorectal cancer, [19], and some other cancers [10, 20].

It is reported that the proportion of $CD8^+$ T cells to Tregs is correlated with survival [21, 22]. Several studies also identified the increased prevalence of $CD8^+$ T cells, and a high ratio of $CD8^+/CD4^+$ or $CD8^+$/Tregs is a significant predictor of better survival in many cancers [23–25]. Because of the complexity of the interaction of immune cells with tumor cells, the mechanisms by which regulatory factors hamper effective immune responses in the microenvironment of tumors are very complex and not completely known.

This study tried to analyze the possible impact of intratumoral or peritumoral Tregs and $CD8^+$ T cells on clinicopathologic characteristics and survival of a cohort of patients undergoing pancreatectomy for pancreatic ductal adenocarcinoma (PDAC).

Patients and methods

Patients

Ethical approval was obtained from the research ethics committee of Zhongshan Hospital, Fudan University, China. Participants of this study were selected from all patients undergoing pancreaticoduodenectomy (PD) or distal pancreatectomy (DP) for PDAC in our hospital between February 15, 2007, and September 7, 2011, with complete survival data. Other lesions, such as ampullary, duodenal, or distal bile duct adenocarcinomas; mucinous cyst adenocarcinomas, or intraductal papillary mucinous neoplasms, were excluded as were those patients with unresectable lesions, distant metastasis, peritoneal seeding or those requiring vascular reconstruction because of the invasion of portal vein, superior mesenteric vein, superior mesenteric artery, common hepatic artery, or celiac trunk. Patients with a gross residual tumor or microscopically positive resection margins, immunodeficiency disease, autoimmune disease, or any preoperative anticancer therapies were also excluded. The final study cohort comprised 92 patients. All patients underwent pancreatectomy for curative purpose. The clinical pathology of the tumors was determined according to the Union for International Cancer Control (UICC) staging classification of pancreatic cancer (6th edition).

The 92 eligible patients comprised 68 men and 24 women, with a median age of 61 years. The 72 patients (78.3 %) with lesions in the pancreatic head underwent pylorus-preserving pancreaticoduodenectomy (PPPD) or classic PD; the other 20 patients (21.7 %) with lesions in the pancreatic body or tail underwent DP with splenectomy. According to the UICC TNM system, 25 (27.2 %), 41 (44.5 %), and 26 patients (28.3 %) were diagnosed as stage I, stage II, and stage III, respectively. Tumors were identified as a histologic grading of 1 in 4 patients (4.3 %), 2 in 57 patients (62.0 %), and 3 in 31 patients (33.7 %). The demographics and clinicopathologic characteristics of the patients are summarized in Table 1.

Table 1 Patient demographics and clinicopathologic factors ($n = 92$)

	No. of patients	%
Age (years)		
Median	61	
Range	35–81	
Sex		
Male	68	73.9
Female	24	26.1
Tumor location		
Head	72	78.3
Body or tail	20	21.7
UICC stage		
I	25	27.2
II	41	44.5
III	26	28.3
Histologic grading		
1	4	4.3
2	57	62.0
3	31	33.7
Perineural invasion	72	78.3
Vascular invasion	9	9.8
Lymphatic invasion	32	34.8

UICC Union for International Cancer Control

Surgery

Patients underwent PPPD, classic PD, or DP. All operations were performed by one of the four surgeons (D.J., W.L., D.W. and W.W.). For the tumor in the pancreatic head, the standard surgical procedure was PPPD with lymphadenectomy on the right side of the portal vein. In patients with the tumor invading pylorus or duodenum, a Whipple's procedure was performed. For cancer of the pancreatic body or tail, patients underwent DP plus splenectomy. If the carcinoma was close to (within 1 mm) or present at the final pancreatic neck, uncinate process, bile duct, duodenal or retroperitoneal soft tissue, the patient was not enrolled in this study.

Postoperative treatment and follow-up

Postoperatively, the patients remained in the recovery room for 1–2 h. In cases of ventilatory disorder or perioperative infection, patients were transported to the intensive care unit for 1–3 days, and then sent back to the surgical ward. The drainage tubes were removed 5–11 days after surgery.

After surgery, patients received a regimen of adjuvant chemotherapy with GEMOX for 6–12 cycles: gemcitabine 1000 mg/m^2 and oxaliplatin 100 mg/m^2 on day 1, and then one cycle every 14 days. Follow-up was completed on January 1, 2014. Postsurgery follow-up was performed in our hospital on an outpatient basis. Follow-up examinations comprised physical examinations, abdominal ultrasonography or computed tomography (CT) scans and thoracic CT scans, as well as routine blood analysis including serum CA 19–9 every 3–6 months. All patients received CT scans every 3 months in the first year after surgery and every 6 months in the second year. If the follow-up data suggested a recurrence or metastasis, CT and/or magnetic resonance imaging (MRI) was/were used to verify the condition. The treatment after relapse varied based on patients' situations. Nine patients with recurrence gave up treatment because of poor liver function. Most common causes of death were recurrence, metastasis, or complications related to liver cirrhosis. Overall survival (OS) was defined as the interval between surgery and death or the last observation for surviving patients. Disease-free survival (DFS) was defined as the time between surgery and the first treatment failure (either recurrence or death). All patients who died of events unrelated to PDAC were also excluded.

Pathological examination

After tumor resection, pathology protocols were performed on all specimens to ensure consistent interpretation of tumor location, histology, resection margin, UICC stage, histologic grading, perineural invasion, vascular invasion, and lymphatic invasion.

Immunohistochemical staining

$CD4^+$, $Foxp3^+$, $CD4^+Foxp3^+$, and $CD8^+$ T cells in the resectional regimen were analyzed through immunohistochemical (IHC) staining. The mouse monoclonal antihuman Foxp3, CD8 (Abcam Biotech Co, Cambridge, UK), CD4, and granzyme B (GrB, Novocastra Biotech Co, Cambridge, UK) antibodies were used. The Novolink Polymer Detection System (Leica Biosystems, Newcastle, UK) was used to allow the identification of these antigens following the manufacturer's instructions. IHC of formalin-fixed, paraffin-embedded 3-μm sections was carried out using the Bond-max system automated IHC (Leica Biosystems, Newcastle, UK). IHC Protocol F program was used for single IHC staining. Double staining using IHC Protocol F DOUBLE program was done on CD4 and Foxp3. In brief, sections were deparaffinized with xylene and then hydrated with gradient ethanol. After heat-induced epitope retrieval in citrate buffer, tissues were blocked with 0.3 % H_2O_2 (RE7101) and protein block (RE7102). The slides were then serial incubated with primary antibodies, postprimary block (RE7111), and horseradish peroxidase-conjugated goat anti-mouse antibody (RE7112). Finally, the antigens were identified with diaminobenzidine solution (RE7105), and slides were counterstained with hematoxylin (RE7112,

all the reagents above are included in the Novolink Polymer Detection System). Slides incubated without primary antibodies were used as the negative control, and the tissues from autoimmune pancreatitis were used as the positive control for GrB staining.

The number of T cells was counted by two authors (L.L. and Y.R.) who were blinded to the patient's clinicopathologic information. Lymphocytes count was done with at least five independent 400× high-power fields (HPF). The results are shown as the mean ± SE number of cells in one field.

The influence of a low versus high level of intratumoral or peritumoral $CD4^+Foxp3^+$ Tregs and $CD8^+$ T cells was evaluated. The median numbers were used as the cutoff, and patients were classified into either low group or high group.

Statistical analysis

Associations of the number of lymphocytes with various clinicopathologic factors were assessed by Chi-squared or Fisher's exact tests. Survival rates were calculated by Kaplan–Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. $p < 0.05$ was considered statistically significant. All statistical analyses were performed with Stata 10.0 (Stata Corp LP, College Station, TX, USA).

Results

Characteristics of T lymphocytes in the tumor microenvironment

The T lymphocytes in the peritumoral and intratumoral stroma were investigated in patients with pancreatic ductal adenocarcinoma. $CD4^+$ and $CD8^+$ T cells displayed a cytoplasmic staining pattern despite being either intratumoral (Fig. 1a, g) or peritumoral (Fig. 1b, h). $Foxp3^+$ cells displayed a nuclear staining, which reflects the function of Foxp3 in transcription (Fig. 1c, d). The $CD4^+$ and $Foxp3^+$ double-staining cells represented the Tregs (Fig. 1e, f). Either $CD4^+$ (25.23 ± 8.21 vs. 16.25 ± 4.56 cells/HPF, $p = 0.026$) or $CD8^+$ T cells (36.68 ± 9.45 vs. 16.07 ± 6.25 cells/HPF, $p < 0.001$) were more frequent in the peritumoral microenvironment than in the intratumoral microenvironment, respectively; $CD4^+Foxp3^+$ Tregs, although their numbers were far less than those of $CD4^+$ or $CD8^+$ T cells, were more frequent in the intratumoral than in the peritumoral microenvironment (4.05 ± 5.86 vs. 2.56 ± 2.31 cells/HPF, respectively; $p < 0.001$, Table 2). Furthermore, no GrB^+ cells were found in either intratumoral or peritumoral tissues.

Correlation between $CD8^+$ T cells and $CD4^+Foxp3^+$ Tregs and clinicopathologic factors

The relationship between clinicopathologic factors and $CD8^+$ T cells as well as between clinicopathologic factors and Tregs in either intratumoral or peritumoral tissues is shown in Supplementary Table S1. There was no significant relationship to age, sex, tumor location, UICC stage, histologic grading, perineural invasion, vascular invasion, and lymphatic invasion of $CD8^+$ T cells or Tregs in the tumor environment in either intratumoral or peritumoral tissue ($p > 0.05$).

Survival and prognostic factors

The OS and DFS rates of all 92 patients who underwent pancreatectomy for PDAC were 73.7 and 63.9 % at 1 year and 50.0 and 24.1 % at 2 years, respectively.

Among the 92 patients, the prognostic impact of intratumoral or peritumoral $CD4^+Foxp3^+$ Tregs and $CD8^+$ T cells was investigated by evaluating the factors associated with OS and DFS using univariate and multivariate analyses. By univariate analysis (Table 3), age, sex, tumor location, perineural invasion, and intratumoral $CD8^+$ T showed no prognostic significance for OS or DFS. There were six prognostic factors significantly correlated with OS as follows: histologic grading ($p = 0.045$), UICC stage ($p < 0.001$), lymphatic invasion ($p = 0.041$), peritumoral $CD8^+$ T cells (Fig. 2c, $p < 0.001$), intratumoral Tregs (Fig. 2e, $p < 0.001$), and peritumoral Tregs (Fig. 2g, $p = 0.004$). Meanwhile, the following five factors were correlated with DFS: UICC stage ($p = 0.013$), vascular invasion ($p = 0.027$), peritumoral $CD8^+$ T cells (Fig. 2d, $p < 0.001$), intratumoral Tregs (Fig. 2f, $p < 0.001$), and peritumoral Tregs (Fig. 2h, $p = 0.022$).

All of these factors ($p < 0.1$) were enrolled into multivariate analysis using a Cox proportional hazards model (Table 4). Peritumoral $CD8^+$ T cells ($p < 0.001$) remained independently associated with overall survival. The median OS of patients with high peritumoral $CD8^+$ T cells was significantly longer than that of patients with low peritumoral $CD8^+$ T cells (31.0 vs. 14.2 months, respectively; $p < 0.001$, Fig. 2c). Vascular invasion ($p < 0.001$) and intratumoral Tregs ($p = 0.024$) also remained independently associated with DFS. The median DFS of patients with low intratumoral Tregs was significantly longer than that of patients with high intratumoral Tregs (22.2 vs. 11.2 months, respectively; $p < 0.001$, Fig. 2f).

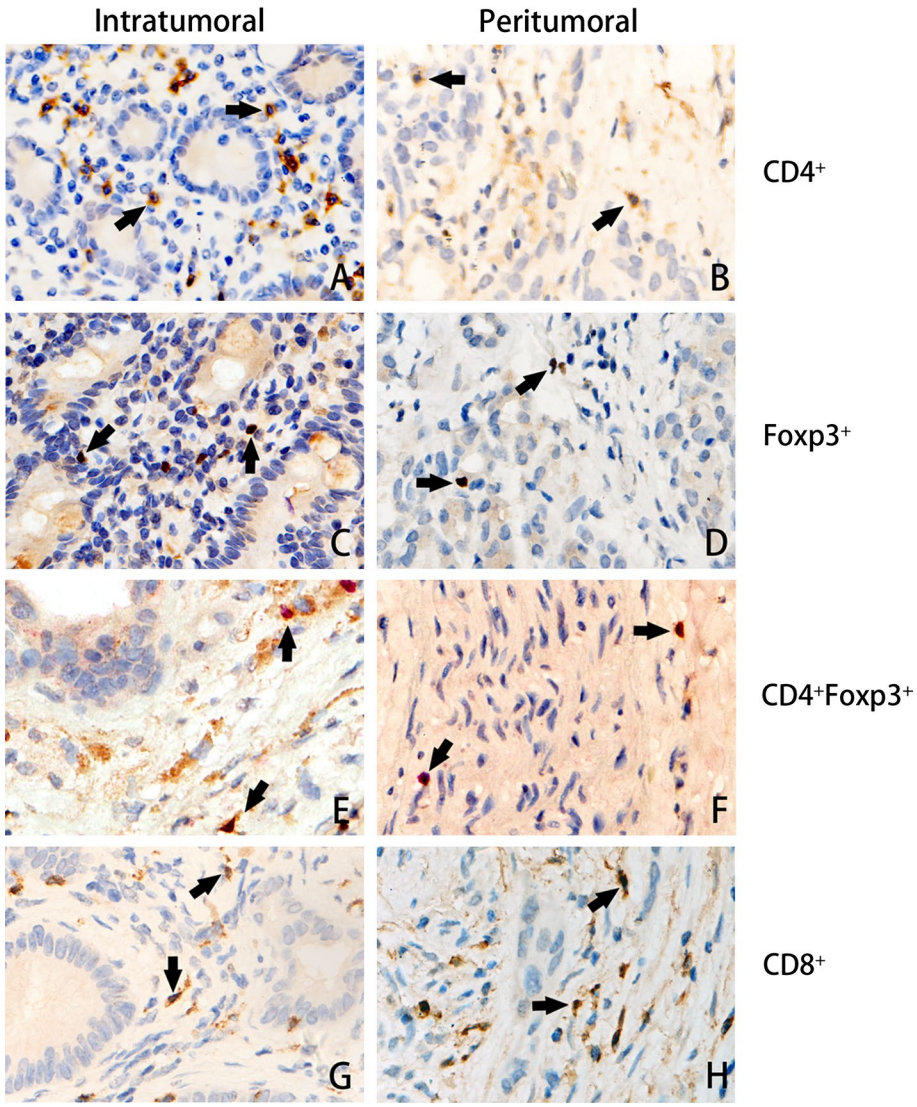

Fig. 1 Tumor-infiltrating $CD4^+$, $Foxp3^+$, $CD4^+Foxp3^+$, and $CD8^+$ cells. Consecutive sections were used for immunohistochemical study. $CD4^+$ and $CD8^+$ T cells displayed a cytoplasmic staining pattern despite intratumoral (**a**, **g**) or peritumoral (**b**, **h**). $Foxp3^+$ cells displayed a nuclear staining, which reflects the function of Foxp3 in transcription (**c**, **d**). The $CD4^+$ and $Foxp3^+$ double staining representing the Tregs are shown in **e**, **f** (original ×400 magnification)

Table 2 Intratumoral and peritumoral T lymphocytes in patients undergoing pancreatectomy for PDAC ($n = 92$)/high-power field (×400 magnification)

	Mean	SE	Median	Range	*p* value
$CD4^+$ T					0.026*
Intratumoral	16.25	4.56	8.20	4–125	
Peritumoral	25.23	8.21	15.34	10–189	
$CD8^+$ T					<0.001*
Intratumoral	16.07	6.25	10.23	0–95	
Peritumoral	36.68	9.45	25.45	5–136	
$CD4^+Foxp3^+$ Tregs					<0.001*
Intratumoral	4.05	5.86	3.15	0–75	
Peritumoral	2.56	2.31	2.02	0–55	

Tregs regulatory T cells, *SE* standard error

* $p < 0.05$

Discussion

Recent researches paid attention to the immune cells in the PDAC microenvironment and their prognostic value through IHC or flow cytometry [8, 13, 26], but none of these studies discussed immune cells in intratumoral or peritumoral sites as a prognostic indicator, respectively. There is much evidence indicating that high T cell infiltration leads to a favorable outcome in a variety of human cancers [14, 27]. Although flow cytometry might provide more accurate data for the prevalence of TILs [26], it cannot be used to show the location of cells in tumor tissues.

There were some limitations in our study. First, we excluded patients with immunodeficiency disease, autoimmune disease, or any preoperative anticancer therapies to avoid changes in the immune microenvironment unrelated

Table 3 Univariate overall survival and disease-free survival analysis of prognostic factors for patients undergoing pancreatectomy for PDAC ($n = 92$)

	OS			DFS		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
Age (years) (≤60 vs. >60)	1.53	0.84–2.80	0.160	1.11	0.69–1.80	0.666
Sex (female vs. male)	1.02	0.50–2.06	0.959	0.90	0.51–1.59	0.721
Tumor location (head vs. body or tail)	1.65	0.69–3.92	0.253	0.69	0.38–1.27	0.236
Histologic grading (1 + 2 vs. 3)	1.87	1.01–3.45	0.045*	1.11	0.66–1.88	0.679
UICC stage (I + II vs. III)	1.30	0.68–2.49	0.424	1.37	0.80–2.34	0.250
Perineural invasion (yes vs. no)	1.83	0.77–4.32	0.168	1.67	0.85–3.28	0.135
Vascular invasion (yes vs. no)	0.59	0.18–1.92	0.383	2.23	1.09–4.52	0.027*
Lymphatic invasion (yes vs. no)	1.87	1.02–3.41	0.041*	1.64	0.99–2.71	0.056
Intratumoral $CD8^+$ t (low vs. high)[a]	0.88	0.49–1.59	0.672	0.89	0.55–1.45	0.634
Peritumoral $CD8^+$ T (low vs. high)[a]	0.15	0.07–0.32	<0.001*	0.28	0.17–0.49	<0.001*
Intratumoral Tregs (low vs. high)[a]	3.27	1.76–6.07	<0.001*	3.97	2.29–6.87	<0.001*
Peritumoral Tregs (low vs. high)[a]	2.47	1.31–4.62	0.005*	1.79	1.08–2.96	0.024*

OS overall survival, *DFS* disease-free survival

* $p < 0.05$

[a] Using median values as cutoff

to cancer. Nevertheless, some comorbidities such as diabetes that can alter immunity were not completely excluded in this study, which might influence the results. A recent study showed that neoadjuvant chemoradiotherapy can significantly increase the number of $CD4^+$ and $CD8^+$ cells [28], but it is not known whether the results would be influenced if this kind of patients had been enrolled in our study. In addition, only resectable patients were included in our study, and this might cause bias because these patients have relatively favorable prognoses. Otherwise, patients with malignance developed from chronic pancreatitis or other diseases which may alter the immune microenvironment of the pancreas have not been analyzed here. Taken together, this study comprising 92 patients draws attention to the importance of the immune system in the pancreatic cancer microenvironment and provokes speculation about the roles and mechanisms involved.

Tregs play a crucial role in impeding immune surveillance and preventing overactive antitumor immunity [29]. In this study, both the intratumoral and peritumoral areas were assessed, and more Tregs were found in the intratumoral areas than in the peritumoral areas (4.05 vs. 2.56 cells/HPF, respectively, $p < 0.001$, Table 2) which was determined by another study as well [8]. This might suggest that tumor cells can mobilize Tregs to evade antitumor immunity. Our study also showed that higher intratumoral Tregs were significantly correlated with poor DFS, which could be an independent prognostic factor in multivariate analysis. In fact, this result is somewhat different from that of the study mentioned above, which showed that higher Treg levels were associated with not only shorter DFS but also shorter OS [8]. In addition, a recent study showed that the intratumoral density of $Foxp3^+$ cells is an independent prognostic factor for OS of the PDAC patient, but the relationship with DFS has not been determined [26]. A high incidence of Tregs in the tumor microenvironment, representing the dominance of immunosuppression, might help cancer cells successfully escape from immune surveillance [30] and into distant organs or lymph nodes through the vascular or lymphatic pathway; therefore, intratumoral Tregs might be related to the cancer's "ability to escape." At the point of resection, patients with a high number of intratumoral Tregs should have a higher number of metastatic cancer cells, remaining in drained lymph nodes, residual organs, or circulating blood, which leads to an earlier relapse with shorter DFS; however, the relationship between intratumoral Tregs and OS remains undetermined, it is highly possible that because most patient in this study are relatively early stages (71.7 % patients are UICC stage I or II).

In contrast, $CD8^+$ T cells are the most prominent lymphocytes acting against cancer cells and are the potential targets for Tregs. Infiltration of $CD8^+$ T cells decreased from the peritumoral (mean 36.68 cells/HPF) to intratumoral area (mean 16.07 cells/HPF) (Table 2), which was confirmed by Ino et al. [8]. Because activated Tregs can suppress cytotoxic T cells (CTLs) in both an antigen-independent and a suppressive cytokine-dependent manner [12], it is not unusual that less $CD8^+$ T cells are found in intratumoral sites where more Tregs exist. Our study showed that the peritumoral $CD8^+$ T cells were significantly correlated with only OS in the multivariate survival analysis. It can be reasonably speculated that peritumoral $CD8^+$ T cells are related to the "ability to prevent" tumor progression of

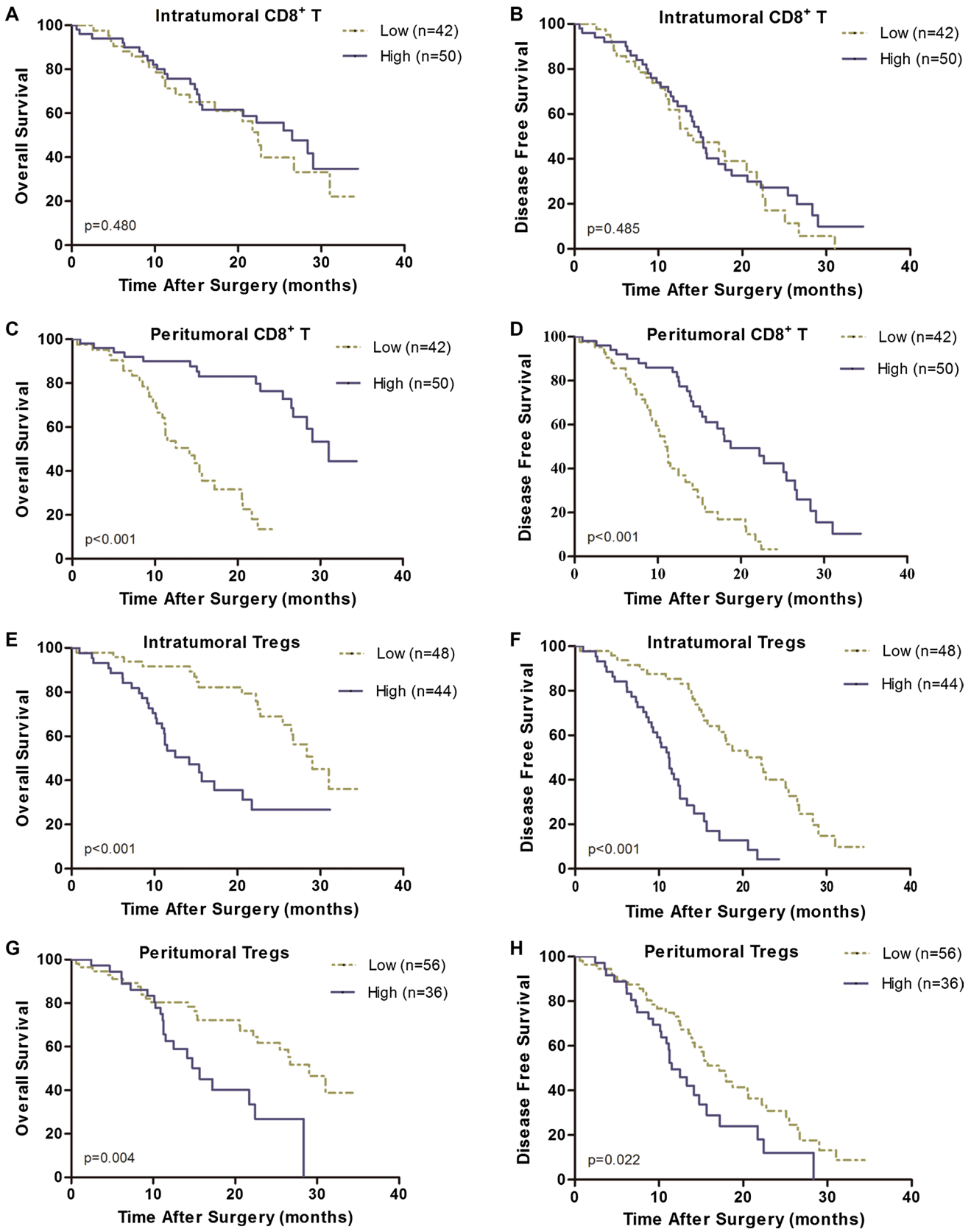

Fig. 2 Kaplan–Meier analysis of overall survival (OS) and disease-free survival (DFS) for tumor-infiltrating Tregs and $CD8^+$ T cells. Intratumoral $CD8^+$ T cells showed no prognostic significance for OS (**a**) and DFS (**b**). High peritumoral $CD8^+$ T cells showed benefit of OS (**c**) and DFS (**d**). Neither high intratumoral (**e**, **f**) nor peritumoral Tregs (**g**, **h**) correlated with OS and DFS

Table 4 Multivariate overall survival and disease-free survival analysis of prognostic factors for patients undergoing pancreatectomy for PDAC ($n = 92$)

	Median (months)		HR	95 % CI	*p* value
OS					
Histologic grading (1 + 2 vs. 3)	26.7	15.7	1.72	0.92–3.22	0.093
Lymphatic invasion (yes vs. no)	15.3	26.5	2.29	1.21–4.33	0.011*
Peritumoral $CD8^+$ T (low vs. high)[a]	14.2	31.0	0.10	0.03–0.31	<0.001*
Intratumoral Tregs (low vs. high)[a]	29.0	14.2	0.96	0.38–2.44	0.929
Peritumoral Tregs (low vs. high)[a]	29.0	15.7	0.63	0.29–1.38	0.249
DFS					
Peritumoral $CD8^+$ T (low vs. high)[a]	11.2	18.8	0.41	0.16–1.04	0.061
Intratumoral Tregs (low vs. high)[a]	22.2	11.2	3.39	1.33–8.61	0.010*
Peritumoral Tregs (low vs. high)[a]	17.2	11.5	0.58	0.30–1.11	0.099
Vascular invasion (yes vs. no)	8.6	15.4	3.94	1.85–8.44	<0.001*

OS overall survival, *DFS* disease-free survival, *Tregs* regulatory T cells, *HR* hazard ratio, *CI* confidence interval

* $p < 0.05$

[a] Using median values as cutoff

the immune system; therefore, patients with high peritumoral $CD8^+$ T cells could more effectively suppress tumor growth, whether more or less tumor burden remained just after resection, and then prolong OS. Ino et al. [8] claimed that their detailed analysis indicated that the infiltration of the tumor by $CD8^+$ T alone is not sufficiently associated with longer survival because this is also positively related to the infiltration of $CD4^+$ T cells. Because $CD8^+$ T cells and $CD4^+$ T cells are differentiated from $CD3^+$ T cells, we speculate that there is a positive relationship between these two kinds of different T cells and the total number of $CD3^+$ T cells which reflect the immune status not just the tumor microenvironment; therefore, we could still regard the infiltrated $CD8^+$ T cells as an indicator for antitumor immunity in the PDAC microenvironment. In fact, melanoma patients with a higher number of $CD8^+$ T cells in both the tumor and the invasive margin responded better to therapeutic PD-1 blockade [31], which indicates infiltrating $CD8^+$ T cells might also be a predictor for immunotherapy.

In addition, a specific type of $CD8^+$ cells, activated $CD8^+$ CTLs, could be positively correlated with survival [21]. GrB expression might serve as a marker of CTL activation and elevated as an indicator for disease activity, providing additional evidence for immune activation [32, 33]. Higher GrB expression or $GrB^+/Foxp3^+$ has also been regarded as a predictor of longer survival in colorectal cancer, lung cancer, and ovarian cancer [34–36]. A decreased expression of GrB has been identified in lung cancer, breast cancer, and colorectal cancer [34, 35, 37]. Protease inhibitor-9 (PI-9, serpin 9), a natural inhibitor of GrB, plays an important role in the suppression of GrB activity by prostate cancer and leukemia cells [38, 39]. The suppression of GrB might also refer to prostaglandin E2/cyclooxygenase 2 pathways and some other mechanisms [34, 40]. However, no GrB^+ cells were found in the intratumoral or peritumoral tissues, which is absolutely out of our exception. The exact reason still has not been determined, but the GrB^+ cells was found in our positive control shown in the Supplementary Figure S1, which may exclude any suspected technical problems. Our findings raised the question of why the GrB^+ $CD8^+$ CTLs disappear and how the Tregs affect $CD8^+$ T cells in PDAC. It could be speculated that the PDAC cells in our study exhibited such malignant biologic behavior that GrB^+ cells were too few to be detected, or that Tregs in the tumor microenvironment profoundly suppress $CD8^+$ CTLs [22]. As a result, the perforin pathway was somewhat blocked, and CTLs may kill pancreatic cancer cells mainly through the Fas/FasL pathway [17]. Additional studies must be conducted to confirm this.

In conclusion, a high number of intratumoral Tregs, which is related to the ability of cancer cells to escape, were a predictor of the poor prognosis for patients with PDAC. Under these conditions, Tregs could be a mediator of tumor cells' inhibitory aggression on the immune system, suppressing its antitumor function. Tregs would determine the decrease in the number of the activated antitumor lymphocytes including activated $CD8^+$ T cells [41]. The cell infiltration of peritumoral $CD8^+$ T cells, which is related to the ability of immune surveillance, was positively correlated with patient OS. These results might provide new independent predictors of prognosis and suggest that the depletion of Tregs might be an effective strategy in pancreatic cancer immunotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no potential conflicts of interest.

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