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Prognostic relevance of tertiary lymphoid organs following neoadjuvant chemoradiotherapy in pancreatic ductal adenocarcinoma

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Abstract

The efficacy of preoperative neoadjuvant chemoradiotherapy (NAC) in cases of pancreatic cancer with extremely poor prognoses has been reported. In this study, we aimed to identify novel biomarkers that reflect prognoses following chemoradiotherapy using tertiary lymphoid organs (TLO) expressed in the tumor microenvironment. Resected tumor specimens were obtained from 140 pancreatic cancer patients. We retrospectively investigated the clinical relevance of TLO by categorizing patients into those who underwent upfront surgery (surgery first [SF]) and those who received NAC. The immunological elements within TLO were analyzed by immunohistochemistry (IHC). In the IHC analysis, the proportions of CD8+ T lymphocytes, PNAd+ high endothelial venules, CD163+ macrophages and Ki-67+ cells within the TLO were higher in the NAC group than in the SF group. In contrast, the proportion of programmed cell death-1+ immunosuppressive lymphocytes within TLO was lower in the NAC group than in the SF group. The NAC group demonstrated favorable prognoses compared with the SF group. In the multivariate analysis, the TLO/tumor ratio was determined as an independent predictive prognostic factor. In conclusion, the administration of preoperative chemoradiotherapy may influence the immunological elements in the tumor microenvironment and result in favorable prognoses in pancreatic ductal adenocarcinoma patients.

KEYWORDS
chemoradiotherapy, immunology, neoadjuvant, pancreatic cancer, tumor microenvironment

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor that shows aggressive growth and causes local recurrence and distant metastasis. The prognosis of PDAC is very poor, and the overall 5-year survival rate of patients with PDAC who have undergone curative resection is 15%-25%.[1] PDAC is the fifth leading cause of cancer-related death worldwide and is expected to be the second

leading cause by 2030 in Western countries[1,2]; therefore, the development of a new multidisciplinary treatment strategy is an urgent requirement. The possibility of improving treatment outcomes through the performance of multidisciplinary neoadjuvant chemotherapy or chemoradiotherapy, which results in the reduction of tumor size and rate of lymph node metastasis accompanied by an increase in the R0 resection rate, has been reported.[3] Therefore, identifying novel biomarkers for the prediction of treatment outcomes is essential to further improve patient prognoses.

Thus far, several biomarkers have been reported following preoperative treatment focusing on serum protein levels,[4] specific gene mutations[5] based on next-generation sequencing, and pathological improvement[6] in the resected specimens based on criteria such as the Evans and College of American Pathologists classifications. In addition to serological, biological and histopathological assessments for the identification of biomarkers, immunological assessment has been recently accepted because of its ability to reflect host immune response to the tumor based on the cancer immunity cycle[7] in the tumor microenvironment (TME), which comprises various components, such as tumor cells as well as blood vessels, fibroblasts, host immune cells and extracellular matrix.[8]

In this study, we investigated the potential of tertiary lymphoid organs (TLO; alternately, ectopic lymphoid tissues) as immunological biomarkers in reflecting the effect of neoadjuvant chemoradiotherapy (NAC). TLO are organized lymphoid structures similar to secondary lymphoid organs (SLO), characterized by B-cell follicles, T-cell zones and specialized vessels known as high endothelial venules (HEV), although they are not encapsulated and supplied by afferent lymphatics; their roles in the immune system are similar to those of SLO, characterized by recognition of antigens and production of antigen-specific antibodies.[9] Initially, TLO were identified in studies focusing on chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's diseases and *Helicobacter pylori* infection.[10] However, it was recently found that TLO are also present in malignant tissues,[11-13] and their clinical relevance has been reported in pancreatic cancer. Previous studies found that patients with high expression of intratumoral TLO in cancer tissues had favorable prognoses in terms of overall survival (OS) and disease-free survival (DFS), among patients who had undergone curative resection without preoperative treatment.[14]

In the present study, we investigated the clinical relevance of TLO by observing the differences between patients who received NAC and those who underwent curative surgical resection (surgery first [SF]). This study aimed to investigate TLO expression, functional differences in immunological aspects and differences in prognoses between patients belonging to the 2 groups in the TME, and to identify novel biomarkers for the reflection of prognoses following chemoradiotherapy.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

The present study included 146 patients diagnosed between January 2009 and December 2015 with pancreatic cancer who underwent surgical resection at the Department of Gastroenterological Surgery II in Hokkaido University Hospital. Among these, 4 patients who received adjuvant chemotherapy for other types of cancer, such as gastric cancer or colon cancer, and 2 patients whose lesions disappeared after preoperative treatment were excluded. The remaining 140 patients were divided into 2 groups: 93 who underwent SF and 47 who received NAC as a preoperative treatment. Clinical and pathological data were obtained through a detailed retrospective review of the medical records of all patients. All tissue sections obtained from formalin-fixed paraffin blocks were retrospectively analyzed for the formation of TLO using a microscope according to their clinical history with or without preoperative NAC. Then, the proportion of TLO in the TME was calculated and compared with the total tumor load. To further analyze the functional changes in the immunological elements within the TLO influenced by NAC, we performed an immunohistochemical test.

Finally, the clinicopathological factors of the 2 groups were statistically compared, including chemotherapy response and OS. The present study was approved by the Institutional Review Board of Hokkaido University Hospital. Informed consent was obtained from all the patients included. All tumors were staged according to the 7th TNM classification system of the Union for International Cancer Control.[15]

2.2 | Evaluation of tertiary lymphoid organs

The resected specimens of all 140 patients were formalin-fixed. In the present study, we selected tissue slides with the maximum divided surface, which were stained with H&E, and then detected the tumor area and identified TLO by microscopic observation. The microscopic images were imported as digital photo files using a NanoZoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu, Japan). We measured the area of the TLO (mm^2), and the total area of the PDAC tissue (mm^2) using Image J software (NIH, Bethesda, MD, USA). Then, we calculated the TLO area/total PDAC tissue area ratio (TLO/tumor ratio) to compensate for the bias affecting TLO due to the tumor size of the SF group and the NAC group.

2.3 | Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Heat-induced antigen retrieval was carried out in a high or low-pH antigen retrieval buffer (DakoCytomation, Glostrup, Denmark). Endogenous peroxidase was blocked by incubating tissue sections in 3% H_2O_2 for 5 minutes. The primary antibodies against CD4 as a marker for helper T cells (1:500, EPR6855; Abcam, Cambridge, UK), CD8 as a maker for cytotoxic T lymphocytes (1:500, EP1150Y; Abcam), CD20 as a marker for pan B cells (1:50, L26; Abcam), Foxp3 as a marker for regulatory T cells (Treg) (1:300, 236A/E7; Abcam), PNAd as a marker for HEV (1:100, MECA-79; BD Pharmingen™), programmed cell death (PD)-1 as a

marker for immune checkpoint molecules (1:50, NAT105; Abcam), Ki-67 as a marker for cell proliferation (1:100, SP6; Abcam), CD80 as a marker for M1 macrophages (1:1000, EPR1157(2); Abcam) and CD163 as a marker for M2 macrophages (1:500, EPR11598; Abcam) were applied for 30 minutes. These sections were visualized using the HRP-labeled polymer method (EnVision FLEX System, Dako). Immunostained sections were counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. These data are summarized in Table S1.

2.4 | Evaluation criteria of the immune cells in the tertiary lymphoid organs

The lymphocytes or immune cells stained diffusely or partly with antibodies, such as CD4, CD8, CD20, CD80 and CD163, in the TLO were measured to determine the area (mm^2) occupied by the positive lymphocytes within the TLO, and the lymphocytes stained individually with antibodies such as Foxp3, PD-1 and Ki-67 were counted for the number of positive cells within the TLO. In the case of HEV, the area of the vascular lumen was measured, and then, finally, the proportion of lymphocytes, immune cells or vascular lumen stained with these antibodies in the TLO was calculated. All imaging analyses were performed using Image J software (NIH, Bethesda, MD, USA) or BZ-X analyzer (Keyence, Osaka, Japan).

2.5 | Statistical analysis

Differences between the 2 groups were analyzed using Mann-Whitney *U*-tests. Categorical variables were compared using Fisher's exact test or a χ^2-test. The association between 2 continuous variables was calculated using Spearman's rank correlation analysis. OS was defined as the time from surgery to death from any cause in the SF group and the time from the start of NAC to death from any cause in the NAC group. The proportion of OS was calculated using the Kaplan-Meier method. Comparisons between the 2 groups were performed using a log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards model. All differences with a *P*-value of <0.05 were considered significant. Statistical analyses were performed using JMP 13 (SAS Institute Inc., Cary, NC, USA).

3 | RESULTS

3.1 | Clinicopathological features of the patients

The demographic background characteristics of all patients in this study are listed in Table 1. There were no significant differences in the background characteristics between the SF and NAC groups in terms of age, sex, tumor localization, lymph node and distant metastasis, pathological staging, histological differentiation, and presence or absence of adjuvant therapy. Data on the preoperative treatment regimens in the NAC group are summarized in Table S2. Sixteen and four patients received S-1 or gemcitabine alone, respectively; 8 patients received both S-1 and gemcitabine, and 2 patients received FOLFIRINOX, which comprised 5-fluorouracil (5-FU), oxaliplatin

TABLE 1 Clinicopathological background characteristics of the patients

Characteristics	Number of patients	SF	NAC	*P*-value
All cases	140	93	47	
Age				
<60	29	19	10	0.9071
≥60	111	74	37	
Sex				
Male	83	60	23	0.0764
Female	57	33	24	
Tumor-located area				
Ph	90	55	35	0.0739
Pbt	50	38	12	
Tumor status				
Tis	1	0	1	0.2426
T1	11	6	5	
T2	3	3	0	
T3	125	84	41	
T4	0	0	0	
Node status				
N0	53	31	22	0.1206
N1	87	62	25	
Metastasis status				
M0	137	92	45	0.2608
M1	3	1	2	
Stage				
0	1	0	1	0.2055
IA	7	4	3	
IB	1	1	0	
IIA	45	27	18	
IIB	83	60	23	
III	0	0	0	
IV	3	1	2	
Histological grade				
G1	49	31	18	0.5349
G2	84	56	28	
G3	7	6	1	
Adjuvant therapy				
+	111	76	35	0.3173
–	29	17	12	

There were no significant differences in terms of the background characteristics between the surgery first and the neoadjuvant chemoradiotherapy group.
Pbt, pancreas body and tail; Ph, pancreas head.
[a]χ^2-tests or Fisher's exact tests.

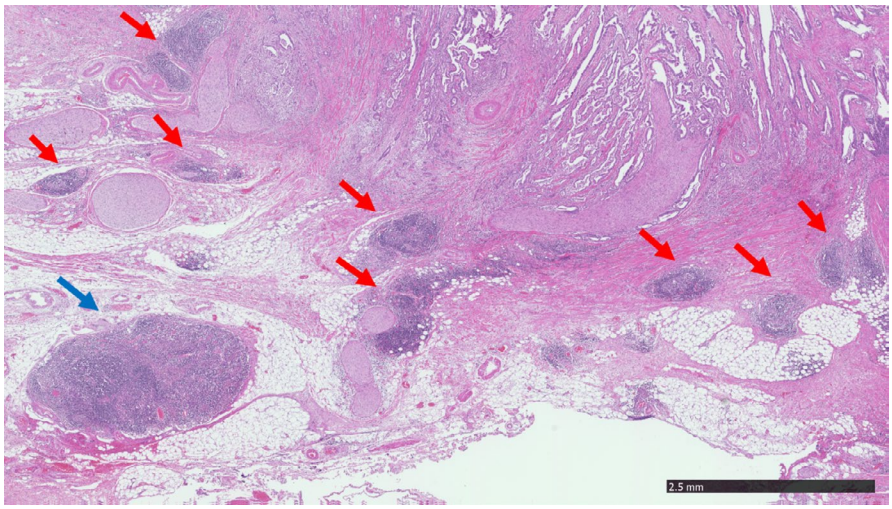

FIGURE 1 Characterization of the lymph nodes and TLO expressed in the tumor microenvironment. The representative picture obtained from the digital slide of the resected pancreatic cancer showed lymph nodes (blue arrow) and TLO (red arrow) expressed in the tumor microenvironment. Morphologically, lymph nodes had a capsule but TLO had no capsule. Scale bar, 2.5 mm. TLO, tertiary lymphoid organ

(L-OHP), irinotecan (CPT-11) and leucovorin (LV). Sixteen patients underwent combined radiotherapy 11 +gemcitabine +S-1, 1 +gemcitabine, 3 +S-1, 1 +FOLFIRINOX. Only 1 patient underwent intra-arterial chemotherapy using 5-FU and gemcitabine.

3.2 | Comparisons of the total tumor area, tertiary lymphoid organ area, and tertiary lymphoid organ/tumor ratio between the surgery first and neoadjuvant chemoradiotherapy groups

We measured the total area of the tumor (mm^2) and the area of the TLO (mm^2) using Image J software (NIH), and calculated the TLO/tumor ratio in the SF group and the NAC group. Microscopy images stained with H&E imported as digital files represented the lymph nodes and lymphoid follicles that were expressed adjacent to the pancreatic cancer cells. TLO are morphologically defined as histological structures that have lymphocyte aggregation without a capsule.[14] Unlike lymph nodes, these lymphoid follicles are not encapsulated (Figure 1).

The TLO expression rate was 91.4% (128/140) and there was no significant difference between the SF (88/93 94.6%) group and the NAC (40/47 85.1%) group ($P = 0.0575$). In patients with TLO expression, the total area of the tumor and the area of the TLO were significantly smaller in the NAC group than those in the SF group (Table S3). There was a weak correlation between the total tumor area and the TLO area ($r = 0.36$, $P < 0.001$) (Figure S1). Differences in the total tumor area between the SF and NAC groups were thought to be associated with the shrinkage effect of NAC. Differences in the TLO area between the SF and NAC groups were thought to be a result of modification bias influenced by tumor reduction due to anticancer drugs. Based on these data, the TLO/tumor ratio was considered appropriate as a parameter to compensate for TLO by tumor size.

3.3 | Immunohistochemical analysis

To analyze the components inside the TLO from an immunological viewpoint, we investigated the functional differences between the SF group and the NAC group in the TME by immunostaining lymphocytes or immune cells or lymphatic or blood vessels composed of TLO. Immunohistochemical staining was performed for 88 patients from the SF group and 40 patients from the NAC group with positive TLO expression. The lymphocytes positively stained with CD4, CD8 and CD20 were diffusely dominated within the TLO; in contrast, the immune cells positively stained with CD80 and CD163 were partly accounted for within the TLO. In addition, the lymphocytes positively stained with Foxp3, PD-1 and Ki-67 were individually expressed, which could be counted as 1 cellular unit within the TLO. The specially organized vascular endothelial cells stained with PNAd called HEV were also included within the TLO. These data are shown in Figure 2.

The proportions of CD8+ T cells and PNAd+ HEV were significantly higher in the NAC group than in the SF group (12.4% vs 21.7%, $P = 0.0046$; 1.7% vs 2.9%, $P = 0.0149$) and the proportion of PD-1+ lymphocytes was significantly higher in the SF group (4.6% vs 2.2%, $P = 0.0004$). There were no significant differences between the 2 groups with regard to the proportion of CD4+ T cells, CD20+ B cells and Foxp3+ Treg cells (20.0% vs 23.4%, $P = 0.3738$; 43.7% vs 38.1%, $P = 0.3015$; 3.9% vs 4.0%, $P = 0.9754$). The proportions of Ki-67+ lymphocytes and the CD163+ cells were significantly higher in the NAC group than in the SF group (2.4% vs 4.7%, $P = 0.0003$; 1.9% vs 3.7%, $P < 0.0001$). The proportion of CD80+ cells was higher in the NAC group than in the SF group (0.37% vs 0.54%, $P = 0.0532$) (Table 2).

Furthermore, we performed a subgroup analysis dividing each group of patients into "high" and "low" groups with a median TLO/tumor ratio value (as shown in Figure S2). For the NAC group, the proportion of CD20+ B cells was significantly higher among those with a high TLO/tumor ratio than in those with a low TLO/tumor ratio (42.7% vs 23.2%, $P = 0.0133$). Moreover, the proportion of PNAd+ HEV was significantly higher in the high TLO/tumor ratio group (3.5% vs 1.8%, $P = 0.0009$). In contrast, the proportions of Foxp3+ Treg cells and PD-1 positive lymphocytes, both acting immunosuppressively, were significantly higher in the low TLO/tumor ratio group than in the high TLO/tumor ratio group (2.9% vs 6.1%, $P = 0.0208$; 1.5% vs 5.1%, $P = 0.0086$). There was no significant

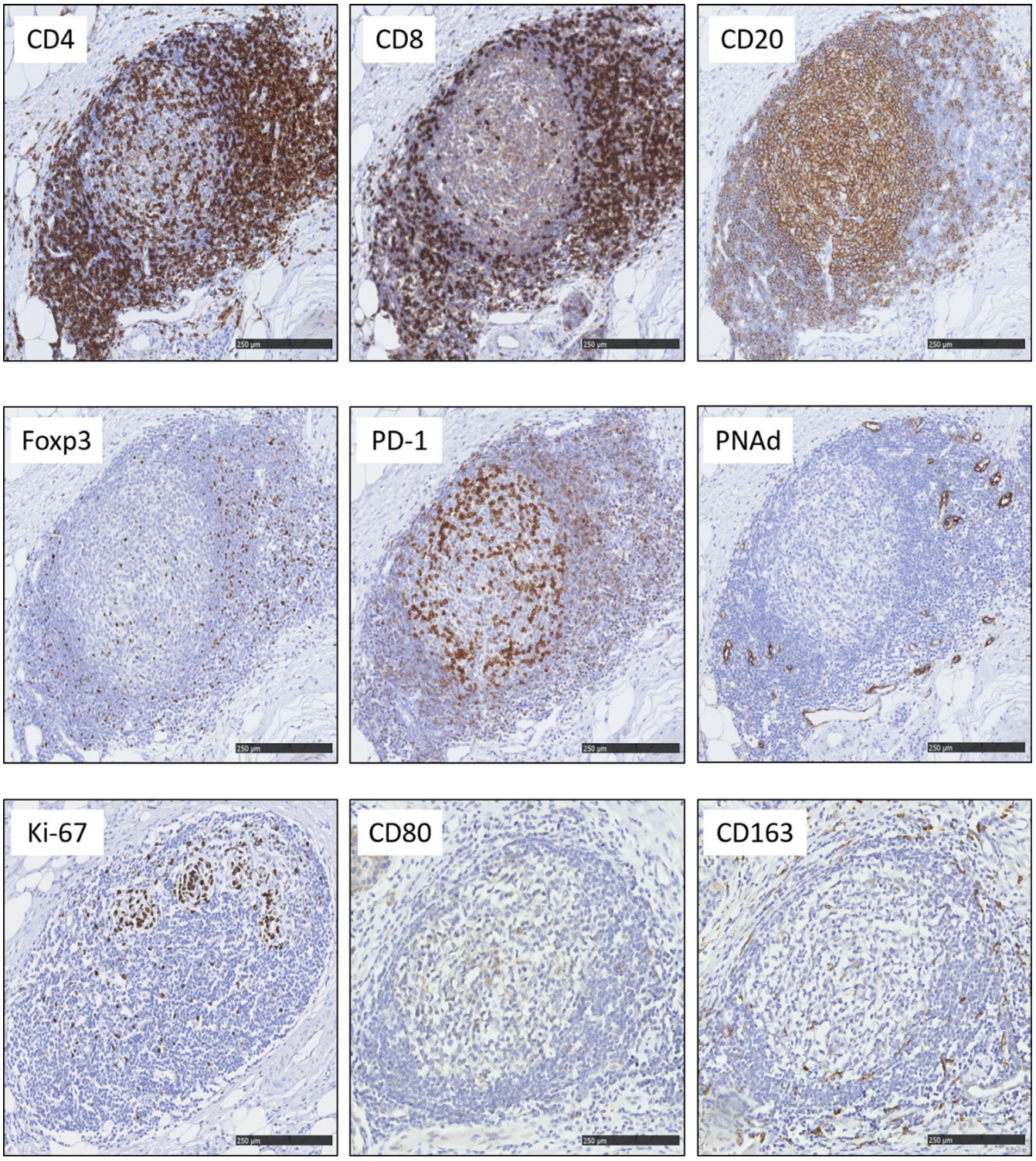

FIGURE 2 Intratumoral TLO observed in PDAC patients. In the immunohistochemical staining analysis, the TLO were composed of T cells (CD4, CD8), B cells (CD20), Treg cells (Foxp3), PD-1 positive cells, PNAd+ HEV and antigen-presenting cells (CD80, CD163). Cell-proliferation was shown by Ki-67+ cells. All positive signals are in brown. Scale bars, 250 μm. HEV, high endothelial venule; PDAC, pancreatic ductal adenocarcinoma; TLO, tertiary lymphoid organ

difference between the 2 groups with regard to the proportions of CD4+ and CD8+ T cells (24.3% vs 22.5%, $P = 0.1756$; 21.7% vs 21.6%, $P = 0.7119$) and the proportions of CD80+ and CD163+ cells (0.47% vs 0.56%, $P = 0.4040$, 3.7% vs 3.7%, $P = 0.6715$). The proportion of Ki-67+ lymphocytes tended to be higher in the low TLO/tumor ratio group, but the difference was not significant (3.1% vs 6.8%, $P = 0.0609$) (Table S4A). In the SF group, there was no significant difference between the high TLO/tumor ratio group and the low TLO/

TABLE 2 Comparison of the proportions of several immune components within the TLO between the SF and the NAC group

	SF (n = 88)	NAC (n = 40)	*P*-value[a]
CD4	20.0 (3.3-45.2)	23.4 (0.7-59.3)	0.3738
CD8	12.4 (3.0-47.5)	21.7 (3.4-52.6)	0.0046
CD20	43.7 (3.5-71.4)	38.1 (0.1-69.9)	0.3015
Foxp3	3.9 (0.6-12.2)	4.0 (0.3-28.7)	0.9754
PD-1	4.6 (0.3-19.1)	2.2 (0.2-16.7)	0.0004
PNAd	1.7 (0.4-4.7)	2.9 (0.3-12.0)	0.0149
Ki-67	2.4 (0.3-14.0)	4.7 (0.8-23.9)	0.0003
CD80	0.37 (0.02-3.72)	0.54 (0.04-15.2)	0.0532
CD163	1.9 (0.2-7.3)	3.7 (0.7-11.7)	<0.0001

The proportions of CD8+ T cells and PNAd+ HEV were significantly higher in the NAC group than in the SF group, and the proportion of PD-1+ lymphocytes was significantly higher in the SF group. There were no significant differences between the 2 groups with regard to the proportions of CD4+ T cells, CD20+ B cells and Foxp3+ Treg cells. The proportions of Ki-67+ lymphocytes, CD163+ cells and CD80+ cells were significantly higher in the NAC group. Data: median (range).
HEV, high endothelial venule; NAC, neoadjuvant chemoradiotherapy; SF, surgery first; TLO, tertiary lymphoid organ.
[a]Mann-Whitney *U*-tests.

tumor ratio group except in the case of CD163+ cells, the proportion of which was significantly higher in the low TLO/tumor ratio group than in the high TLO/tumor group (1.6% vs 2.3%, $P = 0.0340$) (Table S4B).

3.4 | Prognostic analysis

In terms of OS, the NAC group had a significantly better prognosis than the SF group ($P = 0.0017$) (Figure 3A). The median follow-up time was 749.5 days, and the 2-year and 5-year survival rates of the SF group were 51.9% and 17.7%, while those of the NAC group were 82.0% and 44.2%, respectively. Regarding for the postoperative adjuvant therapy, there was no significant difference in overall survival with and without adjuvant therapy, therefore it suggested that adjuvant therapy had no impact on patients' overall survival in both SF and NAC group (data not shown). In the subgroup analysis, Kaplan-Meier curving plots showed that patients in the NAC group with high TLO/tumor ratios had better prognoses than those with low TLO/tumor ratios ($P = 0.0328$); there were no corresponding significant differences in the SF group ($P = 0.6719$) (Figure 3B,C). The demographic background characteristics of the patients in each group are listed in Table S5A (NAC) and Table S5B (SF). We performed univariate and multivariate analyses by adding the TLO/tumor ratio into the clinicopathological factors. In the multivariate analysis, the absence of lymph node metastasis (hazard ratio [HR]: 0.029, 95% confidence interval [CI]: 0.003-0.163, $P < 0.0001$) and a high TLO/tumor ratio (HR: 0.056, 95% CI: 0.006-0.297, $P < 0.0001$) were determined as independent favorable prognostic factors (Table 3).

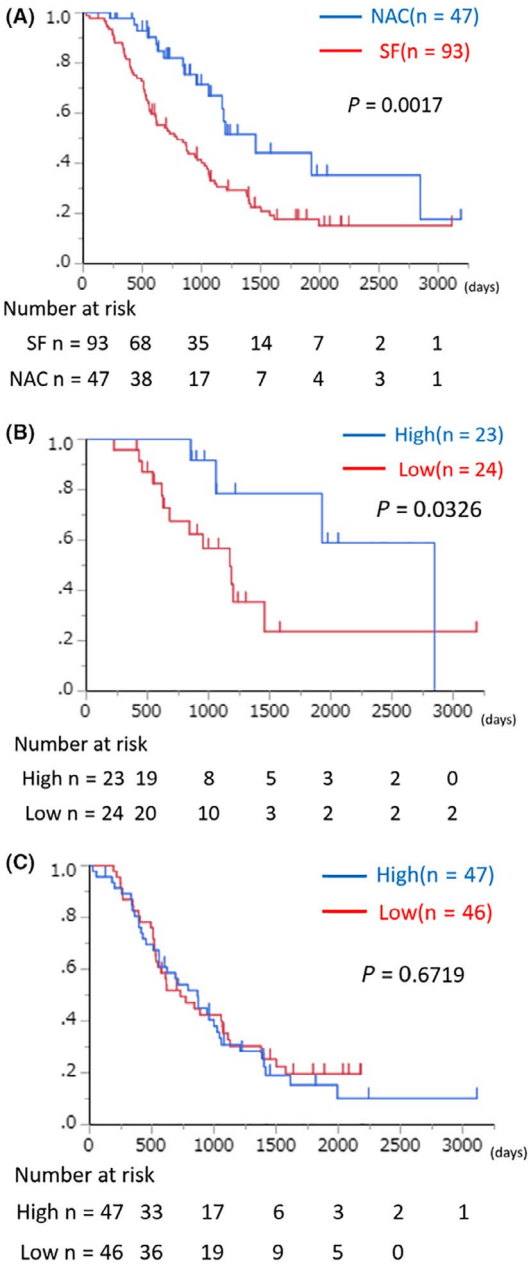

FIGURE 3 Overall survival of the 140 patients with pancreatic cancer according to the performance of neoadjuvant chemoradiotherapy or upfront surgical resection (A), and differences in the overall survival between the SF group and the NAC group according to the patients' TLO/tumor ratio. B, In the NAC group. C, In the SF group. The OS curves were estimated using the Kaplan-Meier method, and differences between the 2 groups were evaluated using a log-rank test. The number of patients at risk is shown under the OS curves. HEV, high endothelial venule; NAC, neoadjuvant chemoradiotherapy; OS, overall survival; SF, surgery first; TLO, tertiary lymphoid organ

4 | DISCUSSION

In this study, we investigated the clinical relevance of TLO in PDAC following chemoradiotherapy. TLO were originally defined as lymphoid organs characterized by the expression of lymphocyte

aggregates without a capsule. TLO formation is associated with the generation of encapsulated SLO.[16] However, TLO formation is distinct from the preprogrammed ontogenic processes that are associated with secondary lymphoid organogenesis and it does not occur in all patients.[16] During chronic inflammatory reactions, lymphocytes can accumulate and lead to the de novo formation of B cell follicles and T cell areas.[9,10] This phenomenon has been termed "tertiary lymphoid neogenesis" or "TLO formation."[10,16] Despite the presence of structural differences between SLOs and TLO, various mechanisms that control the initial development, cellular composition and functional maintenance of these structures are shared.[9-13,16]

In cancer, the presence of tumor-associated TLO correlates with patients' prognoses depending on the tumor types and the cell composition inside the TLO, and it is suggested that they coordinate the endogenous antitumor immune responses that can improve patient survival positively.[17] Recently, several studies focusing on different types of tumors, including pancreatic cancer,[14,18] colorectal cancer,[19] non–small cell lung cancer,[20,21] gastric cancer[22] and breast cancer,[23,24] have used approaches such as the measurement of TLO density and analysis of TLO-related gene expression.

With regard to the clinical relevance of TLO in pancreatic cancer, Hiraoka et al[14] reported that the presence of intratumoral TLO in PDAC tissues appeared to be an independent prognostic factor; moreover, it represented a TME that was less vulnerable to cancer invasiveness, being associated with an active immune reaction in patients who underwent upfront surgery. In another study on preoperative treatment, Lutz et al[18] tested the hypothesis that the administration of vaccine-based immunotherapy can convert PDAC tumors from a "nonimmunogenic" form to an "immunogenic" form with infiltrating effector lymphocytes. They showed that the administration of immune-based therapy with an irradiated, granulocyte-macrophage colony-stimulating factor-secreting, allogenic PDAC vaccine (GVAX) induced the significant development of tertiary lymphoid aggregates within the PDAC tissues, suggesting that GVAX triggered antigen-specific immune response at the tumor site. Furthermore, microdissection and gene array analyses demonstrated that decreased Treg and increased Th17 immune effector signatures within the vaccine-induced intratumoral lymphoid aggregates were associated with enhanced systemic post–vaccination T cell response and longer patient survival.

Here, we demonstrated for the first time the development of TLO following chemoradiotherapy based on novel data associated with the functional mechanisms behind the immunological compartments inside the TLO and how TLO affect patients' prognoses. In our data, the proportions of CD8+ T lymphocytes and PNAd+ HEV composed of TLO were higher in the NAC group than in the SF group. In contrast, the proportion of PD-1+ lymphocytes inside the TLO was lower in the NAC group. Moreover, the proportions of Ki-67+ lymphocytes and CD163+ M2 macrophages were higher in the NAC group than in the SF group. These data suggest that lymphocytes and antigen-presenting cells (APC) were actively induced into the TME by an increase in the HEV levels, which caused the activation of cellular immunity by CD8+ killer T cells; in addition, decreased immunosuppressive PD-1+ cell levels may have led to the favorable prognoses in the NAC group.

The detailed mechanisms behind the immune network initiated by antigen stimulation have been reported in several tumors. The current paradigm is that the presence of abundant tumor-infiltrating immune cells, especially cytotoxic T cells, confers a positive prognostic value in the majority of human cancers.[25] With regard to the relationship between CD4+ T cells and CD8 T cells, it was shown in a mouse model that CD4+ T cells could produce IFN-γ in the tumor

TABLE 3 Univariate and multivariate analyses of OS of the clinicopathological variables and TLO/tumor ratio in the NAC group

	Univariate analysis[a]		Multivariate analysis[a]	
N = 47	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (>60/≤60)	1.530 (0.486-6.738)	0.493		
Gender (M/F)	2.539 (0.952-7.476)	0.063		
Location (Ph/Pbt)	3.527 (1.077-16.095)	0.036	1.375 (0.358-6.782)	0.656
Tumor size (>2.0 cm/≤2.0 cm)	4.019 (1.416-14.310)	0.008	1.017 (0.280-4.258)	0.981
Pathologic node status (N0/N1)	0.152 (0.041-0.457)	<0.001	0.029 (0.003-0.163)	<0.001
Histological grade (G1/G2, G3)	0.633 (0.200-1.719)	0.381		
Evans classification (1, 2a/2b, 3)	2.160 (0.746-7.766)	0.162		
Lymphatic invasion (+/−)	1.042 (0.322-2.901)	0.94		
Venous invasion (+/−)	1.161 (0.420-3.700)	0.782		
Neural invasion (+/−)	1.794 (0.610-6.501)	0.3		
TLO/tumor (high/low)	0.306 (0.085-0.884)	0.028	0.056 (0.006-0.297)	<0.001

In the multivariate analysis, the absence of lymph node metastasis and a high TLO/tumor ratio were considered as independent favorable prognostic factors.

CI, confidential interval; HR, hazard ratio; NAC, neoadjuvant chemoradiotherapy; OS, overall survival; Pbt, pancreas body and tail; Ph, pancreas head; TLO, tertiary lymphoid organ.

[a]Cox-regression proportional hazards model.

milieu, leading to the recruitment, proliferation and possible survival of CD8+ T cells.[26] Collectively, our data showed that the proportion of CD8+ T lymphocytes infiltrating the TLO was much higher in the NAC group than in the SF group, and this may have led to better prognoses in PDAC patients who underwent preoperative treatment. Although there was no significant difference in the proportion of CD4+ T lymphocytes between the SF group and the NAC group, a correlation between the proportion of CD4+ and CD8+ T lymphocytes in each group was observed (SF; $r = 0.55$, NAC; $r = 0.58$). This finding suggested that these cells may have interacted with each other in the TME. Taken together, our data support the findings of previous studies in that CD8+ T cells were found to be good prognostic indicators in human cancers.

The present study's results also demonstrated that patients with a high density of TLO in the TME, that is, those with a high TLO/tumor ratio, showed higher infiltration of CD20+ B cells into the TLO than those with a low TLO/tumor ratio, and this might have been conducive to favorable prognoses in the NAC group only. With regard to the relevance of B cells in TLO, their efficacy as prognostic indicators has been reported in several types of cancers. Generally, B cells have 2 major roles in anti–tumor immunity, both as APCs and tumor antigen-specific antibody-secreting cells.[27] In a study on gastric cancer, the presence of a high number of CD20+ B cells was associated with significantly better OS.[22] Previous studies demonstrated that the organization of intratumoral B cells into B-cell follicles was associated with the development of antigen-specific humoral immunity and that the density of follicular B cells was highly predictive of survival in non–small cell lung cancer.[28] The organization of B cells into the TLO B-cell follicles may, thus, better reflect the initiation of local anti–tumor B-cell mediated immunity.[22,27,28] However, the capacity of B cells to cross-present antigens to CD8+ T cells has also been well established.[29] According to the present study, B cells may similarly serve as APC in cancer and, thus, promote anti–tumor response. Tumor-infiltrating CD20+ B cells are often found in close proximity to CD8+ T cells, and the presence of both CD20+ and CD8+ lymphocytes was associated with markedly prolonged survival in ovarian cancer patients.[29] This is because the high infiltration of TLO into the TME may be related to the development of CD20+ B cell follicles, and preoperative chemoradiotherapy could promote this phenomenon; these findings are consistent with those of previous studies on breast cancer.[30] Furthermore, in addition to B cells, the role of other APC and the mechanisms of antigen presentation in TLO have been discussed.[31] Macrophages are generally categorized into 2 subsets known as classically activated macrophages (M1 like) and alternatively activated macrophages (M2 like), respectively. In general, M1 cells exhibit a pro–inflammatory effect, while M2 cells facilitate the resolution of inflammation and promote tissue repair.[32] In this study, patients in the NAC group who received preoperative chemoradiotherapy had a higher proportion of CD163+ M2 macrophages within the TLO than the patients in the SF group, who did not receive preoperative treatment. This finding suggested that the administration of preoperative treatment could facilitate the recognition of the tumor-associated antigens released by tumor destruction and promote antitumoral immunity.

Finally, with regard to HEV, the detailed mechanisms of the de novo formation of TLO have been described in some tumors. Intratumoral PNAd+ HEV are exclusively associated with TLO, and blood T cells enter the TLO via HEV, which represent a new gateway for T cells to enter the tumor environment.[33] With regard to the clinical relevance of HEV, high densities of tumor HEV were found to be associated with increased numbers of poorly differentiated T cells, such as tumor infiltrating naïve and central memory T cells and activated effector memory T cells and significantly better prognoses in breast cancer.[33] Through their ability to recruit large numbers of circulating lymphocytes, tumor HEV may represent new attractive targets for both cancer diagnosis and therapy.[34] Similar to the findings of a previous study, our data also showed the efficacy of PNAd+ HEV, the proportion of which was higher in the NAC group than in the SF group, resulting in favorable prognoses. In the subgroup analysis using the TLO/tumor ratio, patients with a high TLO/tumor ratio showed a higher proportion of HEV within the TLO and significantly better prognoses than those with a low TLO/tumor ratio (as shown in Table S4A, Figure 3B). This result suggested that the abundance of lymphocytes, which were induced to infiltrate into the TME by HEV, could play a role in cellular or humoral immunity.

In the process of investigating effector mechanisms, we also focused on the immunosuppressive molecules within the TLO. The infiltration of Treg cells into many tumor types correlates with poor prognoses, but the mechanisms of intratumoral Treg function remain unknown. Treg function was investigated in a genetically engineered mouse model of lung cancer. Results showed that Treg cells suppressed the anti–tumor response in TLO.[35] In this study, although there was no significant difference between NAC and SF groups in the proportions of Foxp3+ Treg cells within the TLO, we found that the proportion of PD-1 within the TLO was significantly lower in the NAC group than in the SF group, signifying better prognoses, and this finding supports the results of previous studies. It was suggested that the administration of preoperative chemoradiotherapy effectively reduced the proportion of immunosuppressive molecules regardless of the type of regimen.

To date, many studies have focused on the infiltration of T cells into the TME following chemoradiotherapy. However, little is known about the immunological and functional mechanisms of the immune components inside the TLO after chemoradiotherapy. Previous studies demonstrated that administration of chemoradiotherapy preoperatively can prove useful in reducing Treg cell levels[36] or induce the accumulation of CD4+ and CD8+ cells in the TME; a high accumulation of CD8+ cells might be a good prognostic marker in pancreatic cancer.[37] Although the association between TIL and TLO, one of the limitations of this study, should be investigated in future analysis, our data are in agreement with those of previous studies suggesting that TLO may be activated by the tumor-associated antigen released by tumor destruction following chemoradiotherapy, accompanied by B cell infiltration,

promotion of the proliferation of immune cells, development of antigen presentation, and recognition and suppression of PD-1+ cells, and leading to the angiogenesis of HEV.

In conclusion, we showed the influence of preoperative chemoradiotherapy on the immunological elements in the TME, which resulted in favorable prognoses in PDAC patients.

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DISCLOSURE

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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