Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer

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Background: The host immune reaction is represented by immune/inflammatory cell infiltrates. Here we systematically analysed tumour-infiltrating immune/inflammatory cells in pancreatic ductal carcinoma (PDC) and evaluated their clinicopathological impact.

Methods: Using immunohistochemistry, we examined tumour-infiltrating $CD68^+$ pan-macrophages, $HLA\text{-}DR^+CD68^+$ M1 macrophages (M1), $CD163^+$ or $CD204^+$ M2 macrophages (M2), $CD66b^+$ neutrophils (Neu), $CD4^+$ T cells ($CD4^+T$), $CD8^+$ T cells ($CD8^+T$), and $FOXP3^+CD4^+$ regulatory T cells (Treg) in 212 cases of PDC, and conducted correlation and survival analyses using the Kaplan–Meier method and Cox proportional hazards model.

Results: Higher levels of tumour-infiltrating pan-macrophages, M2, Neu, or the ratio of Tregs to $CD4^+T$ (%Treg) were significantly associated with shorter survival, whereas higher levels of tumour-infiltrating $CD4^+T$, $CD8^+T$, or the ratio of M1 to pan-macrophages (%M1) were significantly associated with longer survival. Survival analysis of pairs of these variables revealed that some of the resulting patient groups had exclusively longer survival. We then connected the apparently related factors, and two significant variables emerged: tumour-infiltrating $CD4^+T^{high}/CD8^+T^{high}/\%Treg^{low}$ and tumour-infiltrating $\%M1^{high}/M2^{low}$. Multivariate survival analysis revealed that these variables were significantly correlated with longer survival and had a higher hazard ratio.

Conclusion: Tumour-infiltrating $CD4^+T^{high}/CD8^+T^{high}/\%Treg^{low}$ and $\%M1^{high}/M2^{low}$ are independent prognosticators useful for evaluating the immune microenvironment of PDC.

The tumour microenvironment, comprising multiple cellular and molecular factors, has important roles in the biological behaviour of cancer (Hanahan and Weinberg, 2011). The host immune reaction, as represented by tumour-infiltrating immune/inflammatory cells, is one of the leading players in the tumour microenvironment (Dunn *et al*, 2004; Sica and Bronte, 2007; Biswas and Mantovani, 2010; Ferrone and Dranoff, 2010; Grivennikov *et al*, 2010; Hiraoka *et al*, 2011). Recent extensive studies using animal models have helped to clarify the molecular and cellular mechanisms that define the roles of immune/inflammatory cells in the tumour microenvironment. Such knowledge of the human cancer microenvironment in real terms is important for the development and application of cancer therapy, especially immunotherapy. The microenvironment of human cancer is complex, and often shows different characteristics according to the tissues from which tumours develop, the carcinogenetic pathways involved, and tumour stages. In colorectal and ovarian cancers, a high proportion of $CD8^+$ T cells or

CD45RO$^+$ memory T cells infiltrating the cancer tissue can be a favourable prognostic indicator (Zhang et al, 2003; Pages et al, 2005). In contrast, several kinds of tumour-infiltrating immune/inflammatory cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and alternatively activated macrophages, support tumour growth and extension by suppressing host immune responses, and accelerating angiogenesis and tissue remodelling (Dunn et al, 2004; Hiraoka et al, 2006; Sica and Bronte, 2007; Biswas and Mantovani, 2010; Grivennikov et al, 2010). Macrophages are heterogeneous and can be divided into classically activated macrophages (alternatively M1 macrophages, M1) that are activated mainly by Th1 cytokines and alternatively activated macrophages (alternatively M2 macrophages, M2) that are activated mainly by Th2 cytokines (Mantovani et al, 2002; Mosser and Edwards, 2008; Biswas and Mantovani, 2010; Sica and Mantovani, 2012). Thus, it is anticipated that tumour-infiltrating immune/inflammatory cells would be useful hallmarks for evaluating the characteristics of the tumour immune microenvironment and for immune monitoring of tumour tissues in both non-treated and treated patients. In addition to the clinicopathological impact of individual tumour-infiltrating cell types and subsets of immune/inflammatory cells, it is important to observe the interactions among these infiltrating cells to understand the actual conditions within the tumour microenvironment, as the effects of specific cell combinations may also be of clinicopathological significance. Therefore, systematic analysis of several tumour-infiltrating immune/inflammatory cells characteristic of innate or adaptive immune responses in the same type of cancer tissue would appear to be of potential importance. However, very few such studies have yet been performed.

Pancreatic cancer (pancreatic ductal carcinoma (PDC)) is the fourth and fifth leading cause of cancer-related death in the United States and Japan, respectively (Center for Cancer Control and Information Services & National Cancer Center, 2009; Siegel et al, 2011). PDC is one of the most aggressive cancers, with almost equivalent incidence and mortality rates, because of its aggressive growth and early metastatic dissemination (Hruban et al, 2010). PDC has a characteristically desmoplastic stroma and immune/inflammatory infiltrates. The development of predictive biomarkers to assist selection of patient subsets is useful for studies aimed at reducing the mortality of PDC patients, especially clinical studies designed to evaluate various therapeutic approaches (Philip et al, 2009).

The aim of this study was to examine whether tumour-infiltrating immune/inflammatory cells represent actual conditions of the immune microenvironment by analysing their clinicopathological impact. We then performed immunohistochemistry to detect tumour-infiltrating M1 and M2, neutrophils (Neu), CD4$^+$ T cells (CD4$^+$T), CD8$^+$ T cells (CD8$^+$T), and FOXP3$^+$ CD4$^+$ Treg using 212 cases of PDC and examined their clinicopathological influences. Furthermore, we investigated the prognostic significance of combinations of these tumour-infiltrating immune/inflammatory cells as indicators of the immune microenvironment.

MATERIALS AND METHODS

Patients and samples. This study was approved by the Ethics Committee of the National Cancer Center, Japan. Informed consent was obtained from all participants involved in this study and all clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki. Clinical and pathological data were obtained through a detailed retrospective review of the medical records of all 212 patients with PDC who had undergone initial surgical resection between 1990 and 2005 at the

National Cancer Center Hospital, Japan. The patients enrolled in the study were those for whom sufficient immunohistochemical analyses were available. None of the patients had received any therapy before surgery. The operative procedures included 149 classical pancreatoduodenectomies or pylorus-preserving pancreatoduodenectomies, 56 distal pancreatectomies, and 7 total pancreatectomies. All the patients included in this study underwent macroscopic curative resection. All of the cases were conventional ductal carcinomas and intraductal papillary-mucinous neoplasm-related cancers, secondary tumours, and post-neoadjuvant cases were excluded. The clinicopathological characteristics of the patients are summarised in Supplementary Table 1. All M1 patients had nodal metastasis around the abdominal aorta, without any other form of metastasis. The median follow-up period after surgery for the patients as a whole and for the living patients were 18.8 (2.6–201) and 54.5 (2.6–201) months, respectively. Every patient was followed up in the outpatient clinic every 1–3 months during the first postoperative year, and every 6–12 months thereafter. Patients underwent physical examination, laboratory tests, chest radiography, abdominal computed tomography, and/or ultrasonography, unless there was a confirmed relapse. The tumour markers carcinoembryonic antigen and carbohydrate antigen 19–9 were also measured until relapse. Recurrence was suspected when a new local or distant metastatic lesion was found on serial images and an increase in tumour marker levels was recognised. When progression of the disease was confirmed by repeated imaging studies, the date of the first suspicious radiologic finding was used as the date of initial disease recurrence. At the census date (June 2009), we checked whether all our patients were dead or alive from our medical records and family registers administered by the Japanese government, 49 patients (23.1%) were alive, 141 (66.5%) had died of pancreatic cancer, and 22 (10.3%) had died of other causes. Information on post-resection adjuvant therapy was available for 206 patients, of whom 8 received chemotherapy and radiotherapy, 84 chemotherapy only, and 2 radiotherapy only; 112 patients did not receive any post-resection therapy.

Pathological examination. All of the cases were pathologically reexamined and classified according to the World Health Organization classification (Hruban et al, 2010). the International Union Against Cancer tumour-node-metastasis classification (Sobin et al, 2009), and the Classification of Pancreatic Carcinoma of the Japan Pancreas Society (Japan Pancreas Society, 2011). Tumour necrosis was judged according to the definition reported previously (Hiraoka et al, 2010).

Immunohistochemistry. Immunohistochemistry was performed on the formalin-fixed, paraffin-embedded tissue sections using the avidin–biotin complex method as described previously (Hiraoka et al, 2006). We used 4-μm-thick sections of representative blocks with antibodies listed in Supplementary Table 2.

Quantitative evaluation of tumour-infiltrating immune/inflammatory cells. After immunohistochemistry, the microscopic images were imported as digital photo files using a NanoZoomer Digital Pathology (NDP) system (Hamamatsu Photonics, Hamamatsu, Japan), and we selected three areas at low magnification in which the immunolabeled cells had infiltrated into the tumour most densely (Supplementary Figure 1). The selected areas were those in which it had been confirmed at high magnification that invasive proliferation of cancer cells was present. We did not select areas into which infiltrated immune/inflammatory cells had been recruited as a result of secondary tumour effects, such as pancreatitis, necrosis, ulceration, or mucus flooding of the tissue. Using NDP view at a magnification of $\times$ 200, immunolabeled lymphocytes, neutrophils (except intravascular neutrophils), or macrophages were then counted by two

independent investigators (YI and NH). These observers were blinded to each other and also not provided with any clinical information on the outcome of the patients. The average counts for each of the three areas made by the two observers were then compared, and when the difference between their counts was $<20\%$ of the maximum value, the average of the two was used as the final count; if the difference exceeded 20%, the observers discussed the reasons for the difference and performed recounts until the difference became $<20\%$. For the survival and correlation analyses, patients were divided into two groups showing high and low cell infiltration, representing values higher and lower than the median for tumour-infiltrating immune/inflammatory cells.

Statistical analysis. Comparisons of variables were performed using Fisher's exact test based on their categorical data (Table 1). The postoperative proportion of overall survival (OS) and disease-free survival (DFS) was calculated by the Kaplan–Meier method. Univariate analysis was performed for prognostic factors using the log-rank test. The factors found to be significant by univariate analysis were subjected to multivariate analysis using the Cox proportional hazards model (backward elimination method). For examining combinations of variables and performing Kaplan–Meier analysis, patients were divided into four groups based on combinations of two groups of two factors (i.e., $X^{high}Y^{high}$, $X^{high}Y^{low}$, $X^{low}Y^{high}$, and $X^{low}Y^{low}$). The profiles of the resulting Kaplan–Meier curves were divisible into two types, which we termed type A and type B; type A was the pattern associated with the longest surviving group with a combination of both conditions for whom longer survival was expected from their individual factors, one shortest survival group with a combination of both conditions for whom shorter survival was expected from their individual factors, and an intermediate survival group with combinations of conditions other than the previous two groups; in type B, there was one longest (or shortest) survival group, and the other three groups showed similar survival curves. Differences at $P<0.05$ were considered statistically significant. Statistical analyses were performed with StatView-J 5.0 software (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Tumour-infiltrating immune/inflammatory cells. We used the following antigens as markers for detecting each of the cell types: CD68 for pan-macrophages, CD163 or CD204 for M2, CD66b for Neu, CD4, CD8, and FOXP3$^+$CD4$^+$ (Treg) for subsets of T cells. M1 were detected as HLA-DR$^+$CD68$^+$ cells (Figure 1). In 78% of the cases, M2 were predominant among tumour-infiltrating macrophages. In contrast, M1 infiltrated chronic pancreatitis (non-cancerous pancreas) predominantly. The majority of the macrophages that had infiltrated into areas of necrosis were M2. The density of tumour-infiltrating macrophages, CD4$^+$T, and CD8$^+$T was higher near the invasive front of the tumour, and that of T cells was lower at the centre of the tumour. Preliminary tests revealed that rather than the absolute numbers of infiltrating cells, it would be better if we used the ratio of FOXP3$^+$CD4$^+$ T cells to CD4$^+$T (%Treg) and the ratio of M1 to pan-macrophages (%M1) as variables, as they were more representative of the biological characteristics of the respective infiltrating cells. Comparison of immune/inflammatory cells infiltrating into normal pancreas, chronic pancreatitis (non-cancerous pancreas), and PDC is shown in Figure 1I.

Interrelationships between clinicopathological variables. We analysed the interrelationships among clinicopathological variables of PDC containing tumour-infiltrating immune/inflammatory cells (Table 1). Tumour-infiltrating pan-macrophages, CD163$^+$ M2, CD204$^+$ M2, and Neu, showed close correlations with each other.

All of them showed significant and negative correlations with %M1. Tumour-infiltrating CD4$^+$T and CD8$^+$T showed a significant positive correlation with each other, but no significant correlation was evident between tumour-infiltrating myeloid cells and tumour-infiltrating CD4$^+$T or CD8$^+$T. In contrast, tumour-infiltrating %Treg were closely correlated with tumour-infiltrating macrophages, Neu, and CD4$^+$T. Most of the conventional clinicopathological variables did not demonstrate a close correlation with tumour-infiltrating immune/inflammatory cells. The presence of tumour necrosis was significantly correlated with all types of tumour-infiltrating immune/inflammatory cells. The presence of venous invasion was closely correlated with tumour-infiltrating pan-macrophages, CD204$^+$ M2, and %Treg.

Prognostic impact of individual tumour-infiltrating immune/inflammatory cells. Kaplan–Meier survival analyses revealed that higher numbers of tumour-infiltrating pan-macrophages, CD163$^+$ or CD204$^+$ M2, and Neu, and lower %M1 were significantly associated with both shorter OS and DFS in PDC patients (Figure 2 and Supplementary Figure 2). More marked infiltration of CD4$^+$T or CD8$^+$T into tumour tissues was significantly associated with both longer OS and DFS, whereas higher %Treg was closely associated with both shorter OS and DFS (Figure 2 and Supplementary Figure 2).

When each of the parameters of tumour-infiltrating immune/inflammatory cells was subjected to Cox regression multivariate analysis, together with the conventional clinicopathological variables that had been found to be significant by univariate analysis (Supplementary Tables 3 and 4), all of the tumour-infiltrating immune/inflammatory cells were found to be independent predictors of OS and DFS (data not shown). Tumour-infiltrating CD163$^+$ and CD204$^+$ M2 showed comparable results, and we adopted CD163$^+$ macrophages as M2 thereafter.

Prognostic impact of combinations of tumour-infiltrating immune/inflammatory cells. To clarify more precisely the characteristics of tumour-infiltrating immune/inflammatory cells in the immune microenvironment, we then examined various combinations of tumour-infiltrating immune/inflammatory cells and performed Kaplan–Meier analysis (see Materials and Methods, Figure 3). Most of the combinations were found to be type A, as expected. However, four combinations (tumour-infiltrating CD4$^+$T and CD8$^+$T, tumour-infiltrating CD4$^+$T and %Treg, tumour-infiltrating CD8$^+$T and %Treg, and tumour-infiltrating %M1 and M2) showed type B. For the combination of tumour-infiltrating CD4$^+$T and CD8$^+$T, only the group with higher numbers of tumour-infiltrating CD4$^+$T (CD4$^+$T^{high}) and higher numbers of tumour-infiltrating CD8$^+$T (CD8$^+$T^{high}) showed long survival, and the other three groups showed similar degrees of shorter survival even if the number of either CD4$^+$T or CD8$^+$T was higher. Similarly, the group with CD4$^+$T^{high} and lower numbers of tumour-infiltrating %Treg (%Treg^{low}) in the combination of tumour-infiltrating CD4$^+$T and %Treg, the group with CD8$^+$T^{high} and %Treg^{low} in the combination of tumour-infiltrating CD8$^+$T and %Treg, and the group with higher numbers of tumour-infiltrating %M1 (%M1^{high}) and lower numbers of tumour-infiltrating M2 (M2^{low}) in the combination of tumour-infiltrating %M1 and M2 were the only group that showed long survival, whereas the other three groups in each of the combinations showed similar degrees of shorter survival. These results indicated that specific groups with long survival could be detected more precisely by combination of variables. In addition to the combination of CD4$^+$T^{high} and CD8$^+$T^{high}, a lower tumour-infiltrating %Treg (%Treg^{low}) was important for a close association with longer survival. In fact, one group with CD4$^+$T^{high}, CD8$^+$T^{high}, and %Treg^{low} showed exclusively long survival (Supplementary Figure 3).

**Table 1.** Interrelationship between clinicopathological variables

Variables	Gender	Tumour size	Pathologic tumour status	Pathologic node status	Pathologic metastasis status	Histological grade	Tumour necrosis	Lymphatic invasion[a]	Venous invasion[a]	Intrapancreatic neural invasion[a]	Nerve plexus invasion[a]	CD68+ M	CD163+ M2	CD204+ M2	%M1[b]	CD66b+ Neu	CD4+ T	CD8+ T	%Treg[c]
Age (≥60 years/<60 years)	NS	NS	NS	NS	0.033	NS	NS	0.011	NS	0.078	NS	NS	NS	NS	NS	NS	NS	NS	NS
Gender (male/female)		NS	NS	NS	NS	NS	0.017	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Tumour size (<30 mm/≥30 mm)			0.042	0.004	NS	NS	0.010	0.0002	0.002	0.034	0.030	0.075	NS	0.023	NS	NS	NS	NS	NS
Pathologic tumour status (T1+T2/T3)				NS	NS	NS	NS	0.007	0.021	NS	0.017	NS	NS	0.058	NS	NS	NS	NS	NS
Pathologic node status (N0/N1)					0.010	0.044	0.057	<0.0001	<0.0001	0.003	<0.0001	NS	NS	NS	NS	NS	NS	NS	NS
Pathologic metastasis status (M0/M1)						NS	NS	0.006	0.040	NS	NS	0.041	NS	NS	NS	NS	NS	NS	NS
Histological grade (G1/G2, G3)							0.004	0.057	<0.0001	NS	NS	NS	0.082	NS	NS	NS	0.018	NS	0.0008
Tumour necrosis (negative/positive)								NS	0.002	NS	NS	0.001	0.003	0.001	0.002	0.001	0.042	0.019	0.002
Lymphatic invasion (0, 1/2, 3)[a]									<0.0001	0.0009	0.008	0.035	NS	NS	NS	NS	NS	NS	NS
Venous invasion (0, 1/2, 3)[a]										0.0004	0.028	0.003	NS	0.048	NS	NS	0.067	NS	0.034
Intrapancreatic neural invasion (0, 1/2, 3)[a]											<0.0001	NS	NS	NS	NS	NS	NS	NS	0.072
Nerve plexus invasion (absence/presence)[a]												NS	NS	NS	NS	NS	NS	NS	NS
CD68+ M (low/high)													<0.0001	<0.0001	<0.0001	<0.0001	NS	NS	0.019
CD163+ M2 (low/high)														<0.0001	<0.0001	<0.0001	NS	NS	0.039
CD204+ M2 (low/high)															0.0003	0.013	NS	NS	0.006
%M1[b] (low/high)																0.002	NS	NS	0.002
CD66b+ Neu (low/high)																	NS	NS	0.019
CD4+ T (low/high)																		0.074	0.004
CD8+ T (low/high)																			0.002
%Treg[c] (low/high)																			NS

Nonsignificant (NS), P>0.10; bold value, P<0.05; underlined value, negatively correlated.
[a]Classified according to the classification of pancreatic carcinoma of Japan Pancreas Society.
[b]Ratio of CD68+HLA-DR+ M1 to CD68+ M.
[c]Ratio of CD4+FOXP3+ Treg to CD4+ T.
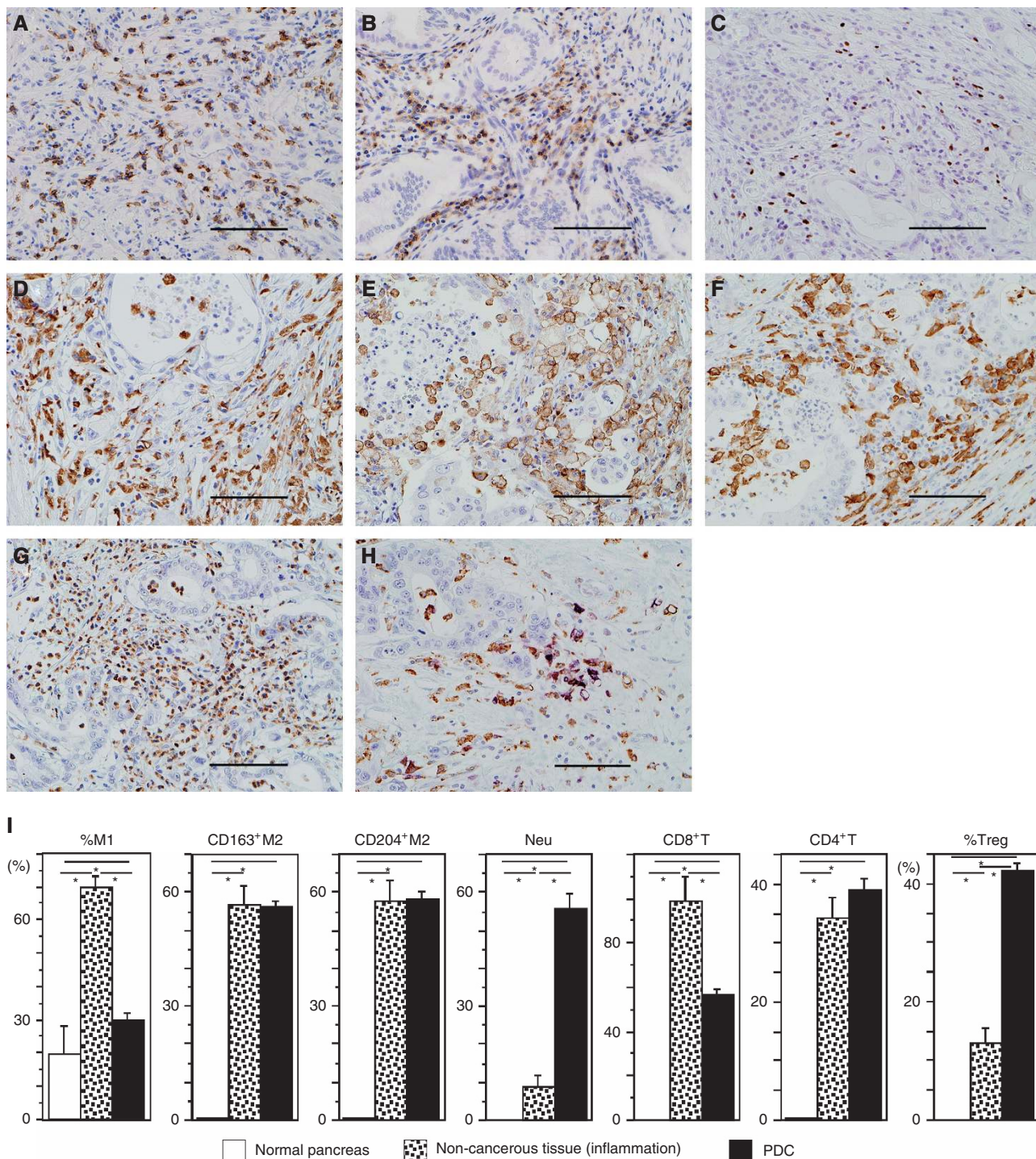
Figure 1. Immunohistochemical features of tumour-infiltrating immune/inflammatory cells. The representative photos are shown: (A) CD8$^+$ T cells, (B) CD4$^+$ T cells, (C) FOXP3$^+$ T cells, (D) CD68$^+$ pan-macrophages, (E) CD163$^+$ M2 macrophages, (F) CD204$^+$ M2 macrophages, (G) CD66b$^+$ neutrophils, and (H) CD68$^+$HLA-DR$^+$ M1 macrophages (CD68 in brown and HLA-DR in purple). Scale bars show 10 μm in length. A comparison of infiltrating immune/inflammatory cells into normal pancreas, chronic pancreatitis (non-cancerous pancreas, and PDC is shown in I. Columns and bars show mean (n = 20) and standard error, respectively. Significance values were *P<0.05 and **P<0.01.

To represent the effects of tumour-infiltrating immune/inflammatory cells more precisely, we then connected these factors to make two new variables: tumour-infiltrating CD4$^+$T^{high}/CD8$^+$ T^{high}/%Treg^{low} and tumour-infiltrating %M1^{high}/M2^{low}. Multivariate analysis together with conventional clinicopathological variables that had been found to be significant by univariate analysis revealed that these two variables were independently prognostic for OS and DFS with higher hazard ratio values (Table 2 and Supplementary Table 4).

DISCUSSION

Host immune responses to tumour cells are represented by the features of tumour-infiltrating immune/inflammatory cells (Dunn et al, 2004; Sica and Bronte, 2007; Biswas and Mantovani, 2010; Ferrone and Dranoff, 2010; Grivennikov et al, 2010; Hiraoka et al, 2011). We investigated the profiles of tumour-infiltrating immune/inflammatory cells using over 200 cases of PDC that had been
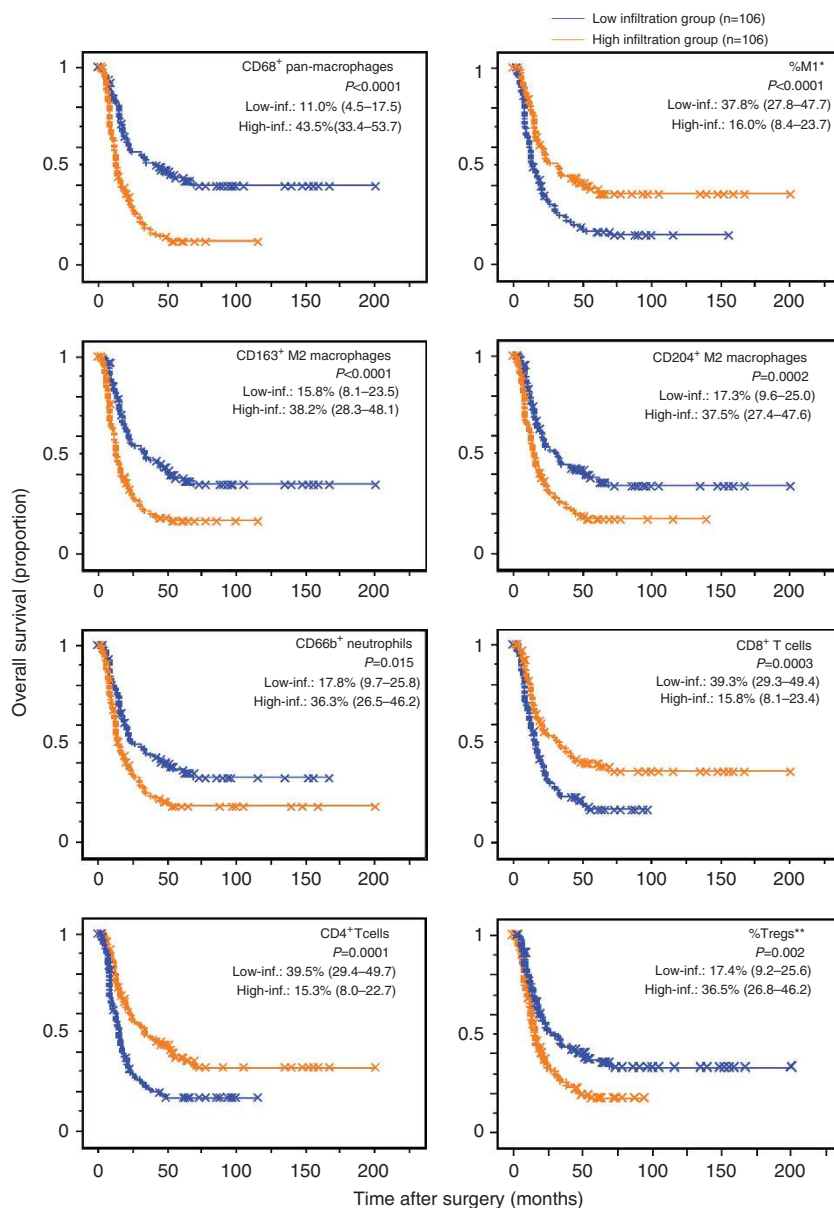
Figure 2. Kaplan–Meier survival curves showing comparison of overall survival between high (orange) and low (blue) infiltration (inf.) of immune/ inflammatory cells. *P*-values obtained from log-rank test. The 'x' and ' + ' represent censoring and failure, respectively. The proportions of 5-year survival with 95% confidence intervals (in parentheses) are shown. *Ratio of CD68 + HLA-DR + M1 macrophages to CD68 + pan-macrophages, **ratio of FOXP3 + CD4 + Tregs to CD4 + T cells.

surgically resected in one hospital. We found that tumour-infiltrating pan-macrophages, CD163 + or CD204 + M2, and Neu had positive correlations with each other, and that all of them were negatively correlated with tumour-infiltrating %M1 (Table 1). Tumour-infiltrating CD4 + T and CD8 + T showed a positive correlation with tumour-infiltrating CD8 + T and a negative correlation with tumour-infiltrating %Treg (Table 1). No significant correlation was found between tumour-infiltrating myeloid cells and T cells, except for the close correlation between tumour-infiltrating myeloid cells and %Treg. These findings suggest that tumour-infiltrating myeloid cells and T cells are regulated independently, and that infiltration by each subset of myeloid cells or Treg is closely interrelated. Univariate and multivariate survival analyses revealed that tumour-infiltrating CD4 + T, CD8 + T, and %M1 were independently prognostic of OS and DFS and that tumour-infiltrating pan-macrophages, M2, Neu,

and %Treg were independent prognosticators of poor OS and DFS. When pairs of these six variables (%M1, M2, Neu, CD4 + T, CD8 + T, and %Treg) were combined and subjected to survival analyses, the four combinations (CD4 + T and CD8 + T, CD4 + T and %Treg, CD8 + T and %Treg, and %M1 and M2) showed that only one specific patient group (CD4 + T^{high} and CD8 + T^{high}, CD4 + T^{high} and %Treg^{low}, CD8 + T^{high} and %Treg^{low}, and %M1^{high} and M2^{low}) had longer survival, whereas the other three groups had shorter survival with similar degrees. These results suggest that systematic analysis of several tumour-infiltrating immune/inflammatory cells is important for providing the findings of effects of specific cell combination in immune microenvironment more precisely. The factors involved in these combinations were then connected, and two new variables emerged: tumour-infiltrating CD4 + T^{high}/CD8 + T^{high}/%Treg^{low} and %M1^{high}/M2^{low}. These corrected for the unbalanced patient distribution, and represented
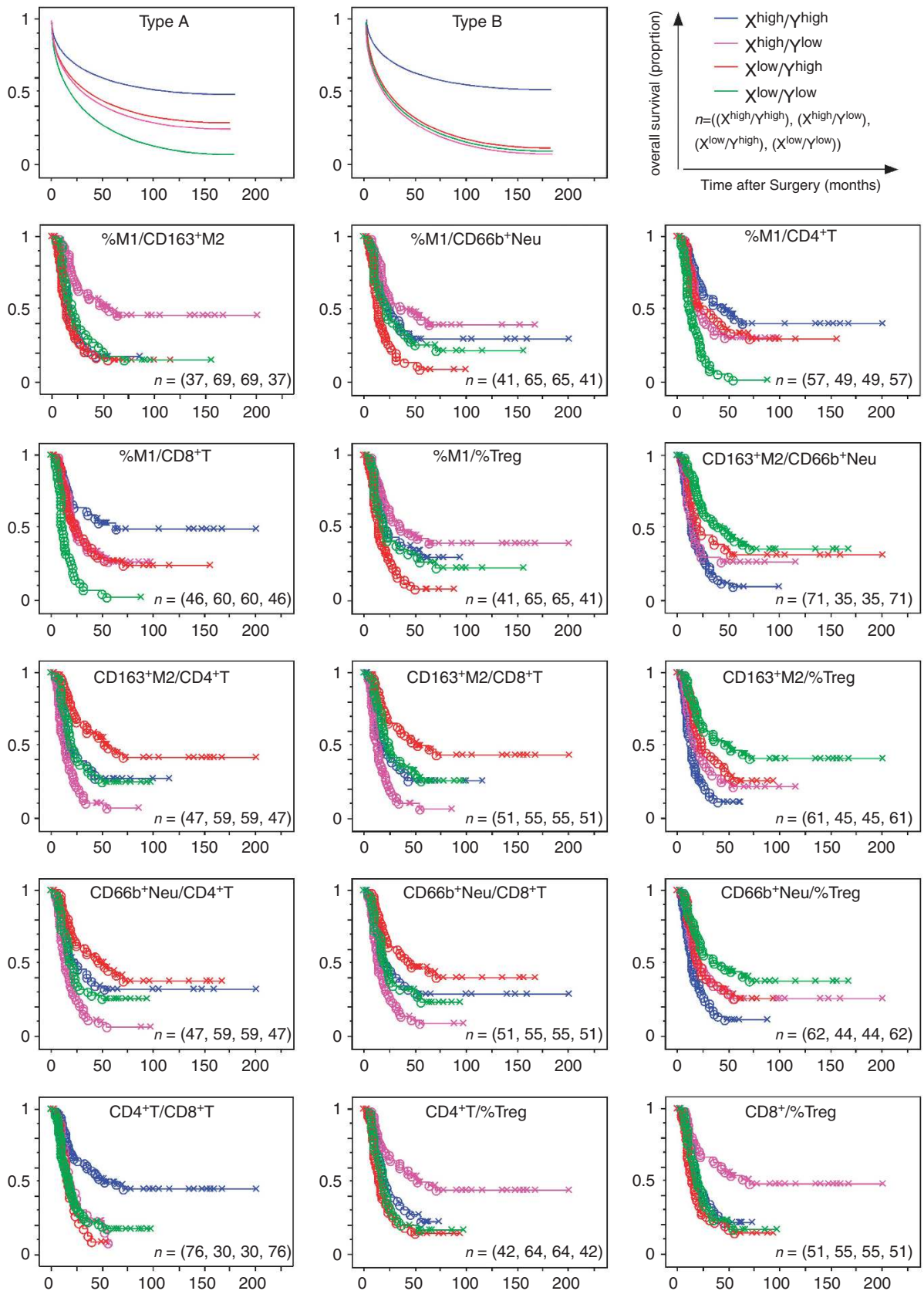
Figure 3. Kaplan–Meier survival curves showing comparison of overall survival among four groups of patients divided on the basis of combinations of two variables with high and low infiltration of immune/inflammatory cells. A and B are representative profiles. The four groups are 1st variable[high] and 2nd variable[high] in blue, 1st variable[high] and 2nd variable[low] in pink, 1st variable[low] and 2nd variable[high] in red, and 1st variable[low] and 2nd variable[low] in green. The 'x' and 'circle' represent censoring and failure, respectively.

Table 2. Univariate and multivariate analyses of tumour-infiltrating immune/inflammatory cells as prognostic factors associated with (a) overall survival and (b) disease-free survival in patients with ductal carcinoma of the pancreas

	Univariate analysis		Multivariate analysis	
Variables	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
(a)				
Age ($\geqslant$60 years/$<$60 years)	0.862 (0.612–1.215)	0.400		
Gender (male/female)	1.197 (0.848–1.690)	0.306		
Tumour size ($<$30 mm/$\geqslant$30 mm)	1.905 (1.257–2.887)	**0.002**		
Pathologic tumour status (T1 + T2/T3)	5.249 (0.733–37.6)	0.099		
Pathologic node status (N0/N1)	2.131 (1.337–3.396)	**0.002**		
Pathologic metastasis status (M0/M1)	2.572 (1.594–4.150)	**0.0001**	1.932 (1.169–3.192)	**0.010**
Histological grade (G1/G2, G3)	1.585 (1.068–2.353)	**0.022**		
Tumour margin status (negative/positive)	1.526 (1.050–2.218)	**0.027**		
Nerve plexus invasion (absence/presence)[a]	1.593 (1.114–2.278)	**0.011**	1.600 (1.100–2.328)	**0.014**
Lymphatic invasion (0, 1/2, 3)[a]	2.495 (1.662–3.746)	**$<$0.0001**	2.052 (1.354–3.110)	**0.0007**
Venous invasion (0, 1/2, 3)[a]	1.932 (1.355–2.755)	**0.0003**		
Intrapancreatic neural invasion (0, 1/2, 3)[a]	1.739 (1.234–2.450)	**0.002**		
$CD4^+T^{high}/CD8^+T^{high}/\%Treg^{low}$ (yes/no)	3.501 (2.093–5.856)	**$<$0.0001**	3.206 (1.903–5.402)	**$<$0.0001**
$\%M1^{high}/M2^{low}$ (yes/no)	2.781 (1.877–4.119)	**$<$0.0001**	2.577 (1.726–3.846)	**$<$0.0001**
$CD66b^+$ Neu (low/high)	1.703 (1.221–2.374)	**0.002**	1.486 (1.045–2.113)	**0.028**
(b)				
Age ($\geqslant$60 years/$<$60 years)	0.864 (0.619–1.206)	0.390		
Gender (male/female)	1.082 (0.780–1.502)	0.637		
Tumour size ($<$30 mm/$\geqslant$30 mm)	1.962 (1.313–2.932)	**0.001**	1.631 (1.082–2.459)	**0.020**
Pathologic tumour status (T1 + T2/T3)	4.788 (0.669–34.27)	0.119		
Pathologic node status (N0/N1)	2.166 (1.375–3.412)	**0.0009**		
Pathologic metastasis status (M0/M1)	2.585 (1.624–4.114)	**$<$0.0001**	2.046 (1.269–3.297)	**0.003**
Histological grade (G1/G2, G3)	1.572 (1.077–2.295)	**0.019**		
Tumour margin status (negative/positive)	1.288 (0.893–1.856)	0.176		
Nerve plexus invasion (absence/presence)[a]	1.510 (1.072–2.126)	**0.018**		
Lymphatic invasion (0, 1/2, 3)[a]	1.924 (1.326–2.793)	**0.0006**		
Venous invasion (0, 1/2, 3)[a]	2.115 (1.499–2.985)	**$<$0.0001**	1.619 (1.142–2.295)	**0.007**
Intrapancreatic neural invasion (0, 1/2, 3)[a]	1.633 (1.175–2.269)	**0.004**		
$CD4^+T^{high}/CD8^+T^{high}/\%Treg^{low}$ (yes/no)	3.123 (1.958–4.981)	**$<$0.0001**	2.667 (1.661–4.281)	**$<$0.0001**
$\%M1^{high}/M2^{low}$ (yes/no)	2.526 (1.743–3.660)	**$<$0.0001**	2.252 (1.545–3.284)	**$<$0.0001**
$CD66b^+$ Neu (low/high)	1.445 (1.051–1.986)	**0.024**		

Abbreviations: CI = confidence interval; HR = hazard ratio. Bold value, $P<0.05$.
[a]Classified according to the classification of pancreatic carcinoma of Japan Pancreas Society.

the effects of the immune-related factors more precisely. In fact, multivariate survival analyses showed that these two variables were independent prognosticators of OS and DFS with higher hazard ratio values than those of individual sets of tumour-infiltrating cells (Table 2). Therefore, tumour-infiltrating $CD4^+T^{high}/CD8^+T^{high}/\%Treg^{low}$ and tumour-infiltrating $\%M1^{high}/M2^{low}$ are suitable variables for evaluating the immune microenvironment of PDC.

Various types of myeloid cells affect the immune microenvironment. Though some studies have obtained opposite results (Forssell *et al*, 2007), several studies have reported that a high amount of tumour-infiltrating macrophages is correlated with a poor outcome in various cancers (Komohara *et al*, 2008; Shabo *et al*, 2008) containing pancreas cancer (Kurahara *et al*, 2011). We speculated that this discrepancy in previous results might have been attributable to the fact that most of these studies had measured tumour-infiltrating macrophages as pan-macrophages and/or M2, and not M1 independently. M1 have strong tumoricidal activity, being opposite to the effect of M2. Tumour-infiltrating macrophages were predominantly M2, whereas M1 was predominant macrophage in the non-cancerous inflammatory region surrounding the area of cancer cell infiltrates. Therefore, analysis of tumour-infiltrating M1 and M2, as well as their

locations, separately is important for assessing the infiltration of macrophages.

Recently, a study using mice demonstrated that tumour-associated Neu were polarised into two types: the N1 antitumour phenotype and the N2 protumour phenotype, similar to the M1 and M2 macrophages (Fridlender et al, 2009). In humans, tumour-infiltrating Neu were significantly correlated with shorter survival in renal cell carcinoma (Wislez et al, 2003; Jensen et al, 2009) and significantly correlated with longer survival in gastric carcinoma (Caruso et al, 2002). We have found that almost all tumour-infiltrating Neu in PDC express arginase 1 (Ino and Hiraoka, unpublished data), which hydrolyses arginine to ornithine and urea, resulting in a reduction of arginine in the tumour microenvironment. It has been reported that adequate levels of arginine in the extracellular milieu are crucial for T-cell proliferation and activity, and that reduction of arginine suppresses T-cell responses (Rodriguez et al, 2004; Rodriguez and Ochoa, 2008). These results, combined with findings that higher levels of tumour-infiltrating Neu are significantly correlated with shorter survival of patients, suggest that tumour-infiltrating Neu act as a pro-tumour factor. Therefore, it is suggested that N2 cells are the predominant infiltrating type in PDC, although currently there are no markers for distinguishing the two human Neu phenotypes, N1 and N2.

MDSCs represent a heterogeneous population of cells that comprise myeloid progenitor cells and immature myeloid cells and have immune suppressor function (Gabrilovich and Nagaraj, 2009). In contrast to extensive studies of murine MDSCs, the corresponding cells in humans have been inadequately characterised because of the lack of uniform markers. Human MDSCs have a common $Lin^{-/low}HLA-DR^{-/low}CD11b^{+}CD33^{+}$ phenotype and comprise two subsets: a $CD14^{+}$ monocytic subpopulation (Mo-MDSCs) and a $CD15^{+}$ granulocytic subpopulation (G-MDSCs) (Filipazzi et al, 2012). G-MDSCs have a Neu-like polymorphonuclear morphology and also express CD66b. G-MDSCs may have been included in the Neu counts in our present study. Although MDSCs were not identified directly in our study, tumour-infiltrating myeloid cells were closely correlated both with each other, and also with %Treg. These findings, together with a recent report by Corzo et al (2010) indicating that hypoxia causes MDSCs to differentiate rapidly into tumour-associated macrophages, suggest that tumour-infiltrating MDSCs have a positive correlation with tumour-infiltrating M2 and Neu, and a negative correlation with %M1. Thus, our evaluation system using $\%M1^{high}/M2^{low}$ might also cover tumour-infiltrating MDSCs.

In our study both tumour-infiltrating $CD4^{+}T$ and $CD8^{+}T$ were independent prognostic factors, however, the detailed analysis indicated that infiltration of the tumour by $CD4^{+}T$ or $CD8^{+}T$ alone was not sufficiently associated with longer survival, and that infiltration by higher numbers of both $CD4^{+}T$ and $CD8^{+}T$ was prerequisite for longer survival. This result was similar to that of a previous study that included a smaller number of cases (Fukunaga et al, 2004). It was also reported that De Monte et al (2011) found that Th2 rather than Th1 cells predominantly infiltrated in PDC, and that the ratio of tumour-infiltrating Th2/Th1 cells was independently predictive of patient survival. These findings together with our present results suggest that $CD4^{+}T^{high}/CD8^{+}T^{high}$ is the hallmark of an immune microenvironment where a strong Th1 response might be present.

In conclusion, tumour-infiltrating $CD4^{+}T^{high}/CD8^{+}T^{high}/\%Treg^{low}$ and tumour-infiltrating $\%M1^{high}/M2^{low}$ were independent prognosticators with a higher hazard ratio. Therefore, these variables appear to be suitable for monitoring the immune microenvironment of PDC, and we are now planning to use them for this purpose, with a view to devising some more effective form of immunotherapy.

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