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Article

Tumor Microenvironment Immune Response in Pancreatic Ductal Adenocarcinoma Patients Treated With Neoadjuvant Therapy

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Abstract

Background: Neoadjuvant folinic acid, fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) and chemoradiation have been used to downstage borderline and locally advanced pancreatic ductal adenocarcinoma (PDAC). Whether neoadjuvant therapy-induced tumor immune response contributes to the improved survival is unknown. Therefore, we evaluated whether neoadjuvant therapy induces an immune response towards PDAC. **Methods:** Clinicopathological variables were collected for surgically resected PDACs at the Massachusetts General Hospital (1998-2016). Neoadjuvant regimens included FOLFIRINOX with or without chemoradiation, proton chemoradiation (25 Gy), photon chemoradiation (50.4 Gy), or no neoadjuvant therapy. Human leukocyte antigen (HLA) class I and II expression and immune cell infiltration ($CD4^+$, $FoxP3^+$, $CD8^+$, granzyme B^+ cells, and M2 macrophages) were analyzed immunohistochemically and correlated with clinicopathologic variables. The antitumor immune response was compared among neoadjuvant therapy regimens. All statistical tests were 2-sided. **Results:** Two hundred forty-eight PDAC patients were included. The median age was 64 years and 50.0% were female. HLA-A defects were less frequent in the FOLFIRINOX cohort ($P = .006$). HLA class II expression was lowest in photon and highest in proton patients ($P = .02$). The FOLFIRINOX cohort exhibited the densest $CD8^+$ cell infiltration ($P < .001$). FOLFIRINOX and proton patients had the highest $CD4^+$ and lowest T regulatory ($FoxP3^+$) cell density, respectively. M2 macrophage density was statistically significantly higher in the treatment-naïve group ($P < .001$) in which dense M2 macrophage infiltration was an independent predictor of poor overall survival. **Conclusions:** Neoadjuvant FOLFIRINOX with or without chemoradiation may induce immunologically relevant changes in the tumor microenvironment. It may reduce HLA-A defects, increase $CD8^+$ cell density, and decrease T regulatory cell and M2 macrophage density. Therefore, neoadjuvant FOLFIRINOX therapy may benefit from combinations with checkpoint inhibitors, which can enhance patients' antitumor immune response.

For the last decade, neoadjuvant therapy with folinic acid, fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) followed by radiotherapy has been used for patients with borderline resectable (BR) or locally advanced (LA) pancreatic ductal adenocarcinoma (PDAC) with the expectation of converting them to resectable (1). Patients with BR or LA PDAC who undergo surgery

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ARTICLE

following neoadjuvant FOLFIRINOX treatment have a longer overall survival (OS) compared with upfront resectable patients (1). Whether a neoadjuvant therapy–induced tumor immune response contributes to the improved survival is unknown. Chemo- and/or radio-therapy have been shown to increase susceptibility of tumor cells to cytotoxic T lymphocyte recognition and destruction and/or to enhance the antitumor activity of cytotoxic T lymphocytes in vitro, in animal models, and some cases of patients with various types of cancer (2–5). These preclinical data prompted us to 1) analyze the infiltration of tumors by immune cells, which reflects a patient's immune response in the tumor microenvironment (TME) (6); 2) evaluate human leukocyte antigen (HLA) expression by PDAC cells, given that these molecules play a crucial role in the interactions of tumor cells with the host's immune system (7, 8); and 3) correlate the immunohistochemical (IHC) results with clinicopathologic variables.

Methods

Patients

With institutional review board approval and after obtaining written informed consent, surgically removed PDACs between 1998 and 2016 at the Massachusetts General Hospital were evaluated. Based on specimen availability, 248 specimens that were readily available for analysis were obtained. This study population represents only a fraction of all resected tumors at the Massachusetts General Hospital during the study period. Of those, 63 received neoadjuvant FOLFIRINOX alone (n = 6) or were followed by either proton or photon radiotherapy (n = 57) between 2012 and 2016, 30 received neoadjuvant proton radiotherapy (short course, 5 days × 5 Gy) between 2007 and 2011, 18 received neoadjuvant photon radiotherapy (50.4 Gy) between 1998 and 2010, and 137 received no neoadjuvant therapy between 1998 and 2011. Proton and photon therapy were administered as described (9–11). Detailed methods regarding tissues, monoclonal antibodies (mAb), and IHC staining are presented in the Supplementary Material (available online).

Statistical Analysis

Continuous variables are presented as medians (interquartile range [IQR]). The inter-rater reliability between investigators who scored the staining and between the manual and automated assessment of immune cell counts was analyzed using Cohen's κ. The correlation between continuous variables was assessed with Spearman's ρ. Comparison of categorical variables among groups was performed using Fisher's exact test. Comparison of continuous variables between groups was performed using the Mann-Whitney U test or the Kruskal-Wallis method, as appropriate, and within groups with the Wilcoxon signed rank test. OS was measured as disease-specific survival. Time was defined as the interval between the date of diagnosis and the date of disease-specific death (event) or the date of last follow-up (patient censored). Survival curves were plotted using the Kaplan-Meier method. Differences in OS among groups were analyzed by the log-rank test. Multivariable survival analyses were performed using the Cox proportional hazards model; the assumption of proportionality was verified by graphical assessment of Kaplan-Meier curves.

The difference in time interval between the day of operation and our IHC analysis among groups is due to the evolving paradigm of care for patients with PDAC. Neoadjuvant proton therapy began as part of a clinical trial in 2006 (12). Systemic FOLFIRINOX was established as the standard of care for metastatic PDAC in 2011 (9). To account for the time difference that could be a potential confounder, we calculated the time interval (days) between day of operation and day of IHC analysis. We then determined whether this operation-to-IHC interval was associated with any of the immune parameters within each treatment group.

P less than .05 was considered statistically significant. All tests were 2-tailed. Bonferroni-corrected P values were calculated when appropriate. Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 24.0 (IBM Corporation, Armonk, NY) and GraphPad Prism, version 8.0 for Windows (GraphPad Software, La Jolla, CA).

Results

Clinicopathological Features

A total of 248 surgically resected PDACs were evaluated. The median age was 64 years, and one-half of the patients were women (Table 1). The patient cohort included 63 FOLFIRINOX patients (6 received FOLFIRINOX only and 57 received FOLFIRINOX followed by proton or photon radiotherapy), 30 proton, 18 photon, and 137 neoadjuvant treatment-naïve patients. Patients who went directly to the operating room and proton patients were considered resectable at the time of presentation by our institutional multidisciplinary team. Neoadjuvant FOLFIRINOX (with or without radiotherapy) and photon chemoradiation patients were considered either BR or LA at presentation.

Of the 63 FOLFIRINOX patients, 22 (34.9%) had pathological stage IIB disease compared with 24 (80.0%) of the proton, 9 (50.0%) of the photon, and 98 (71.5%) of the treatment-naïve patients ($P < .001$). Negative resection margins were achieved more frequently in the FOLFIRINOX (84.1%) and proton groups (90.0%) than in the photon (66.7%) or treatment-naïve groups (62.0%) ($P < .001$). Median OS from the time of diagnosis of FOLFIRINOX, proton, photon, and treatment-naïve patients was 31.6 (IQR = 21.2-55.5), 28.3 (IQR = 16.7-N/A), 31.5 (IQR = 17.1-37.7), and 21.4 (IQR = 11.8-40.5) months, respectively.

Frequency of HLA Class I Defects in PDAC Tumors

Both HLA-A and HLA-B/C expression were defective (negative or heterogeneous) in more than 60.0% of PDACs (Table 2; Figure 1). The frequency of HLA-A defects in FOLFIRINOX patients was statistically significantly lower than in the other cohorts (Bonferroni-corrected $P = .006$ for FOLFIRNOX vs no therapy and for FOLFIRINOX vs photon; Bonferroni-corrected $P = .04$ for FOLFIRINOX vs proton; Figure 2A). The latter did not differ from each other. In contrast, the frequency of HLA-B/C defects did not differ among the 4 cohorts (Figure 2B). Among FOLFIRINOX patients, the frequency of HLA-B/C defects was statistically significantly higher in those who received FOLFIRINOX + proton radiotherapy than in those who received FOLFIRINOX alone (Bonferroni-corrected $P = .009$; Supplementary Figure 1B available online).

Table 1. Clinicopathological characteristics of 248 pancreatic ductal adenocarcinomas surgically removed from patients treated with neoadjuvant FOLFIRINOX alone or followed by radiotherapy (photon or proton) (n = 63), photon radiotherapy (n = 18), or proton radiotherapy (n = 30) and from patients who received no neoadjuvant therapy (n = 137)

Clinicopathologic characteristics	FOLFIRINOX ± proton or photon radiation No. (%)	Proton radiation No. (%)	Photon radiation No. (%)	No neoadjuvant No. (%)	Overall No. (%)	P[a]
Median age (IQR), y	62 (56-67)	62 (56-70)	66 (59-73)	70 (60-76)	64 (58-73)	<.001
Sex						.95
Male	31 (49.2)	14 (46.7)	10 (55.6)	69 (50.4)	124 (50.0)	
Female	32 (50.8)	16 (53.3)	8 (44.4)	68 (49.6)	124 (50.0)	
Pathological stage						<.001
IA	6 (9.5)	1 (3.3)	2 (11.1)	2 (1.5)	11 (4.4)	
IB	4 (6.3)	1 (3.3)	3 (16.7)	11 (8.0)	19 (7.7)	
IIA	31 (49.2)	4 (13.3)	4 (22.2)	26 (19.0)	65 (26.2)	
IIB	22 (34.9)	24 (80.0)	9 (50.0)	98 (71.5)	153 (61.7)	
Resection margins						<.001
Positive	10 (15.9)	3 (10.0)	6 (33.3)	52 (38.0)	71 (28.6)	
Negative	53 (84.1)	27 (90.0)	12 (66.7)	85 (62.0)	179 (72.3)	
Lymphovascular invasion						<.001
Positive	21 (33.3)	14 (46.7)	12 (66.7)	89 (65.0)	136 (54.8)	
Negative	42 (66.7)	16 (53.3)	6 (33.3)	48 (35.0)	112 (45.2)	
Perineural invasion						<.001
Positive	49 (77.8)	25 (83.3)	15 (83.3)	126 (92.0)	215 (86.7)	
Negative	14 (22.2)	5 (16.7)	3 (16.7)	11 (8.0)	33 (13.3)	

[a] P values derived from Fisher's exact test or Kruskal-Wallis test, as appropriate. FOLFIRINOX = folinic acid, fluorouracil, irinotecan, and oxaliplatin; IQR = interquartile range.

Table 2. HLA-A, HLA-B/C, and HLA class II expression in 248 pancreatic ductal adenocarcinomas surgically removed from patients treated with neoadjuvant FOLFIRINOX alone or followed by radiotherapy (photon or proton) (n = 63), photon radiotherapy (n = 18), or proton radiotherapy (n = 30) and from patients who received no neoadjuvant therapy (n = 137)

HLA component	FOLFIRINOX ± proton or photon radiation No. (%)	Proton radiation No. (%)	Photon radiation No. (%)	No neoadjuvant No. (%)	Overall No. (%)	P[a]
HLA-A						<.001
Negative	1 (1.6)	4 (13.3)	5 (27.8)	23 (17.6)	33 (13.7)	
Heterogeneous	37 (58.7)	22 (73.4)	11 (61.1)	76 (58.0)	146 (60.3)	
Positive	25 (39.7)	4 (13.3)	2 (11.1)	32 (24.4)	63 (26.0)	
HLA-B/C						.86
Negative	5 (7.9)	2 (6.7)	1 (5.6)	6 (4.6)	14 (5.8)	
Heterogeneous	35 (55.6)	20 (66.7)	11 (61.1)	72 (55.4)	138 (57.3)	
Positive	23 (36.5)	8 (26.7)	6 (33.3)	52 (40.0)	89 (36.9)	
HLA class II						.07
Negative	13 (20.6)	4 (13.8)	8 (50.0)	34 (28.1)	59 (25.8)	
Heterogeneous	44 (69.8)	20 (69.0)	7 (43.8)	65 (53.7)	136 (59.4)	
Positive	6 (9.5)	5 (17.2)	1 (6.2)	22 (18.2)	34 (14.8)	

[a] P values derived from Fisher's exact test. Staining was not obtained for all slides for all markers. FOLFIRINOX = folinic acid, fluorouracil, irinotecan, and oxaliplatin; HLA = human leukocyte antigen.

HLA Class II Expression in PDAC Tumors

In agreement with the literature (13, 14), HLA class II was undetectable on peritumoral normal ductal and exocrine pancreatic cells. In contrast, it was expressed on more than 70.0% of tumor cells from both the FOLFIRINOX and proton groups as well as treatment-naïve patients, but in only 50.0% of photon-treated tumors (Table 2). HLA class II expression was lowest in photon and highest in proton patients (Bonferroni-corrected $P = .02$; Figure 2C).

Tumor-Infiltrating Immune Cells

The inter-rater reliability between scoring investigators for the assessment of tumor-infiltrating immune cells as well as between manual and automated scoring of immune cells was very high (Cohen $\kappa = 0.81$, $P < .001$; Cohen $\kappa = 0.83$, $P = .001$, respectively). All PDACs demonstrated tumor infiltrating immune cells (Table 3; Figure 3).

PDACs from FOLFIRINOX patients had a statistically significantly higher $CD8^+$ cell density than those from the other 3 cohorts ($P < .001$ for all between-group comparisons; Figure 4A). In the latter 3, $CD8^+$ cell density was the lowest in the photon cohort ($P = .002$ vs proton, $P = .004$ vs no neoadjuvant). $CD8^+$ cell density in FOLFIRINOX alone patients was statistically significantly higher than that in FOLFIRINOX + photon radiation tumors ($P = .009$; Supplementary Figure 2A available online).

Granzyme B^+ cell density, utilized as a marker of activated $CD8^+$ T cells, was similar in FOLFIRINOX patients to that in treatment-naïve patients. PDACs from FOLFIRINOX patients

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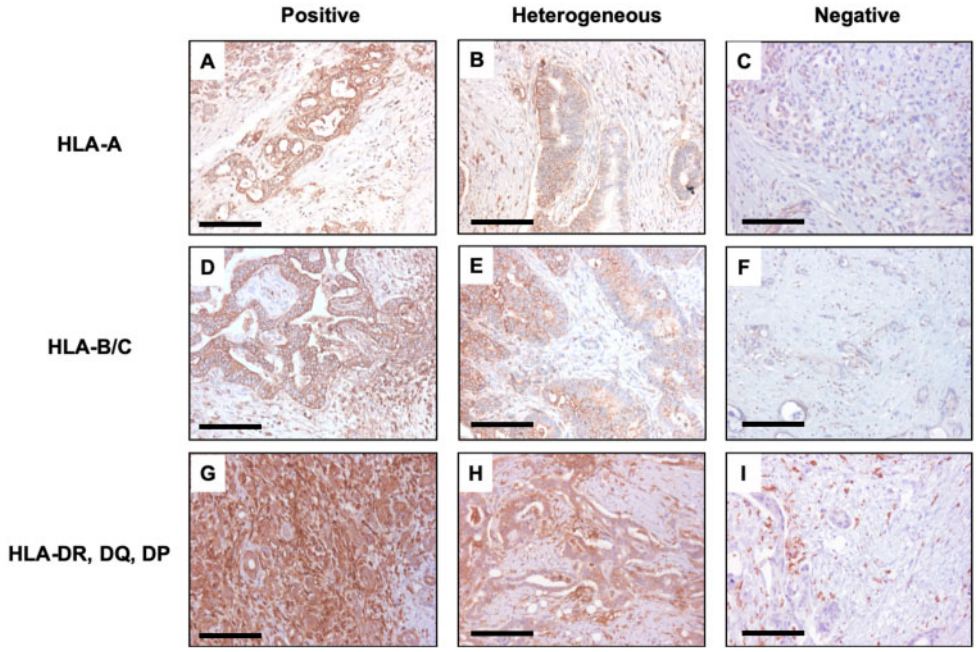

Figure 1. Human leukocyte antigen (HLA) class I and class II expression patterns in pancreatic ductal adenocarcinoma (PDAC). Formalin-fixed, paraffin-embedded PDAC lesions were stained with mouse HLA-A–specific monoclonal antibodies (mAb) HC-A2, HLA-B/C–specific mAb HC-10, and HLA-DR, DQ, DP–specific mAb LGII-612.14. Staining was scored as positive (**A**, **D**, and **G**), heterogeneous (**B**, **E**, and **H**), and negative (**C**, **F**, and **I**). Scale bars = 100μm. ×200 original magnification.

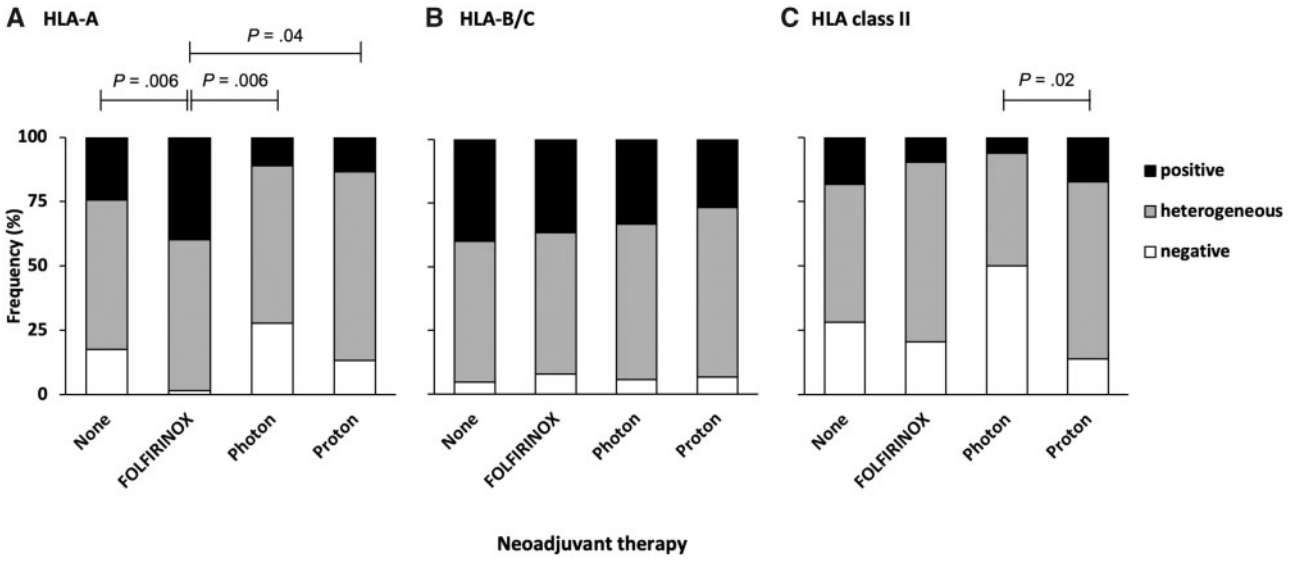

Figure 2. Human leukocyte antigen (HLA) antigen expression in pancreatic ductal adenocarcinomas surgically removed from patients treated with neoadjuvant folinic acid, fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX), proton radiotherapy, or photon radiotherapy and from patients who received no neoadjuvant therapy. Stacked bar graphs show the frequency of negative, heterogeneous, and positive HLA-A (**A**), HLA-B/C (**B**), and HLA class II (**C**) expression in tumors surgically removed from patients treated with neoadjuvant therapy or from neoadjuvant treatment-naïve patients. *P* values were derived from Fisher's exact tests and were adjusted for multiple comparisons using the Bonferroni correction method.

had a statistically significantly higher granzyme B^+ cell density than those from photon patients ($P < .001$; Figure 4B). Granzyme B^+ cell density was statistically significantly higher in treatment-naïve patients than in proton and photon patients ($P = .009$ vs photon; $P = .02$ vs proton). Among FOLFIRINOX patients, granzyme B^+ cell density was statistically significantly higher in FOLFIRINOX alone patients than in FOLFIRINOX + photon patients ($P = .02$; Supplementary Figure 2B available online).

The highest $CD4^+$ cell density was identified in FOLFIRINOX and proton patients, which did not differ from each other. $CD4^+$ cell density in the latter 2 cohorts was statistically significantly higher compared with photon-treated and treatment-naïve patients ($P < .001$ for all between-group comparisons; Figure 4C).

T regulatory (Treg) cells, identified as $FoxP3^+$ cells, were detected in all 4 groups. The lowest $FoxP3^+$ cell density was

Table 3. Immune cell infiltration in 248 pancreatic ductal adenocarcinomas surgically removed from patients treated with neoadjuvant FOLFIRINOX alone or followed by radiotherapy (photon or proton) (n = 63), photon radiotherapy (n = 18) or proton radiotherapy (n = 30), and from patients who received no neoadjuvant therapy (n = 137)

Immune cell type	FOLFIRINOX ± proton or photon radiation Cells per HPF, median (IQR)	Proton radiation Cells per HPF, median (IQR)	Photon radiation Cells per HPF, median (IQR)	No neoadjuvant Cells per HPF, median (IQR)	Overall Cells per HPF, median (IQR)	P[a]
$CD4^+$	58 (27-80)	45 (26-96)	10 (2-18)	7 (1-16)	18 (4-42)	<.001
$FoxP3^+$	1 (0-2)	1 (0-1)	3 (1-4)	3 (1-5)	2 (1-4)	<.001
$CD8^+$	40 (24-80)	24 (12-34)	7 (1-15)	17 (9-30)	21 (11-37)	<.001
Granzyme B^+	18 (11-30)	5 (4-8)	8 (3-11)	12 (7-28)	15 (8-28)	<.001

[a] P values derived from Kruskal-Wallis test. Staining was not obtained for all slides for all markers. FOLFIRINOX = folinic acid, fluorouracil, irinotecan, and oxaliplatin; HPF = high power field; IQR = interquartile range.

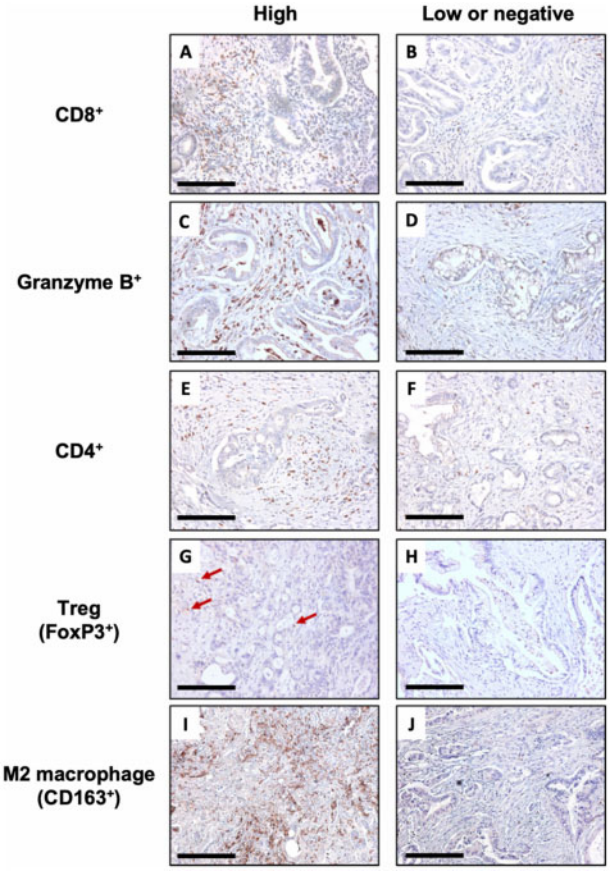

Figure 3. Pancreatic ductal adenocarcinoma (PDAC) fibrous septa infiltration by $CD8^+$ T cells, granzyme B^+ cells, $CD4^+$ cells, Treg cells, and M2 macrophages. Representative patterns of formalin-fixed, paraffin-embedded PDAC lesions stained withmouse anti-CD8 (**A** and **B**), rabbit anti-granzyme B (C and D), rabbit anti-CD4 (**E** and **F**), rabbit anti-FoxP3 (**G** and **H**), and rabbit anti-CD163 (**I** and **J**). High $CD8^+$ cell (**A**), high granzyme B^+ cell (**C**), high $CD4^+$ T cell (**E**), high $FoxP3^+$ cell (**G**) and high M2 macrophage (**I**) infiltration are compared with low $CD8^+$ cell (**B**), low granzyme B^+ cell (**D**), low $CD8^+$ T cell (**F**), negative $FoxP3^+$ cell (**H**) and low M2 macrophage (**J**) infiltration. Scale bars = 100 μm. ×200 original magnification.

found in FOLFIRINOX patients and proton patients, who did not differ from each other. $FoxP3^+$ cell density in FOLFIRINOX and proton-treated patients was statistically significantly lower than that in photon and treatment-naïve patients ($P < .001$ for all between-group comparisons; Figure 4D).

M2 macrophage ($CD163^+$ cells) density was statistically significantly higher in the treatment-naïve group compared with the FOLFIRINOX and proton groups ($P < .001$ for both). M2 macrophage density was not different between FOLFIRINOX and photon or proton patients (Figure 4E).

Association of HLA Class I and Class II Expression in PDAC Tumors With Tumor Infiltrating Lymphocyte Density

In FOLFIRINOX-treated patients, $CD8^+$ and granzyme B^+ cell densities were correlated with HLA-A, HLA-B/C, and HLA class II expression ($CD8^+$: Spearman $\rho = 0.59$, $P < .001$; $\rho = 0.44$, $P = .001$; and $\rho = 0.35$, $P = .004$, respectively; granzyme B^+: $\rho = 0.54$, $P < .001$; $\rho = 0.40$, $P = .001$; and $\rho = 0.37$, $P = .003$, respectively). In contrast, $CD4^+$ cell density was correlated with neither HLA-A nor HLA-B/C expression but was correlated with HLA class II expression ($\rho = 0.34$; $P = .007$). $FoxP3^+$ cell and M2 macrophage densities were correlated with neither HLA class I nor HLA class II expression.

In proton patients, HLA class I and class II expression did not correlate with immune cell density. However, $FoxP3^+$ cell density was inversely correlated with HLA-B/C expression ($\rho = -0.58$, $P = .01$) in photon patients.

In treatment-naïve patients, $CD8^+$ cell density correlated with HLA-A and HLA-B/C expression ($\rho = 0.27$, $P = .002$; and $\rho = 0.57$, $P = .001$, respectively). Granzyme B^+ cell density correlated with HLA-B/C and HLA class II expression ($\rho = 0.25$, $P = .004$; and $\rho = 0.21$, $P = .02$, respectively). In contrast, $CD4^+$ cell and $FoxP3^+$ cell densities correlated with neither HLA-A nor HLA-B/C expression.

Prognostic Value of HLA Expression and Lymphocyte Density

To assess the clinical significance of the IHC analysis, IHC results were correlated with patients' clinicopathologic characteristics. In FOLFIRINOX patients, HLA-A expression defects correlated with higher T stage and perineural invasion ($P = .03$, $P = .002$, respectively; Supplementary Figure 3 available online). Dense M2 macrophage infiltration correlated with higher lymphovascular invasion ($P = .007$). In proton patients, higher $CD4^+$ cell density correlated with higher T stage and perineural invasion ($P = .04$, $P = .04$, respectively). In photon-treated patients, there was no association between the TME immunological characteristics and patients' clinicopathologic characteristics.

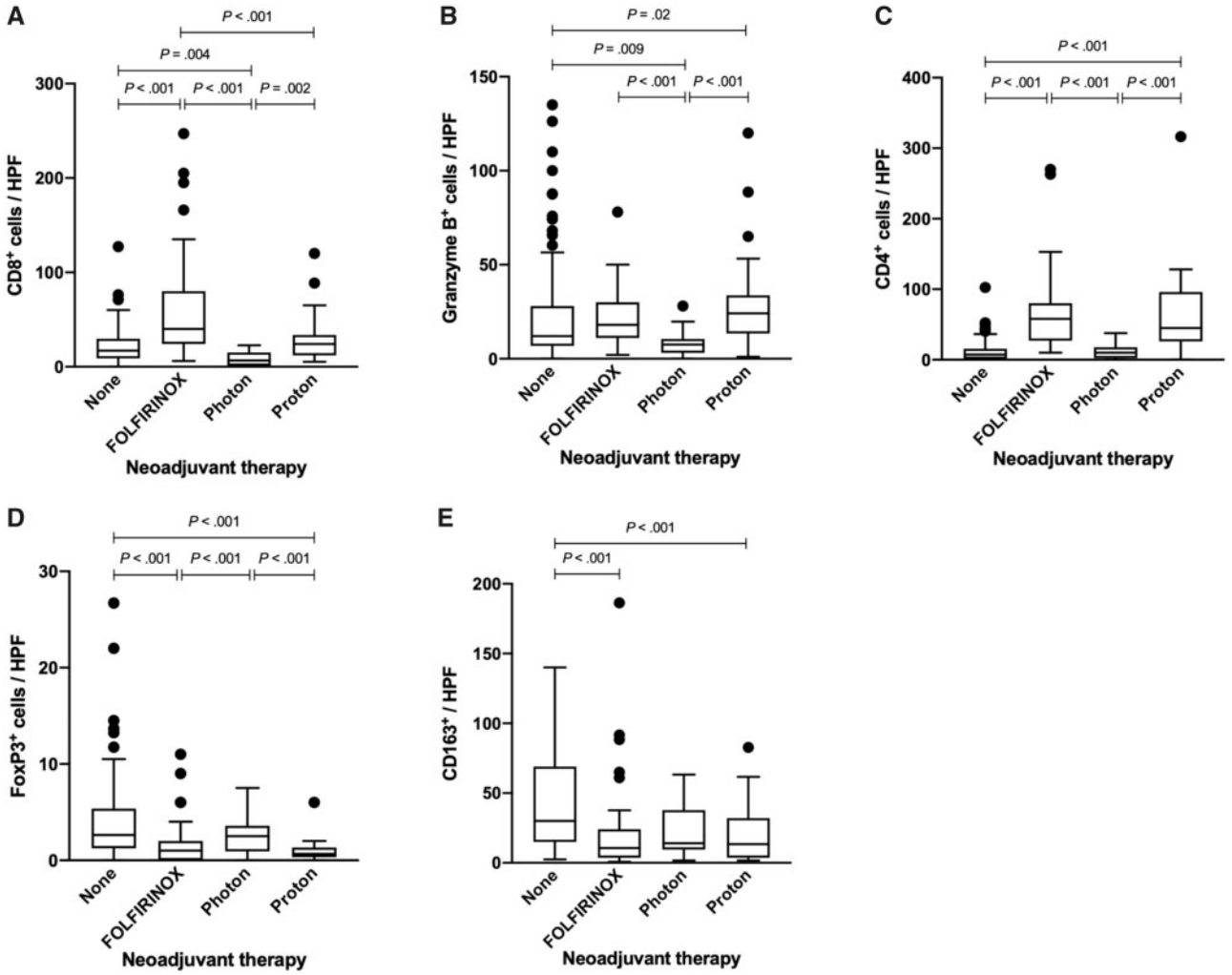

Figure 4. Differential $CD8^+$ cell, granzyme B^+ cell, $CD4^+$ cell, $FoxP3^+$ cell, and M2 macrophage tumor infiltration density in pancreatic ductal adenocarcinoma tumors from patients treated with different neoadjuvant therapy regimens and from neoadjuvant treatment-naïve patients. Data for CD8+ cell, granzyme B+ cell, CD4+ cell, FoxP3+ cell, and M2 macrophage tumor infiltration density are presented in panels **A-E**), respectively. **Boxes** represent the interquartile range (IQR) and the **horizontal lines** across the boxes indicate the median. The **whiskers** extend from the upper and lower edge of the boxes to the highest and lowest values which are no greater or smaller than 1.5× the IQR. Outliers with values greater than 1.5× the IQR are represented by **circles**. FOLFIRINOX = folinic acid, fluorouracil, irinotecan, and oxaliplatin; HPF = high power field; IQR = interquartile range. *P* values were derived from Mann-Whitney U tests.

Only in treatment-naïve patients were HLA class I expression and immune cell density correlated with OS. Lower HLA-B/C expression, concordant negative HLA-A and HLA-B/C expression, and low $CD8^+$ cell and high M2 macrophage densities correlated with poor OS ($P < .001$, $P < .001$, $P = .049$, and $P = .01$, respectively; Figure 5) in univariate analyses. In treatment-naïve patients, a higher T stage (hazard ratio [HR] = 1.54, 95% confidence interval [CI] = 1.06 to 2.23, $P = .02$), positive margins (HR = 1.57, 95% CI = 1.01 to 2.47, $P = .04$), lower HLA-B/C expression (HR = 4.14, 95% CI = 1.75 to 9.80, $P < .001$), and dense M2 macrophage infiltration (HR = 1.79, 95% CI = 1.19 to 2.62, $P = .03$) were independent predictors of poor OS. Results remained similar when time from operation to IHC was introduced in the multivariable analysis models.

Discussion

Our analysis of the immune microenvironment in PDAC treated with various types of neoadjuvant therapies demonstrated that FOLFIRINOX with or without chemoradiation was associated with upregulated HLA-A expression and increased $CD8^+$ T cell density, including activated $CD8^+$ cells; in contrast, the frequency of Tregs and M2 macrophages was reduced. These findings are compatible with a FOLFIRINOX-induced immune response to tumor antigens. This possibility is supported by evidence that FOLFIRINOX components not only exert cytotoxic effects but also stimulate an antitumor immune response in vitro in animal models and in a limited number of human patients (2–5). Indeed, the administration of anthracycline- or platinum-based chemotherapy or neoadjuvant chemoradiotherapy plus fluoropyrimidines has been associated with an increased lymphocyte infiltration in breast, rectal, ovarian, and prostate cancers (15–18). More specifically, fluorouracil plus radiotherapy resulted in increased $CD8^+$ cell infiltration in rectal cancer, which was in turn associated with higher regression rates (19). In a murine pancreatic cancer model, fluorouracil plus interferon (IFN)-α enhanced natural killer cell tumor infiltration and major histocompatibility complex class I expression

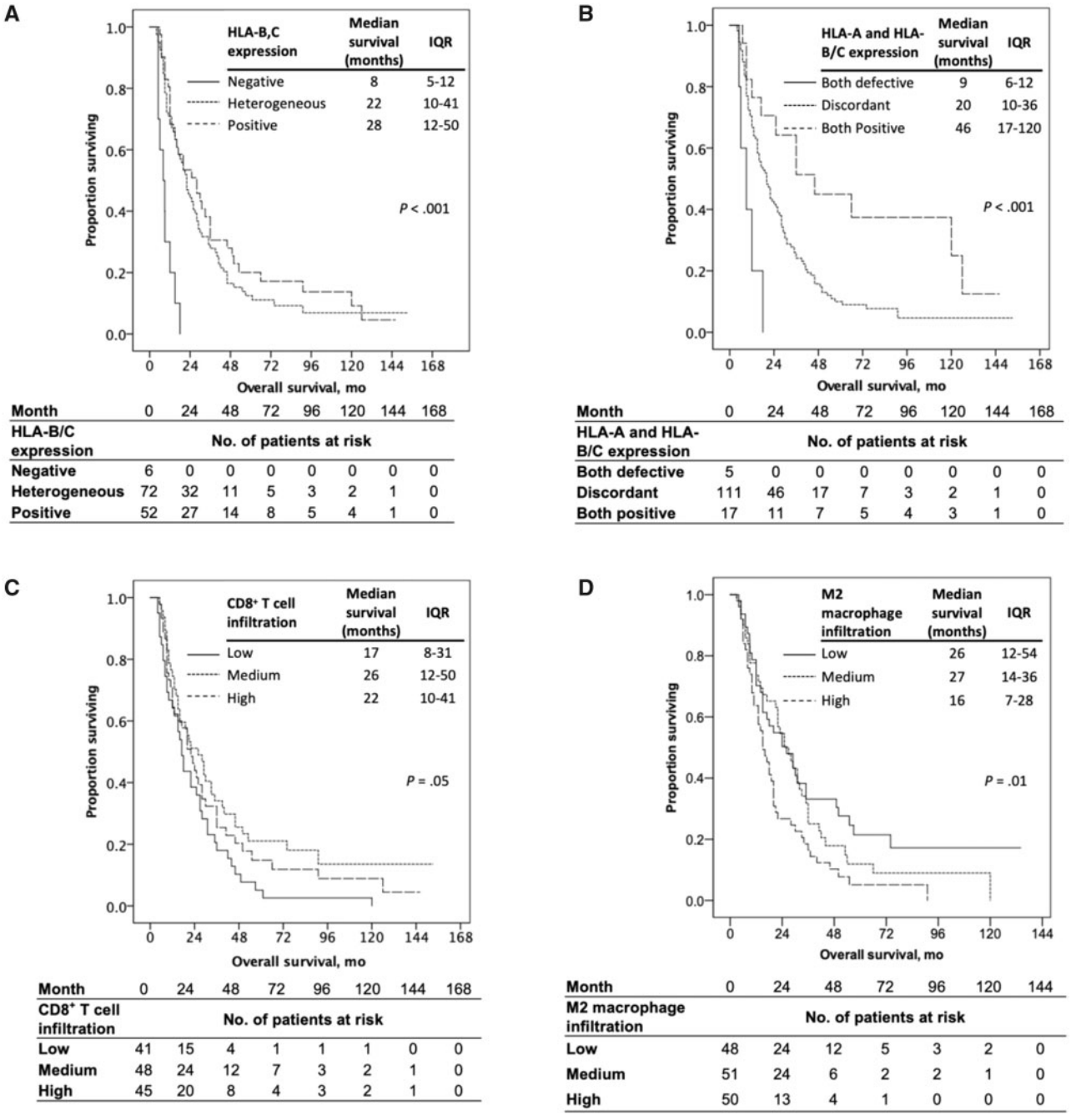

Figure 5. Association of immunological parameters of the pancreatic ductal adenocarcinoma tumor microenvironment with survival of neoadjuvant treatment-naïve patients who underwent resection of their tumors. Kaplan-Meier survival curves for human leukocyte antigen (HLA)-B/C expression (**A**), HLA-A and HLA-B/C expression concordance (**B**), $CD8^+$ T cell infiltration (**C**), and M2 macrophage infiltration (**D**) are presented.

on tumor cells (20). Furthermore, fluorouracil was shown to increase IFN-γ production by tumor-infiltrating $CD8^+$ cells in murine thymoma (21). Oxaliplatin has been shown to promote $CD8^+$ cell antitumor immune response in murine prostate cancers (22), increase the $CD8^+$:Treg ratio in murine colorectal cancers (23), and promote the activity of neutrophils and macrophages in multiple murine malignancies (24). Decreased Treg infiltration may also be induced by FOLFIRINOX (23, 25). Additionally, macrophage density was found to be reduced in PDAC patients treated neoadjuvantly with FOLFIRINOX plus the C-C motif chemokine ligand 2 (CCL2) - C-C motif chemokine receptor 2 (CCR2) axis inhibitor PF-04136309, but no data are available for FOLFIRINOX alone (26).

In this study, firstly, we evaluated PDAC cell HLA expression. HLA expression plays an important role in the interactions of tumor cells with the host's immune system. HLA class I molecules play a crucial role in the presentation of tumor antigen-derived peptides to cognate $CD8^+$ T cells. Therefore, defects in HLA class I expression provide tumor cells with an escape mechanism from recognition and destruction by the immune

system (27). This mechanism may account for the association we found between HLA-B/C downregulation and poor prognosis in treatment-naïve patients. The lowest frequency of HLA class I defects was identified in the FOLFIRINOX cohort. This could be partially attributed to fluorouracil, which is known to upregulate HLA class I expression on tumor cells in vitro (20). Radiation can also upregulate HLA class I expression, possibly via IFN-γ signaling (28, 29); however, we did not observe such an effect with radiation alone.

FOLFIRINOX might have a selective effect on the gene products of the HLA-A locus, as indicated by the statistically significant reduction in the frequency of HLA-A defects in PDACs. This selective upregulation of the HLA-A locus gene products argues against the possibility that this effect is mediated by IFN release, because these cytokines upregulate the HLA-A, B, and C loci gene products to a similar extent. Therefore, FOLFIRINOX may mediate distinct mechanisms that differentially regulate the gene products of classical HLA class I antigen expression. Our results emphasize the usefulness of mAbs which recognize the gene products of HLA class I loci to analyze the phenotype of tumor cells.

Secondly, we demonstrated that HLA class II expression, which can trigger immunosuppressive mechanisms such as activation of Tregs (30–32), was the highest and lowest on PDACs from proton and photon patients, respectively. A previous study demonstrated a lower frequency (11 of 37, 29.7%) of de novo HLA class II expression in PDAC (13). The same study detected HLA class II expression in 42.1% (8 of 19) of PDAC cell lines. The susceptibility of HLA class II expression to modulation by cytokines such as IFN-γ raises the possibility that upregulation in radiated tumors could be mediated by stimulator of interferon genes (STING) activation (5, 33, 34).

We also showed an association of low $CD8^+$ cell density and high M2 macrophage density with poor survival in treatment-naïve patients. Notably, within that group, dense M2 macrophage infiltration was an independent predictor of poor survival. Immune cell infiltration has been associated with outcomes in various cancers. Specifically, dense $CD8^+$ T cell infiltration has been associated with improved prognosis; this likely reflects the ability of $CD8^+$ cells to exert direct cytotoxic effects on tumor cells (35–37). In contrast, Treg and M2 macrophage tumor infiltration has been associated with poor prognosis in multiple malignancies, including PDAC (37–41). Specifically for unresectable PDAC, circulating Treg levels decreased after chemotherapy in patients with stable disease or partial response but increased in patients with progression (42). Furthermore, tumor-associated macrophages have been shown to dampen tumor antigen-specific immune responses in PDAC in both mice and humans (43). Two distinct states of polarization have been described for macrophages: M1 (classically activated) and M2 (alternatively activated). We elected to analyze M2 macrophages because tumor-associated macrophages have been found to predominantly demonstrate an M2-like phenotype (44–46).

We did not compare survival among treatment groups given their heterogeneity in baseline characteristics. However, resected PDAC patients who have received neoadjuvant FOLFIRINOX have a statistically significantly longer survival compared with neoadjuvant therapy-naïve patients with upfront resectable disease (1). The neoadjuvant FOLFIRINOX-treated patients' prolonged survival may result from the ability of FOLFIRINOX to induce or reactivate an adoptive antitumor immunity. If this association reflects a cause-effect relationship, the FOLFIRINOX-treated patients' prolonged survival may be mediated by an increased susceptibility of PDAC cells to immune recognition because of an improved antigen presentation mediated by HLA-A upregulation and/or an enhanced antitumor activity of the patient's immune system. The latter may be due to increased $CD8^+$ T cell density, reduced Treg infiltration, and/or lower M2 macrophage density.

Neoadjuvant FOLFIRINOX and radiotherapy of PDAC may benefit from combination strategies that enhance patients' immune response, unleash cognate tumor antigen-specific T cells, eliminate immunosuppressive cells, and/or augment PDAC cell susceptibility to immune lysis. Of note, a phase II clinical trial evaluating the combination of neoadjuvant FOLFIRINOX, radiotherapy, losartan, and nivolumab, an anti-PD-1 mAb, followed by PDAC resection is currently underway (NCT03563248). Our findings also potentially support the use of neoadjuvant therapy even in PDAC patients with resectable tumors in order to enhance their antitumor immune response.

Our study has some limitations. Given that most of the specimens are not available for further studies and that the majority of pretreatment biopsies are fine-needle aspirates, we did not directly compare the pretreatment with the post-treatment immune milieu within each neoadjuvant treatment group. Instead, our comparisons were performed among heterogeneous groups. The fact that FOLFIRINOX patients had more advanced tumors at baseline but nevertheless exhibited the most prominent immune response supports the idea that FOLFIRINOX has a strong immunomodulatory effect. Importantly, FOLFIRINOX tumors were from patients who responded to neoadjuvant therapy and were rendered resectable, potentially representing tumors with prominent antitumor immune response at baseline. Additionally, neoadjuvant therapy-naïve and FOLFIRINOX-treated patients underwent an operation at different time periods; as a result, FOLFIRINOX patient samples had a shorter interval between operation and IHC analysis. This time interval difference might introduce bias to our results, at least partially due to the potential time-induced antigen degradation (47). However, we found no statistically significant correlation between operation-to-IHC interval and any of the analyzed immune parameters (Supplementary Table 1 available online). Furthermore, differences between FOLFIRINOX patients who did or did not receive neoadjuvant radiotherapy could be attributed to the longer interval from diagnosis to operation for the former group. Lastly, the neoadjuvant treatment-naïve group had 3-5 micro-cores rather than whole slides evaluated. In the literature, these 2 methodologies are felt to be equivalent (48). Despite those limitations, our cohort reflects the recent paradigm shift in the management of PDAC.

In conclusion, neoadjuvant FOLFIRINOX with or without chemoradiation induced the most pronounced changes in the human PDAC TME. The improved OS documented in patients receiving neoadjuvant FOLFIRINOX may partially be due to the induction or enhancement of an antitumor immune response, thus indicating that PDAC patients receiving neoadjuvant FOLFIRINOX may potentially benefit from combination immunotherapeutic strategies that enhance and/or benefit from the favorable FOLFIRINOX-induced TME changes.

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References

1. Michelakos T, Pergolini I, Castillo CF, et al. Predictors of resectability and survival in patients with borderline and locally advanced pancreatic cancer who underwent neoadjuvant treatment with FOLFIRINOX. *Ann Surg*. 2019;269(4):733–740.
2. Ma Y, Yang H, Pitt JM, et al. Therapy-induced microenvironmental changes in cancer. *J Mol Med*. 2016;94(5):497–508.
3. Galluzzi L, Buque A, Kepp O, et al. Immunological effects of conventional chemotherapy and targeted anticancer agents. *Cancer Cell*. 2015;28(6):690–714.
4. Galluzzi L, Zitvogel L, Kroemer G. Immunological mechanisms underneath the efficacy of cancer therapy. *Cancer Immunol Res*. 2016;4(11):895–902.
5. Chacon JA, Schutsky K, Powell DJ. The impact of chemotherapy, radiation and epigenetic modifiers in cancer cell expression of immune inhibitory and stimulatory molecules and anti-tumor efficacy. *Vaccines (Basel)*. 2016;4(4):43.
6. Fridman WH, Zitvogel L, Sautes-Fridman C, et al. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol*. 2017;14(12):717–734.
7. Seliger B, Kloor M, Ferrone S. HLA class II antigen-processing pathway in tumors: molecular defects and clinical relevance. *Oncoimmunology*. 2017;6(2):e1171447.
8. Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. *Semin Cancer Biol*. 2012;22(4):350–358.
9. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;364(19):1817–1825.
10. Hong TS, Ryan DP, Borger DR, et al. A phase 1/2 and biomarker study of preoperative short course chemoradiation with proton beam therapy and capecitabine followed by early surgery for resectable pancreatic ductal adenocarcinoma. *Int J Radiat Oncol Biol Phys*. 2014;89(4):830–838.
11. Katz MH, Shi Q, Ahmad SA, et al. Preoperative modified FOLFIRINOX treatment followed by capecitabine-based chemoradiation for borderline resectable pancreatic cancer: alliance for clinical trials in oncology trial A021101. *JAMA Surg*. 2016;151(8):e161137.
12. Hong TS, Ryan DP, Blaszkowsky LS, et al. Phase I study of preoperative short-course chemoradiation with proton beam therapy and capecitabine for resectable pancreatic ductal adenocarcinoma of the head. *Int J Radiat Oncol Biol Phys*. 2011;79(1):151–157.
13. Pandha H, Rigg A, John J, et al. Loss of expression of antigen-presenting molecules in human pancreatic cancer and pancreatic cancer cell lines. *Clin Exp Immunol*. 2007;148(1):127–135.
14. Lu W, Pipeleers DG, Kloppel G, et al. Comparative immunocytochemical study of MHC class II expression in human donor pancreas and isolated islets. *Virchows Arch*. 1996;429(4-5):205–211.
15. Nardone V, Botta C, Caraglia M, et al. Tumor infiltrating T lymphocytes expressing FoxP3, CCR7 or PD-1 predict the outcome of prostate cancer patients subjected to salvage radiotherapy after biochemical relapse. *Cancer Biol Ther*. 2016;17(11):1213–1220.
16. Lo CS, Sanii S, Kroeger DR, et al. Neoadjuvant chemotherapy of ovarian cancer results in three patterns of tumor-infiltrating lymphocyte response with distinct implications for immunotherapy. *Clin Cancer Res*. 2017;23(4):925–934.
17. Teng F, Meng X, Kong L, et al. Tumor-infiltrating lymphocytes, forkhead box P3, programmed death ligand-1, and cytotoxic T lymphocyte-associated antigen-4 expressions before and after neoadjuvant chemoradiation in rectal cancer. *Transl Res*. 2015;166(6):721–732 e1.
18. Sistigu A, Yamazaki T, Vacchelli E, et al. Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat Med*. 2014;20(11):1301–1309.
19. Lim SH, Chua W, Cheng C, et al. Effect of neoadjuvant chemoradiation on tumor-infiltrating/associated lymphocytes in locally advanced rectal cancers. *Anticancer Res*. 2014;34(11):6505–6513.
20. Khallouf H, Marten A, Serba S, et al. 5-Fluorouracil and interferon-alpha immunochemotherapy enhances immunogenicity of murine pancreatic cancer through upregulation of NKG2D ligands and MHC class I. *J Immunother*. 2012;35(3):245–253.
21. Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res*. 2010;70(8):3052–3061.
22. Shalapour S, Font-Burgada J, Di Caro G, et al. Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy. *Nature*. 2015;521(7550):94–98.
23. Gonzalez-Aparicio M, Alzuguren P, Mauleon I, et al. Oxaliplatin in combination with liver-specific expression of interleukin 12 reduces the immunosuppressive microenvironment of tumours and eradicates metastatic colorectal cancer in mice. *Gut*. 2011;60(3):341–349.
24. Iida N, Dzutsev A, Stewart CA, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science*. 2013;342(6161):967–970.
25. Xynos ID, Karadima ML, Voutsas IF, et al. Chemotherapy +/- cetuximab modulates peripheral immune responses in metastatic colorectal cancer. *Oncology*. 2013;84(5):273–283.
26. Nywening TM, Wang-Gillam A, Sanford DE, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. *Lancet Oncol*. 2016;17(5):651–662.
27. Campoli M, Ferrone S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene*. 2008;27(45):5869–5885.
28. Lugade AA, Sorensen EW, Gerber SA, et al. Radiation-induced IFN-gamma production within the tumor microenvironment influences antitumor immunity. *J Immunol*. 2008;180(5):3132–3139.
29. Gameiro SR, Jammed ML, Wattenberg MM, et al. Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing. *Oncotarget*. 2014;5(2):403–416.

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30. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10(9):942–949.
31. Sasada T, Kimura M, Yoshida Y, et al. CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer*. 2003;98(5):1089–1099.
32. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol*. 2006;24(34):5373–5380.
33. Corrales L, Glickman LH, McWhirter SM, et al. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. *Cell Rep*. 2015;11(7):1018–1030.
34. Weichselbaum RR, Liang H, Deng L, et al. Radiotherapy and immunotherapy: a beneficial liaison? *Nat Rev Clin Oncol*. 2017;14(6):365–379.
35. Stanton SE, Adams S, Disis ML. Variation in the incidence and magnitude of tumor-infiltrating lymphocytes in breast cancer subtypes: a systematic review. *JAMA Oncol*. 2016;2(10):1354–1360.
36. Mei Z, Liu Y, Liu C, et al. Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. *Br J Cancer*. 2014;110(6):1595–1605.
37. Cai L, Michelakos T, Deshpande V, et al. Role of tumor-associated macrophages in the clinical course of pancreatic neuroendocrine tumors (PanNETs). *Clin Cancer Res*. 2019;25(8):2644–2655.
38. Triulzi T, Tagliabue E, Balsari A, et al. FOXP3 expression in tumor cells and implications for cancer progression. *J Cell Physiol*. 2013;228(1):30–35.
39. Yoshikawa K, Mitsunaga S, Kinoshita T, et al. Impact of tumor-associated macrophages on invasive ductal carcinoma of the pancreas head. *Cancer Sci*. 2012;103(11):2012–2020.
40. Kurahara H, Shinchi H, Mataki Y, et al. Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J Surg Res*. 2011;167(2):e211-e219.
41. Hu H, Hang JJ, Han T, et al. The M2 phenotype of tumor-associated macrophages in the stroma confers a poor prognosis in pancreatic cancer. *Tumor Biol*. 2016;37(7):8657–8664.
42. Liu C, Cheng H, Luo G, et al. Circulating regulatory T cell subsets predict overall survival of patients with unresectable pancreatic cancer. *Int J Oncol*. 2017;51(2):686–694.
43. Beatty GL, Chiorean EG, Fishman MP, et al. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science*. 2011;331(6024):1612–1616.
44. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*. 2010;11(10):889–896.
45. Mantovani A, Sozzani S, Locati M, et al. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549–555.
46. Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol*. 2008;180(4):2011–2017.
47. Grillo F, Bruzzone M, Pigozzi S, et al. Immunohistochemistry on old archival paraffin blocks: is there an expiry date? *J Clin Pathol*. 2017;70(11):988–993.
48. Kobierzycki C, Pula B, Wojnar A, et al. Tissue microarray technique in evaluation of proliferative activity in invasive ductal breast cancer. *Anticancer Res*. 2012;32(3):773–777.