Published in final edited form as:

Pancreas. 2017 October ; 46(9): 1180–1187. doi:10.1097/MPA.0000000000000914.

Prognostic Significance of Tumor Infiltrating Lymphocytes in Patients With Pancreatic Ductal Adenocarcinoma Treated With Neoadjuvant Chemotherapy

Reza Nejati, MD^{*}, Jennifer B. Goldstein, MD[†], Daniel M. Halperin, MD[†], Hua Wang, MD, PhD[†], Nazila Hejazi, MD^{*}, Asif Rashid, MD, PhD^{*}, Matthew H. Katz, MD[‡], Jeffrey E. Lee, MD[‡], Jason B. Fleming, MD[‡], Jaime Rodriguez-Canales, MD[§], Jorge Blando, DVM[§], Ignacio I. Wistuba, MD[§], Anirban Maitra, MD^{*,§}, Robert A. Wolff, MD[†], Gauri R. Varadhachary, MD[†], and Huamin Wang, MD, PhD^{*,§}

^{*}Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX

[†]Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX

[‡]Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX

[§]Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX

Abstract

Objectives—To examine tumor infiltrating lymphocytes (TILs) and their prognostic value in patients with pancreatic ductal adenocarcinoma (PDAC) after neoadjuvant therapy.

Methods—Intra-tumoral CD4+, CD8+, and FOXP3+ lymphocytes was examined by immunohistochemistry using a computer-assisted quantitative analysis in 136 PDAC patients who received neoadjuvant therapy and pancreaticoduodenectomy. The results were correlated with clinicopathologic parameters and survival.

Results—High CD4+ TILs in treated PDAC were associated with high CD8+ TILs ($P = 0.003$), differentiation ($P = 0.04$) and a lower frequency of recurrence ($P = 0.02$). Patients with high CD4+ TILs had longer disease-free survival (DFS) and overall survival (OS) than did patients with low CD4+ TILs ($P < 0.01$). The median OS of patients with a high CD8+/FOXP3+ lymphocyte ratio (39.5 [standard deviation, 6.1] months) was longer than that of patients with a low CD8+/FOXP3+ lymphocyte ratio (28.3 [standard deviation, 2.3] months; $P = 0.01$). In multivariate analysis, high CD4+ TILs were an independent prognostic factor for DFS [Hazard ratio (HR): 0.49, 95% CI, 0.30–0.81; $P = 0.005$] and OS (HR, 0.54; 95% CI, 0.33–0.89; $P = 0.02$).

Address correspondence and reprint requests to: Huamin Wang, M.D., Ph.D., Department of Pathology, Unit 085, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030 (hmwang@mdanderson.org). Phone: (713) 563-1846; Fax: (713) 563-1848, or Gauri R. Varadhachary, M.D., Department of Gastrointestinal Medical Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030 (gvaradha@mdanderson.org); Phone: (713) 792-2828.

Disclosure: The authors have no conflicts of interest or funding to disclose

Conclusions—High CD4+ lymphocytes is associated with tumor differentiation, lower recurrence and is an independent prognostic factor for survival in PDAC patients treated with neoadjuvant therapy.

Keywords

pancreatic cancer; tumor infiltrating lymphocytes; CD4; CD8; FOXP3; prognosis

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy and the fourth-leading cause of cancer-related death in the United States.¹ The lack of effective conventional therapies for patients with the disease has prompted researchers to investigate novel treatments and obtain a better understanding of PDAC biology and drug resistance.^{2,3}

The tumor microenvironment plays an important role in the biology of cancers. Among the components of the tumor microenvironment, tumor infiltrating lymphocytes (TILs) have been shown to have prognostic value in patients with a wide variety of cancers.^{4–9} TILs are composed of a heterogeneous population of lymphocytes that promote either tumor immunity or tumor pathogenesis.^{10,11} Dendritic cells, M1 macrophages, Th1 CD4+ T cells, cytotoxic CD8+ T cells, and natural killer cells tend to inhibit tumor growth, whereas M2 macrophages, myeloid-derived suppressor cells, neutrophils, Th2 and Th17 CD4+ T cells, and FOXP3+CD4+ regulatory T cells (Tregs) are suspected to stimulate tumor growth depending on the tumor type.^{10,11} Thus, in predicting tumor behavior and prognosis, both the composition and numbers of the TILs are likely important.

Several studies have shown that TILs have prognostic value in PDAC patients. Fukunaga et al. reported that PDAC patients with high numbers of CD8+ TILs and CD4+ TILs had a significantly longer overall survival (OS) rate than patients with low numbers of TILs.¹² Other studies have shown that in PDAC patients, a high density of FOXP3+ Tregs, which are believed to help suppress T-cell-mediated immune responses and effective antitumor immune responses, is correlated with a high risk of lymph node metastasis and with poor prognosis.^{13–15}

More PDAC patients who are potential candidates for pancreaticoduodenectomy (PD) now receive neoadjuvant chemotherapy prior to the surgery. Increasing evidence in breast cancer suggests that chemotherapeutic agents, which are generally considered to be immunosuppressive, can elicit antitumor immunological responses that enhance the efficacy of the treatment and improve patient survival.^{16,17} It has also been shown that the post-chemotherapy TIL status of residual triple-negative or HER2 positive breast cancer has prognostic value.^{18–20} It has been suggested that chemotherapeutic agents may exert their immunostimulatory effects by inducing immunogenic tumor cell death directly, mitigating immunosuppressive mechanisms, creating lymphopenia and an immunogenic milieu, and/or sensitizing tumor cells to immune destruction.^{16,21–24}

However, few studies have investigated the composition of TILs in PDAC after neoadjuvant chemotherapy.²⁵ The role of TILs in PDAC after neoadjuvant therapy and their effect on

patient prognosis remains unclear. The purpose of the present study was to assess intra-tumoral FOXP3+, CD4+, and CD8+ lymphocytes in patients with PDAC who received neoadjuvant therapy and PD and to correlate the amount of these TILs with patients' survival and clinicopathological features. Our results suggest that targeting the tumor microenvironment to increase the CD4+ lymphocyte count and CD8+/FOXP3+ lymphocyte ratio could improve the clinical outcomes of PDAC patients.

MATERIALS AND METHODS

2.1. Patient Population

The Institutional Review Board of The University of Texas MD Anderson Cancer Center approved this study. Our study population consists of 136 PDAC patients (83 men and 53 women) who underwent neoadjuvant chemotherapy followed by PD between 1999 and 2007. The patients' clinicopathological data, including sex, age, tumor differentiation, histologic subtype, T stage, AJCC stage, and lymph node status, and their follow-up data were extracted from the prospectively maintained database at the Department of Surgical Oncology and validated by reviewing medical records. If necessary, patient deaths were confirmed by review of the U.S. Social Security Death Index. The patients' median age was 63.5 years (range, 42.1–84.2 years). Two pathologists separately reviewed patients' archived tumor specimens for histologic type, differentiation, stage, margin status, regression grade, and lymph node metastasis.

2.2. Tissue Microarray Construction

We used a tissue microarrayer (Beecher Instruments, Sun Prairie, Wis) to construct tissue microarrays from formalin-fixed, paraffin-embedded blocks of archived PDAC specimens as described previously.²⁶ Briefly, a gastrointestinal pathologist (H.W.) reviewed matched hematoxylin-and-eosin (H & E) stained slides to select the best representative tumor region for each case. Three 1.0 mm tissue cores from each selected representative tumor region were used for tissue microarray construction.

2.3. Immunohistochemical Analysis for CD4, CD8, FOXP3

Immunohistochemistry for CD4, CD8, and FOXP3 were performed on 4- μ m unstained sections from the tissue microarray blocks using primary mouse monoclonal antibodies against CD4 (1:25 dilution, SP35; Cell Marque, Rocklin, Calif), CD8 (1:20 dilution from predilute, C8/144B; Thermo Scientific, Fremont, Calif), and FOXP3 (1:50 dilution, clone 206D, BioLegend, San Diego, Calif). The tissue sections were deparaffinized and placed in a 100°C steamer containing 20 mmol citrate buffer (pH 6.0) for 5 minutes for antigen retrieval. The sections were then incubated with the primary antibody at 35°C for 15 minutes, immersed in 3.0% hydrogen peroxidase at 35°C for 5 minutes to block endogenous peroxide activity, immersed in polymer enhancer for 8 minutes, and then incubated with secondary poly-horseradish peroxidase anti-mouse/anti-rabbit immunoglobulin G at 35°C for 8 minutes. The 3,3'-diaminobenzidine was used as a chromogen. Hematoxylin was used for counterstaining.

2.4. Image Analysis of Immunohistochemical Signaling

Digital images of immunohistochemically stained TMA slides were obtained at 20× magnification using a ScanScope Aperio AT Turbo whole-slide scanner (Leica Biosystems, Buffalo Grove, Ill). Tumor regions were manually annotated, and a computer-assisted quantitative analysis was performed using the Aperio ImageScope software program with the custom-made Color Deconvolution v9 algorithm. The amount of TILs was calculated as the percentage of positive staining area versus the total tumor area. For statistical analysis, we classified tumor as CD4+, CD8+, and FOXP3+ low and high using 75 percentile values for each marker as the cut off.

2.4. Statistical Analysis

We used the χ^2 or Fisher's exact test to compare categorical data. Overall survival (OS) was defined as the time from the date of PDAC diagnosis to the date of death or the date of last follow-up if death did not occur. Disease-free survival (DFS) duration was defined as the time from the date of surgery to the date of first recurrence in patients with recurrence or to the date of last follow-up in patients without recurrence. Survival curves were constructed using the Kaplan-Meier method and the log-rank test was used to evaluate the statistical significance of difference in survival. Univariate and multivariate Cox regression analyses were used to assess the prognostic significance of clinicopathological variables. We used the SPSS Statistics for Windows (version 22; IBM SPSS Inc., Armonk, NY) to perform all statistical analyses. We used a 2-sided significance level of 0.05 for all statistical analyses.

RESULTS

3.1. Tumor Infiltrating CD4+, CD8+, and FOXP3+ Lymphocytes in Treated PDAC Samples

For all patients, the mean (standard deviation [SD]) percentages of TILs that were CD4+, CD8+, or FOXP3+ lymphocytes were 0.69% (0.71%), 1.14% (1.13%), or 1.47% (4.47%), respectively. The 75th percentiles of the values of TILs that were CD4+, CD8+, or FOXP3+ lymphocytes were 0.88, 1.44, and 0.82, respectively, which were used as the cutoff values for determining whether tumors had high or low counts of these TILs. Of 136 patients, there were 34 patients whose tumors had high CD4+, CD8+, or FOXP3+ lymphocyte counts. Representative micrographs showing the cases with low and high counts of CD4, CD8 and Foxp3 are shown in Figure 1.

3.2. Correlation Between CD4+, CD8+, and FOXP3+ Tumor Infiltrating Lymphocytes

High CD4+ TILs correlated with high CD8+ TILs. Among the 34 patients whose tumors had high CD4+ TILs, 15 (44%) had tumors that also had high CD8+ TILs; in contrast, of the 102 patients whose tumors had low CD4+ TILs, only 19 (19%) had tumors that also had high CD8+ TILs ($P = 0.003$). We found no significant correlation between FOXP3+ TILs with either CD4+ TILs ($P = 0.18$) or CD8+ TILs ($P = 0.11$).

3.3. Clinicopathological Correlation of CD4+, CD8+, and FOXP3+ Tumor Infiltrating Lymphocytes

Associations between patients' clinicopathological features and CD4+, CD8+, and FOXP3+ TILs are shown in Table 1. High CD4+ TILs was associated with better tumor differentiation. Of the 102 patients whose tumors had low CD4+ TILs, 41 (40.2%) had poorly differentiated PDAC; in contrast, of the 34 patients whose tumors had high CD4+ TILs, only 7 (20.6%) had poorly differentiated PDAC ($P=0.04$). The local recurrence or distant metastasis was present in 79.4% (81/102) patients whose tumors had low CD4+ TILs compared to those whose tumor had high CD4+ TILs (58.8%, 20/34, $P=0.02$). In contrast, high FOXP3+ TILs was associated with a higher recurrence or distant metastasis rate than those patients whose tumors had a low FOXP3+ TILs ($P=0.046$, Table 1).

3.4. Survival Analysis

The median follow-up duration after surgery was 32.5 months (range, 7.6–172.8 months) for all patients and 107.1 months (range, 9.4–172.8 months) for the patients who remained alive at the end of study. Patients whose tumors had high CD4+ TILs had longer DFS and OS than did patients whose tumors had low CD4+ TILs (Figure 2 and Table 2). The median DFS and OS of the patients whose tumors had high CD4+ TILs (24.5 [SD, 4.4] months and 45.5 [SD, 8.5] months, respectively) were significantly longer than those of patients whose tumors had low CD4+ TILs (9.8 [SD, 1.0] months and 28.4 [SD, 3.7] months, respectively; $P<0.01$). Although the associations between high CD8+ TILs or high FOXP3+ TILs with either DFS or OS were not statistically significant ($P>0.05$, data not shown), patients with high CD8+/FOXP3+ lymphocyte ratio had a statistically significant longer median OS than those whose tumor had low CD8+/FOXP3+ lymphocyte ratio (39.5 [SD, 6.1] months vs. 28.3 [SD, 2.3] months; $P=0.01$). Consistent with our previous results,^{27–30} patients with lymph node metastasis had shorter DFS (10.5 [SD, 0.96] months; $P=0.03$) and OS (29.1 [SD, 2.1] months; $P=0.03$) than did patients without lymph node metastasis, whose DFS and OS durations were 22.0 [SD, 10.2] months and 39.5 [SD, 11.1] months respectively (Figure 3 and Table 2). The multivariate analysis revealed that a high CD4+ TILs, lymph node metastasis, and tumor regression grade were independent prognostic factors for DFS and OS (Table 3). The correlation of CD8+/FOXP3+ lymphocyte ratio with either DFS or OS was also significant by multivariate analysis ($P>0.05$).

DISCUSSION

Several studies have investigated the role of TILs and their correlation with clinical outcomes in PDAC patients. Fukunaga et al. showed that the 5-year OS rate of patients with CD8+ tumors (tumors with an average CD8+ T-cell count of 100 for 5 microscopic fields at $\times 400$ magnification) was higher than that of patients with CD8– tumors. They also reported that the OS rate of patients with CD4+ tumors (tumors with an average CD4+ T cell count of 20 for 5 microscopic fields at $\times 400$ magnification) was higher than that of patients with CD4– tumors, but the difference was not statistically significant. They showed that the combined CD8+/CD4+ phenotype was an independent prognostic factor for survival although neither CD8+ nor CD4+ tumor status alone was an independent predictor of survival by multivariate analysis. They thus concluded that the presence of both CD4+ and

CD8+ T cells is crucial to effective tumor immunity in PDAC.¹² Tewari et al evaluated the prognostic significance of CD3+, CD8+, and CD20+ TILs in 81 PDAC patients and found an association between high levels of CD3+ and CD20+ TILs and higher survival rates.³¹ In another study, Ino et al. found that patients with a high CD4+ T-cell count, high CD8+ T-cell count, and low Treg count (defined as values higher and lower than the median for tumor-infiltrating immune cells) had longer survival than those with a low CD4+ T-cell count, low CD8+ T-cell count, and high Treg count.³² A growing body of evidence suggests that the TIL status in the residual tumor after neoadjuvant chemotherapy can predict clinical outcomes of breast cancer patients.^{33,34} However, the prognostic significance of TILs in patients with PDAC who received neoadjuvant therapies and PD is not clear. In this study, we examined 136 such patients and found that a high CD4+ TILs is an independent prognostic factor for DFS and OS in PDAC patients who received neoadjuvant therapy and PD. In addition, we also showed that a high CD8+/FOXP3+ lymphocyte ratio correlated with better clinical outcomes in such patients. In contrast to the previous studies, we did not find significant correlation between CD8+ TILs and survival or other clinicopathological features. This may be due to the fact that all patients in this study received neoadjuvant therapy before PD, which is different from previous studies of TILs in PDAC samples from patients who received no neoadjuvant therapy. It is possible that neoadjuvant therapy may suppress CD8+ TILs and therefore does not impact survival in our study population. In one of the few studies that specifically evaluated the composition of TILs in PDAC patients who had received adjuvant chemoradioimmunotherapy, Karakhanova et al. found that high CD4+ and CD8+ T-cell counts (229 cells/mm² and 95 cells/mm², respectively) were correlated with longer OS and DFS durations. Our finding that a high CD4+ TILs predicts better survival is consistent with this study. However, we did not find any correlation between high CD8+ TILs and OS or DFS.³⁵

Although we did not observe significant correlation between high FOXP3+ TILs and survival, we found that patients with a high FOXP3+ TILs had a higher rate of local recurrence or distant metastasis than those whose tumors had a low FOXP+ TILs. Our finding on the adverse effect of FOXP3+ Tregs is consistent with other studies in pancreatic, ^{15,36} cervical,³⁷ hepatocellular,³⁸ and ovarian³⁹ cancers. Our finding that high CD8+/FOXP3+ lymphocyte ratio is correlated with better OS agrees with other those reported for breast breast,³³ gastric,⁴⁰ esophageal,⁴¹ ovarian,³⁹ and hepatocellular⁴² carcinomas.

Most studies of host immunity following chemotherapy have focused on CD8+ T-cells^{43–45} and the mechanisms by which the therapy mitigates Treg-mediated immune suppression^{46,47} and increases antigen presentation^{22,47–49}. However, despite work showing that tumor-reactive CD4+ T cells have a pivotal role in antitumor immune responses, the role of these cells in host immunity after chemotherapy remains largely unknown. Our study had several potential limitations. It lacked a control arm, and it did not include patients' pre-therapy CD4+ lymphocyte counts or CD8+/FOXP3+ lymphocyte ratios. Despite these limitations, ours is the largest study of TILs in residual PDAC after neoadjuvant chemotherapy.

In summary, our findings suggest that CD4+ lymphocyte status and CD8+/FOXP3+ lymphocyte ratio could be used as prognostic predictors for PDAC patients who have received neoadjuvant therapy and could be beneficial in choosing suitable candidates for

combined chemoimmunotherapy strategies. In addition, although most anticancer immunotherapies currently rely on CD8+ T cells, our study and preclinical studies show the importance of CD4+ T cells in controlling tumor progression and recurrence,^{50,51} which suggests that CD4+ T cell-based immunotherapy could be combined with chemotherapy to effectively treat cancer. The results of our study also suggest that targeting the tumor microenvironment to increase the CD4+ lymphocyte count and CD8+/FOXP3+ lymphocyte ratio could improve the clinical outcomes of PDAC patients. Future studies should aim to develop a practical scoring system that could be easily incorporated into clinical care and gain a better understanding of the role that TIL composition following neoadjuvant therapy has in PDAC progression and prognosis.

Acknowledgments

Supported by the National Institutes of Health grant (1R01 CA196941-01A1) and Khalifa Bin Zayed Al Nahyan Foundation Institute for Pancreatic Cancer Research at The University of Texas M. D. Anderson Cancer Center

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin.* 2016; 66:7–30. [PubMed: 26742998]
2. Oettle H, Post S, Neuhaus P, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA.* 2007; 297:267–277. [PubMed: 17227978]
3. Neoptolemos JP, Stocken DD, Bassi C, et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. *JAMA.* 2010; 304:1073–1081. [PubMed: 20823433]
4. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 2006; 313:1960–1964. [PubMed: 17008531]
5. Clemente CG, Mihm MC Jr, Bufalino R, et al. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer.* 1996; 77:1303–1310. [PubMed: 8608507]
6. Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol.* 2015; 26:259–271. [PubMed: 25214542]
7. Geng Y, Shao Y, He W, et al. Prognostic Role of Tumor-Infiltrating Lymphocytes in Lung Cancer: a Meta-Analysis. *Cell Physiol Biochem.* 2015; 37:1560–1571. [PubMed: 26513143]
8. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med.* 2003; 348:203–213. [PubMed: 12529460]
9. Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med.* 2005; 353:2654–2666. [PubMed: 16371631]
10. Mittal D, Gubin MM, Schreiber RD, et al. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr Opin Immunol.* 2014; 27:16–25. [PubMed: 24531241]
11. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science.* 2011; 331:1565–1570. [PubMed: 21436444]
12. Fukunaga A, Miyamoto M, Cho Y, et al. CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. *Pancreas.* 2004; 28:e26–31. [PubMed: 14707745]
13. Yuan XL, Chen L, Li MX, et al. Elevated expression of Foxp3 in tumor-infiltrating Treg cells suppresses T-cell proliferation and contributes to gastric cancer progression in a COX-2-dependent manner. *Clin Immunol.* 2010; 134:277–288. [PubMed: 19900843]

14. Hinz S, Pagerols-Raluy L, Oberg HH, et al. Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. *Cancer Res.* 2007; 67:8344–8350. [PubMed: 17804750]
15. Jiang Y, Du Z, Yang F, et al. FOXP3+ lymphocyte density in pancreatic cancer correlates with lymph node metastasis. *PLoS One.* 2014; 9:e106741. [PubMed: 25191901]
16. Ma Y, Kepp O, Ghiringhelli F, et al. Chemotherapy and radiotherapy: cryptic anticancer vaccines. *Semin Immunol.* 2010; 22:113–124. [PubMed: 20403709]
17. Denkert C, Loibl S, Noske A, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol.* 2010; 28:105–113. [PubMed: 19917869]
18. Denkert C, von Minckwitz G, Brase JC, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol.* 2015; 33:983–991. [PubMed: 25534375]
19. Dieci MV, Criscitiello C, Goubar A, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: a retrospective multicenter study. *Ann Oncol.* 2015; 26:1518. [PubMed: 26109735]
20. Adams S, Gray RJ, Demaria S, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J Clin Oncol.* 2014; 32:2959–2966. [PubMed: 25071121]
21. Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med.* 2007; 13:54–61. [PubMed: 17187072]
22. Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med.* 2007; 13:1050–1059. [PubMed: 17704786]
23. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol.* 2002; 3:991–998. [PubMed: 12407406]
24. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol.* 2002; 3:999–1005. [PubMed: 12407407]
25. Shibuya KC, Goel VK, Xiong W, et al. Pancreatic ductal adenocarcinoma contains an effector and regulatory immune cell infiltrate that is altered by multimodal neoadjuvant treatment. *PLoS One.* 2014; 9:e96565. [PubMed: 24794217]
26. Wang H, Wang H, Zhang W, et al. Tissue microarrays: applications in neuropathology research, diagnosis, and education. *Brain Pathol.* 2002; 12:95–107. [PubMed: 11770905]
27. Estrella JS, Rashid A, Fleming JB, et al. Post-therapy pathologic stage and survival in patients with pancreatic ductal adenocarcinoma treated with neoadjuvant chemoradiation. *Cancer.* 2012; 118:268–277. [PubMed: 21735446]
28. Fischer LK, Katz MH, Lee SM, et al. The number and ratio of positive lymph nodes affect pancreatic cancer patient survival after neoadjuvant therapy and pancreaticoduodenectomy. *Histopathology.* 2016; 68:210–220. [PubMed: 25945396]
29. Roland CL, Yang AD, Katz MH, et al. Neoadjuvant therapy is associated with a reduced lymph node ratio in patients with potentially resectable pancreatic cancer. *Ann Surg Oncol.* 2015; 22:1168–1175. [PubMed: 25352267]
30. Wang J, Estrella JS, Peng L, et al. Histologic tumor involvement of superior mesenteric vein/portal vein predicts poor prognosis in patients with stage II pancreatic adenocarcinoma treated with neoadjuvant chemoradiation. *Cancer.* 2012; 118:3801–3811. [PubMed: 22180096]
31. Tewari N, Zaitoun AM, Arora A, et al. The presence of tumour-associated lymphocytes confers a good prognosis in pancreatic ductal adenocarcinoma: an immunohistochemical study of tissue microarrays. *BMC Cancer.* 2013; 13:436. [PubMed: 24063854]
32. Ino Y, Yamazaki-Itoh R, Shimada K, et al. Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. *Br J Cancer.* 2013; 108:914–923. [PubMed: 23385730]
33. Miyashita M, Sasano H, Tamaki K, et al. Prognostic significance of tumor-infiltrating CD8+ and FOXP3+ lymphocytes in residual tumors and alterations in these parameters after neoadjuvant

- chemotherapy in triple-negative breast cancer: a retrospective multicenter study. *Breast Cancer Res.* 2015; 17:124. [PubMed: 26341640]
34. Ladoire S, Mignot G, Dabakuyo S, et al. In situ immune response after neoadjuvant chemotherapy for breast cancer predicts survival. *J Pathol.* 2011; 224:389–400. [PubMed: 21437909]
 35. Karakhanova S, Ryschich E, Mosl B, et al. Prognostic and predictive value of immunological parameters for chemoradioimmunotherapy in patients with pancreatic adenocarcinoma. *Br J Cancer.* 2015; 112:1027–1036. [PubMed: 25742476]
 36. Wartenberg M, Zlobec I, Perren A, et al. Accumulation of FOXP3+T-cells in the tumor microenvironment is associated with an epithelial-mesenchymal-transition-type tumor budding phenotype and is an independent prognostic factor in surgically resected pancreatic ductal adenocarcinoma. *Oncotarget.* 2015; 6:4190–4201. [PubMed: 25669968]
 37. Jordanova ES, Gorter A, Ayachi O, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res.* 2008; 14:2028–2035. [PubMed: 18381941]
 38. Gao Q, Qiu SJ, Fan J, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol.* 2007; 25:2586–2593. [PubMed: 17577038]
 39. Barnett JC, Bean SM, Whitaker RS, et al. Ovarian cancer tumor infiltrating T-regulatory (T(reg)) cells are associated with a metastatic phenotype. *Gynecol Oncol.* 2010; 116:556–562. [PubMed: 20006900]
 40. Shen Z, Zhou S, Wang Y, et al. Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. *J Cancer Res Clin Oncol.* 2010; 136:1585–1595. [PubMed: 20221835]
 41. Zingg U, Montani M, Frey DM, et al. Tumour-infiltrating lymphocytes and survival in patients with adenocarcinoma of the oesophagus. *Eur J Surg Oncol.* 2010; 36:670–677. [PubMed: 20510571]
 42. Cai MY, Xu YF, Qiu SJ, et al. Human leukocyte antigen-G protein expression is an unfavorable prognostic predictor of hepatocellular carcinoma following curative resection. *Clin Cancer Res.* 2009; 15:4686–4693. [PubMed: 19584149]
 43. Ercolini AM, Ladle BH, Manning EA, et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med.* 2005; 201:1591–1602. [PubMed: 15883172]
 44. Salem ML, Kadima AN, El-Naggar SA, et al. Defining the ability of cyclophosphamide preconditioning to enhance the antigen-specific CD8+ T-cell response to peptide vaccination: creation of a beneficial host microenvironment involving type I IFNs and myeloid cells. *J Immunother.* 2007; 30:40–53. [PubMed: 17198082]
 45. van der Most RG, Currie AJ, Cleaver AL, et al. Cyclophosphamide chemotherapy sensitizes tumor cells to TRAIL-dependent CD8 T cell-mediated immune attack resulting in suppression of tumor growth. *PLoS One.* 2009; 4:e6982. [PubMed: 19746156]
 46. Lutsiak ME, Semnani RT, De Pascalis R, et al. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood.* 2005; 105:2862–2868. [PubMed: 15591121]
 47. Ghiringhelli F, Menard C, Puig PE, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother.* 2007; 56:641–648. [PubMed: 16960692]
 48. Nakahara T, Uchi H, Lesokhin AM, et al. Cyclophosphamide enhances immunity by modulating the balance of dendritic cell subsets in lymphoid organs. *Blood.* 2010; 115:4384–4392. [PubMed: 20154220]
 49. Salem ML, El-Naggar SA, Cole DJ. Cyclophosphamide induces bone marrow to yield higher numbers of precursor dendritic cells in vitro capable of functional antigen presentation to T cells in vivo. *Cell Immunol.* 2010; 261:134–143. [PubMed: 20036354]
 50. Ding ZC, Blazar BR, Mellor AL, et al. Chemotherapy rescues tumor-driven aberrant CD4+ T-cell differentiation and restores an activated polyfunctional helper phenotype. *Blood.* 2010; 115:2397–2406. [PubMed: 20118405]

51. Ding ZC, Zhou G. Cytotoxic chemotherapy and CD4+ effector T cells: an emerging alliance for durable antitumor effects. *Clin Dev Immunol.* 2012; 2012:890178. [PubMed: 22400040]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 1. Representative micrographs show low and high CD4, CD8, and Foxp3 positive tumor infiltrating lymphocytes in residual pancreatic ductal adenocarcinomas. Original magnification: 400×

FIGURE 2.

Kaplan-Meier curves for disease-free survival (A) and overall survival (B) stratified by CD4⁺ tumor infiltrating lymphocytes in residual pancreatic ductal adenocarcinomas after treated with neoadjuvant chemoradiation followed by pancreaticoduodenectomy.

FIGURE 3.

Kaplan-Meier curves for disease-free survival (A) and overall survival (B) stratified by the CD8/Foxp3+ lymphocytes ratio in residual pancreatic ductal adenocarcinomas after treated with neoadjuvant chemoradiation followed by pancreaticoduodenectomy.

Clinicopathological Correlations of CD4, CD8, and Foxp3 Positive Lymphocytes in PDAC Patients Treated With Neoadjuvant Chemotherapy and Pancreaticoduodenectomy

TABLE 1

Characteristics	CD4-low n (%)	CD4-high n (%)	P	CD8-low n (%)	CD8-high n (%)	P	Foxp3-low n (%)	Foxp3-high n (%)	P
Age (years)			1.00			0.08			0.90
<60	42 (41.2)	14 (41.2)		47 (46.1)	9 (26.5)		41 (40.2)	15 (44.1)	
60–70	36 (35.3)	12 (35.3)		31 (30.1)	17 (50.0)		37 (36.3)	11 (32.4)	
>70	24 (23.5)	8 (23.5)		24 (23.5)	8 (23.5)		24 (23.5)	8 (23.5)	
Sex			0.61			0.08			0.19
Female	41 (40.2)	12 (35.3)		44 (43.1)	9 (26.5)		43 (42.2)	10 (29.4)	
Male	61 (59.8)	22 (64.7)		58 (56.9)	25 (73.5)		59 (57.8)	24 (70.6)	
Tumor differentiation			0.04			1.00			1.00
Well-Moderate	61 (59.8)	27 (79.4)		66 (64.7)	22 (64.7)		66 (64.7)	22 (64.7)	
Poor	41 (40.2)	7 (20.6)		36 (35.3)	12 (35.3)		36 (35.3)	12 (35.3)	
Tumor stage			0.19			0.43			0.79
pT0, pT1 & pT2	5 (4.9)	0 (0.0)		3 (2.9)	2 (5.9)		4 (3.9)	1 (2.9)	
pT3	97 (95.1)	34 (100.0)		99 (97.1)	32 (94.1)		98 (96.1)	33 (97.1)	
Margin			1.00			0.54			1.00
Negative	90 (88.2)	30 (88.2)		91 (89.2)	29 (85.3)		90 (88.2)	30 (88.2)	
Positive	12 (11.8)	4 (11.8)		11 (10.8)	5 (14.7)		12 (11.8)	4 (11.8)	
Tumor Regression Grade			0.26			0.82			0.82
CAP Grade 0 & 1	4 (3.9)	3 (8.8)		5 (4.9)	2 (5.9)		5 (4.9)	2 (5.9)	
CAP Grade 2 & 3	98 (96.1)	31 (91.2)		97 (95.1)	32 (94.1)		97 (95.1)	32 (94.1)	
Lymph Node Status			0.67			0.20			0.40
Negative	34 (33.3)	10 (29.4)		30 (29.4)	14 (41.2)		35 (34.3)	9 (26.5)	
Positive	68 (66.7)	24 (70.6)		72 (70.6)	20 (58.8)		67 (65.7)	25 (73.5)	
Recurrence			0.06			0.34			0.046
No	21 (20.6)	14 (41.2)		23 (22.5)	12 (35.3)		27 (26.5)	8 (23.5)	
Local	22 (21.6)	6 (17.6)		22 (21.6)	6 (17.6)		16 (15.7)	12 (35.3)	
Distant	59 (57.8)	14 (41.2)		57 (55.9)	16 (47.1)		59 (57.8)	14 (41.2)	

Bold values are statistically significant.

TABLE 2
Univariate Cox Regression Analysis of Disease-free and Overall Survival in Relation to Clinicopathologic Features

Characteristics	No. of Patients	Disease-Free Survival			Overall Survival		
		HR (95% CI)	P		HR (95% CI)	P	
CD4+ lymphocytes							
Low (ref)	102	1.00			1.00		
High	34	0.51 (0.31–0.84)	0.008		0.51 (0.31–0.84)	0.007	
CD8/Foxp3 ratio							
Low (ref)	68	1.00			1.00		
High	68	0.69 (0.46–1.02)	0.07		0.61 (0.41–0.90)	0.01	
Age, y			0.06			0.34	
<60 (ref)	56	1.00			1.00		
60–70	48	0.69 (0.44–1.08)	0.11		0.80 (0.51–1.24)	0.31	
>70	32	0.56 (0.33–0.93)	0.03		0.70 (0.42–1.17)	0.18	
Sex							
Female (ref)	53	1.00			1.00		
Male	83	1.13 (0.92–1.37)	0.24		0.83 (0.56–1.24)	0.36	
Pathologic tumor stage							
ypT0,ypT1, ypT2 (ref)	5	1.00			1.00		
ypT3	131	2.52 (0.62–10.25)	0.20		2.69 (0.66–10.94)	0.17	
Tumor differentiation							
Well-Moderate (ref)	88	1.00			1.00		
Poor	48	1.29 (0.86–1.93)	0.23		1.28 (0.85–1.91)	0.24	
Margins							
Negative (ref)	120	1.00			1.00		
Positive	16	1.27 (0.69–2.32)	0.45		1.58 (0.88–2.84)	0.13	
Lymph node status							
Negative (ref)	44	1.00			1.00		
Positive	92	1.63 (1.05–2.51)	0.03		1.60 (1.04–2.46)	0.03	
Tumor Regression Grade							
CAP Grade 0 & 1 (ref)	7	1.00			1.00		

Characteristics	No. of Patients	Disease-Free Survival			Overall Survival		
		HR (95% CI)	P		HR (95% CI)	P	
CAP Grade 2 & 3	129	2.99 (0.95–9.46)	0.06		3.01 (0.95–9.52)	0.06	

Bold values are statistically significant.
Ref indicates reference; HR, hazard ratio; 95% CI, 95% confidence interval

TABLE 3

Multivariate Cox Regression Analysis of Disease-Free and Overall Survival in Relation to Clinicopathologic Features

Characteristics	No. of Patients	Disease-Free Survival		Overall Survival	
		HR (95% CI)	P	HR (95% CI)	P
CD4+ lymphocytes					
Low (ref)	102	1.00		1.00	
High	34	0.49 (0.30–0.81)	0.005	0.54 (0.33–0.89)	0.02
CD8/Foxp3 ratio					
Low (ref)	68	1.00		1.00	
High	68	0.81 (0.54–1.22)	0.32	0.70 (0.47–1.06)	0.09
Lymph node status					
Negative (ref)	44	1.00		1.00	
Positive	92	1.89 (1.22–2.94)	0.004	1.62 (1.04–2.54)	0.04
Tumor Regression Grade					
CAP Grade 0 & 1 (ref)	7	1.00		1.00	
CAP Grade 2 & 3	129	3.37 (1.06–10.73)	0.04	3.42 (1.07–10.92)	0.04

Bold values are statistically significant.

Ref indicates reference; HR: hazard ratio; 95% CI: 95% confidence interval; CAP: College of American Pathologists