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Immunobiological effects of gemcitabine and capecitabine combination chemotherapy in advanced pancreatic ductal adenocarcinoma

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Background: Preclinical studies suggest that chemotherapy may enhance the immune response against pancreatic cancer.

Methods: The levels of granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) and the associated inflammatory marker C-reactive protein (CRP) were assessed in 38 patients receiving gemcitabine and capecitabine combination chemotherapy for advanced pancreatic cancer within the TeloVac trial. Apoptosis (M30) and total immune response (delayed-type hypersensitivity and/or T-cell response) were also assessed and levels of apoptosis induction correlated with immune response. The telomerase GV1001 vaccine was given either sequentially ($n=18$) or concomitantly ($n=24$) with the combination chemotherapy.

Results: There were no differences between baseline and post-treatment levels of CRP ($P=0.19$), IL-6 ($P=0.19$) and GM-CSF ($P=0.71$). There was a positive correlation between post-chemotherapy CRP and IL-6 levels ($r=0.45$, $P=0.005$) and between CRP with carbohydrate antigen-19-9 (CA19-9) levels at baseline ($r=0.45$, $P=0.015$) and post treatment ($r=0.40$, $P=0.015$). The change in CRP and IL-6 levels was positively correlated ($r=0.40$, $P=0.012$). Hazard ratios (95% CI) for baseline CA19-9 (1.30 (1.07–1.59), $P=0.009$) and CRP (1.55 (1.00–2.39), $P=0.049$) levels were each independently predictive of survival. The M30 mean matched differences between pre- and post-chemotherapy showed evidence of apoptosis in both the sequential ($P=0.058$) and concurrent ($P=0.0018$) chemoimmunotherapy arms. Respectively, 5 of 10 and 9 of 20 patients had a positive immune response but there was no association with apoptosis.

Conclusions: Combination gemcitabine and capecitabine chemotherapy did not affect circulating levels of GM-CSF, IL-6 and CRP. Chemotherapy-induced apoptosis was not associated with the immunogenicity induced by the GV1001 vaccine in advanced pancreatic cancer.

Immunotherapy is transforming the management of many cancers. Although single-agent checkpoint inhibition has been disappointing in pancreatic ductal adenocarcinoma (Topalian *et al*, 2012), it is clear that whole-cell vaccines can positively modulate microenvironmental immunity in this disease, upregulate PD-L1 expression (Lutz *et al*, 2014) and synergise with checkpoint blockade (Le *et al*, 2013; Soares *et al*, 2015). Gemcitabine and 5-fluorouracil (5-FU) are commonly used drugs in the management of advanced pancreatic cancer. A number of studies have demonstrated that instead of inhibiting an immune response

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against cancer cells as would be classically expected, these drugs can enhance certain facets of the anticancer immune response. Hence, combination treatment with immunotherapy to effect therapeutic synergy has been proposed and clinically tested (Middleton *et al*, 2014; Emens and Middleton, 2015).

The ultimate outcome of microenvironmental immunity is in part dependent on the balance between antigen-specific T cells and resident immunosuppressive cells, chief of these being the myeloid-derived suppressor cells (MDSCs). In mice harbouring a variety of different tumors, a single dose of gemcitabine significantly reduced the number of splenic MDSCs with no effect on the number of CD4+, CD8+ or B cells (Suzuki *et al*, 2005). In another preclinical study the only other drug, besides gemcitabine, that significantly reduced the number of MDSCs in the spleens and tumor beds of mice was 5-FU (Vincent *et al*, 2010). 5-Fluorouracil triggered dose-dependent apoptosis of MDSCs: thymidylate synthase levels in MDSCs were lower than in splenocytes or tumor cells. Adoptive transfer of MDSCs 1 day after 5-FU blunted the antitumour response.

The importance of tumor-infiltrating MDSCs in PDA was assessed in the KPC model, where a marked increase in Gr1+ CD11b+ cells (the murine MDSC phenotype) was seen during progression from pancreatic ductal intraepithelial neoplasia (PanIN) to invasive adenocarcinoma (Stromness *et al*, 2014). Granulocyte macrophage-colony-stimulating factor (GM-CSF) was upregulated by cancer cells and was a critical survival factor for MDSCs. MDSCs suppressed T-cell proliferation and induced apoptosis in activated T cells, whereas antibody-mediated depletion of MDSCs significantly increased the numbers of proliferating peritumoural CD8+ T cells. Depletion of MDSCs also affected the tumor stroma, resulting in areas of extracellular matrix depletion and enhanced vascular patency. These immunomodulatory and stromal effects were accompanied by a significant increase in tumor cell apoptosis. Knockdown of GM-CSF in an orthotopic model significantly reduced the growth of pancreatic adenocarcinoma, reduced Gr1+CD11b+ cell number and caused a pronounced accumulation of CD8+ T cells that drove tumour cell apoptosis (Pylayeva-Gupta *et al*, 2012). In a study examining the key cytokines in the generation of human MDSCs, the mixture of GM-CSF and interleukin-6 (IL-6) consistently generated MDSCs with the greatest capacity to inhibit autologous T-cell proliferation and IFN-γ production (Lechner *et al*, 2010). Pancreatic stellate cells (PSCs) secrete little GM-CSF but large quantities of IL-6, causing STAT-3 phosphorylation in human peripheral blood mononuclear cells (PBMCs) and thus inducing highly suppressive MDSCs, principally of granulocytic type (Mace *et al*, 2013). Inhibition of STAT-3 significantly reduced the generation of MDSCs when PBMCs were cultured with either PSC supernatants or IL-6/GM-CSF.

GM-CSF also activates JAK2 resulting in STAT-3 activation (Quelle *et al*, 1994). STAT3 is critical in mutant KRAS-induced PanIN formation and progression and the growth of established tumours (Corcoran *et al*, 2011). Both GM-CSF and IL-6 upregulate the inflammatory marker C-reactive protein (CRP) that contains a STAT-3 response element (Deng *et al*, 2006; Nishikawa *et al*, 2008). C-reactive protein is an independent prognostic marker in pancreatic cancer (Szkandera *et al*, 2014). The clinical importance of STAT-3 activation in advanced pancreatic cancer as a result of cancer-induced inflammation was recently demonstrated in a randomised study of second-line capecitabine with or without the JAK2 inhibitor ruxolitinib (Hurwitz *et al*, 2015). Using a cutoff CRP level at the median, there was a significant survival advantage for the addition of ruxolitinib in patients with CRP $>13\,mg\,l^{-1}$, but no benefit in those with CRP $<13\,mg\,l^{-1}$. The ongoing phase III study is including only patients with CRP $>10\,mg\,l^{-1}$. Given the interest in combining chemotherapy with immunotherapy, we thus investigated the impact of the combination of gemcitabine and the oral fluoropyrimidine capecitabine (GemCap) on CRP, GM-CSF and IL-6 levels to investigate whether these two agents modulate the immunosuppressive milieu of pancreatic adenocarcinoma.

We also analysed the relationship between the induction of apoptosis by GemCap as measured using the Apoptosense M30 assay and the development of an immune response to a class II telomerase peptide vaccine (GV1001), given either concomitantly with GemCap chemotherapy or in a sequential approach where 7 weeks of GemCap was followed by GV1001 in the TeloVac Study (Middleton *et al*, 2014). In preclinical models, gemcitabine-induced apoptosis increases antigen cross-presentation and primes the immune system (Nowak *et al*, 2003). Antigen released by apoptosis is available for cross-presentation rather than sequestered from the cross-presentation pathway. The induction of apoptosis is necessary as mice bearing gemcitabine-resistant cells demonstrated no significant difference in the proliferative activity of adoptively transferred antigen-specific lymphocytes when treated with gemcitabine compared with control. 5-Fluorouracil also enhances antigen cross-presentation (Galetto *et al*, 2003). The immunological impact of gemcitabine and 5-FU induced apoptosis in humans is unknown.

MATERIALS AND METHODS

Patients. Patients with advanced pancreatic cancer were recruited for translational studies participating in the TeloVac study (ISRCTN 43482138) (Middleton *et al*, 2014). Venous blood was collected from a subset of patients randomised to chemotherapy with sequential chemoimmunotherapy using the telomerase peptide vaccine GV1001 (Kael Gemvax, Seoul, Korea) or chemotherapy with concurrent chemoimmunotherapy using GV1001 for whom suitable samples were available for T-cell proliferation, CRP, IL-6, GM-CSF and M30 Apoptosense assays (Peviva AB, Strőskarlsva, Sweden). Objective tumor response was measured using RECIST (Therasse *et al*, 2000).

Chemoimmunotherapy. All patients were treated with combination gemcitabine and capecitabine. Gemcitabine was given intravenously at $1000\,mg\,m^{-2}$ weekly $\times 3$ every 4 weeks with orally administered capecitabine at $1660\,mg\,m^{-2}$ per day ($830\,mg\,m^{-2}$ twice daily) for 3 weeks followed by 1 week's rest. In the sequential chemoimmunotherapy arm, patients received an initial 7 weeks of chemotherapy followed by vaccination with GV1001 and GM-CSF (Penn Pharmaceutical Services, Tredegar, UK) as adjuvant as described previously (Middleton *et al*, 2014). In the concurrent chemoimmunotherapy arm, patients received chemotherapy together with vaccine delivered concomitantly from day 1 of therapy. All subjects provided written informed consent for the trial and inclusion criteria stipulated there was no history of autoimmune disease or recent steroid therapy.

DTH testing. Delayed-type hypersensitivity (DTH) testing was undertaken by giving a second intradermal injection of 0.105 mg of GV1001 given simultaneously at the contralateral site on the lower abdomen without concomitant GM-CSF. The site was examined 48 h later and a positive or negative response was recorded.

Blood sample collections. For patients in the sequential chemoimmunotherapy arm, blood was drawn just before the sixth and final gemcitabine infusion before vaccination and 48 h afterwards for the Apoptosense assay. Blood for CRP, IL-6 and GM-CSF assays was drawn before the start of therapy and before the sixth gemcitabine infusion. Peripheral blood mononuclear cells were

drawn at week 18 for T-cell proliferation assays after 10 weeks of GV1001 and GM-CSF administration.

For patients in the concurrent chemoimmunotherapy arm, blood was drawn just before the first gemcitabine infusion at the very start of therapy and 48 h afterwards for the Apoptosense assay. PBMCs were drawn at 10 weeks for T-cell proliferation assays, an equivalent period of vaccination before immunogenicity testing in patients receiving chemotherapy preceding vaccination in the sequential chemoimmunotherapy arm.

Storage and analyses of samples. Blood was drawn into CPT tubes (BD Biosciences, Oxford, UK) and spun at site to isolate PBMCs. The PBMCs were shipped to the biomarker repository at the Cancer Research UK Liverpool Clinical Trials Unit-GCP Laboratory Facility, UK. Viability and cell count of the PBMCs was measured using a ChemoMetec (Allerod, Denmark) Nucleo-Counter NC-100 before freezing in 90% DMSO and 10% human serum to $-80\,^{\circ}C$ overnight. The aliquots were then stored at $-150\,^{\circ}C$ for subsequent batch analysis. Blood for serum was collected into SST tubes (BD Biosciences) centrifuged at 1500 **g** for 10 min, aliquoted and stored at $-80\,^{\circ}C$.

IL-6 and GM-CSF analyses. The levels of IL-6 and GM-CSF along with 25 other analytes were measured in triplicate in patient serum using a commercially available Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay (Bio-Rad Laboratories Ltd, Hercules, CA, USA) on the Bio-Plex 200 System. We report here the analysis of the immunosuppressive cytokines pertinent to MDSC biology and CRP, a surrogate read-out for signalling by these cytokines. Details on the trajectory of the other cytokines have been previously presented (Neoptolemos *et al*, 2014) and a separate manuscript investigating the predictive value of these is in preparation. Initial data analysis was undertaken using Bio-Plex Manager 5.0 Software to determine concentrations. Serially diluted standards (50 μl) and test serum, diluted 1 in 4 in sample diluent (50 μl), was added to a plate containing magnetic antibody-coupled beads for each of the 27 analytes. The samples were incubated at room temperature on a plate shaker at 900 r.p.m. for 1 min followed by 300 r.p.m. for 30 min. Following washing with the Bio Plex Pro Magnetic Plate Washer, the secondary antibodies (25 μl) were added to the plate and incubated as before. The plate was washed again and streptavidin-PE (50 μl) was added and the plate incubated at room temperature on a plate shaker at 900 r.p.m. for 1 min followed by 300 r.p.m. for 15 min. Assay buffer (125 μl) was added to each well of the plate before being analysed on the Bio-Plex 200 machine. Fluorescent intensities obtained for the test samples were read from the standard curve to give pg ml^{-1} values for each of the 27 analytes.

CRP analysis. C-reactive protein was measured in serum samples at the Department of Clinical Biochemistry and Metabolic Medicine at the Royal Liverpool University Hospital (Liverpool, UK). The limit of detection for this assay is 5 mg l^{-1}.

M30 apoptosense analysis. Serum levels of M30 were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Peviva AB), and the results were expressed as U l^{-1} (see Dive *et al*, 2010 for details of CK18 evaluation in pancreatic ductal adenocarcinoma).

T-cell proliferation assay. Thawed PBMCs were seeded in X-VIVO 15 (Lonza, Slough, UK) supplemented with 10% pooled human serum (Innovative Research, Paisely, UK) at 2×10^6 cells per well in 48-well plates (Thermo Fisher Scientific, Asheville, NC, USA) and 20 µg ml^{-1} GV1001 peptide. Media were changed following 3 days of culture and interleukin-2 (Peprotech, London, UK) added to a final concentration of 10 units ml^{-1}. Restimulation was performed on day 11, and the GV1001-enriched cells were harvested and aliquoted into a round-bottom 96-well plate (50 μl, 1×10^5 cells per well). To the GV1001-enriched cells, irradiated (45 Gy) autologous PBMCs (50 μl, 1×10^5cells per well) were added to act as antigen-presenting cells. The GV1001-specific proliferation was tested for by the addition of 100 μl of control media, media containing 20 μg ml^{-1} GV1001 or 5 μg ml^{-1} phytohaemagglutinin (PHA). After 48 h of incubation, ^{3}H-thymidine (1 μCi per well) was added for 16 h before harvesting and counting. A stimulation index above 1·8 with a significant difference in counts per min from four replicates was defined as a positive proliferative response to GV1001.

Positive total immune response. This was defined as a positive DTH test and/or a positive proliferation assay.

Statistical methods. Survival analyses were undertaken using Cox proportional hazards regression and the Kaplan–Meier method. Univariate analyses were carried out for patient characteristics, baseline levels of carbohydrate antigen 19-9 (CA19-9), CRP, IL-6 and GM-CSF dichotomised at median values; GM-CSF was dichotomised into present and absent. Multivariate analysis was carried out using a stepwise method. The Mann–Whitney test was used to analyse dichotomised continuous variables and Fisher's exact for categorical data, and the Wilcoxon test was used for paired analysis.

RESULTS

The trajectory of serum CRP, IL-6 and GM-CSF levels during treatment with GemCap chemotherapy was determined in 38 patients for whom the appropriate longitudinal blood samples were available, and who were treated on the sequential chemoimmunotherapy arm of the TeloVac trial, where patients received 7 weeks of chemotherapy before vaccination with GV1001. The level of these analytes was assessed at baseline and after 7 weeks of chemotherapy, before vaccination with GV1001, at a time that coincided with the first assessment of response by CT scan. The demographics of this subgroup of patients (Table 1) were representative of the patients randomly allocated to sequential chemoimmunotherapy in the TeloVac trial ($n = 350$, median age 64, proportion of males 58%, proportion of locally advanced patients 30%, patients with ECOG performance status 0, 1 and 2, 29%, 60% and 11% respectively; Middleton *et al*, 2014). The median overall survival of the 38 patient subgroup was 7.6 months and baseline median CA19.9 was 961 kU l^{-1}, and of all 350 patients randomised to sequential chemoimmunotherapy, it was 6.9 months and 933 kU l^{-1} respectively (Middleton *et al*, 2014). Table 2 shows the levels of CRP (mg l^{-1}), IL-6 (pg ml^{-1}) and GM-CSF (pg ml^{-1}) pre- and post-GemCap chemotherapy, the percentage change and the radiological response to treatment.

Paired analysis of the log-transformed data showed that there were no significant differences between baseline and post-treatment CRP levels (Wilcoxon signed rank test $P = 0.19$), IL-6 (Wilcoxon signed rank test $P = 0.19$) and GM-CSF levels (McNemar's test $P = 0.71$). There was a significant positive correlation (Spearman's rho) between post-chemotherapy CRP and IL-6 levels ($r = 0.45$, $P = 0.005$) and between both baseline and post-treatment CRP with CA19-9 levels ($r = 0.45$, $P = 0.015$ and $r = 0.40$, $P = 0.015$ respectively). The change in CRP and IL-6 levels was positively correlated ($r = 0.40$, $P = 0.012$). When analysing only the 28 patients with stable disease, again there was no significant difference between pre- and post-chemotherapy CRP levels (Wilcoxon signed rank test $P = 0.4$) and IL-6 (Wilcoxon signed rank test $P = 0.32$). The change in CRP and IL-6 levels in patients with stable disease was also positively correlated ($r = 0.40$, $P = 0.033$).

Table 2 shows that the CRP levels were at or below the limit of detection (5 mg l^{-1}) in 16 out of 38 (42.1%) patients before

Table 1. Demographic features of 38 patients including summary of the IL-6, CRP and GM-CSF levels pre- and post-chemotherapy

Characteristic	$N = 38$
Age	
(years, median, range)	64.0 (44–79)
Gender	
(male/female)	24 : 14 (63% : 37%)
Survival (based on KM)	
(days, median, IQR)	232 (150–366)
Stage	
Locally advanced	16 (42%)
Metastatic	22 (58%)
ECOG performance status	
0	12 (32%)
1	21 (55%)
2	5 (13%)
Baseline CA19-9	
($kU\,l^{-1}$, median, range)	961 (10–53 064)
Baseline CRP	
($mg\,l^{-1}$, median, range)	7 (5–238)
Post-treatment CRP	
($mg\,l^{-1}$, median, range)	10 (5–175)
Baseline IL-6	
($pg\,ml^{-1}$, median, range)	23 (9–1163)
Post-treatment IL-6	
($pg\,ml^{-1}$, median, range)	18 (3–914)
Baseline GM-CSF	
($pg\,ml^{-1}$, median, range)	0 (0–549)
Post-treatment GM-CSF	
($pg\,ml^{-1}$, median, range	0 (0–427)

Abbreviations: CA19-9 = carbohydrate antigen-19-9; CRP = C-reactive protein; ECOG = Eastern Cooperative Oncology Group; GM-CSF = granulocyte macrophage-colony-stimulating factor; IL-6 = interleukin 6; IQR = interquartile range; KM = Kaplan–Meier.

chemotherapy and in 12 out of 38 patients (31.6%) following 7 weeks of chemotherapy. The majority of patients with a normal CRP level after chemotherapy had normal CRP levels to start with, but in just under a half of patients with initially normal CRP levels the level went up with chemotherapy. Nine out of the 12 (75%) patients with CRP levels at or below the limit of detection after chemotherapy also had had CRP levels at or below the limit of detection before chemotherapy. In 7 of the 16 patients (43.8%) who had CRP levels at or below the limit of detection before chemotherapy, the CRP level increased on chemotherapy.

A total of 14 patients had a CRP level $>10\,mg\,l^{-1}$ at baseline and this increased to 19 patients after chemotherapy. There were 10 patients with a CRP level $\leqslant 10\,mg\,l^{-1}$ at baseline that increased to $>10\,mg\,l^{-1}$ following chemotherapy and there were 9 patients with a CRP level $>10\,mg\,l^{-1}$ both pre- and post-chemotherapy (Figure 1). Seven out of 10 patients with a CRP level $\leqslant 10\,mg\,l^{-1}$ at baseline and which had increased to $>10\,mg\,l^{-1}$ during chemotherapy had partial response or stable disease as objective response to chemotherapy. In only 4 patients was the CRP level $>10\,mg\,l^{-1}$ at baseline and $\leqslant 10\,mg\,l^{-1}$ after chemotherapy, and in all of these the IL-6 level fell. If the cut-point of CRP $>13\,mg\,l^{-1}$ was applied as used previously in a randomised study (Brandt *et al*, 2010), 10 patients had elevated CRP levels before chemotherapy and this increased to 17 patients after chemotherapy.

The GM-CSF was detectable in the serum of only 9 out of 38 patients at baseline and 10 out of 38 after chemotherapy. Investigation of positive or negative GM-CSF at baseline and post treatment showed no significant association with response (progressive disease *vs* stable disease) or stage (locally advanced *vs* metastatic).

In summary, there was no evidence that gemcitabine and capecitabine combination chemotherapy reduced the level of clinically meaningful inflammatory markers in patients with advanced pancreatic ductal adenocarcinoma.

Univariate analysis of the impact on survival of clinical features along with logged CRP, IL-6 and GM-CSF levels is shown in Table 3. Objective tumor response ($P = <0.0001$), log CA19-9 ($P = 0.004$) and log baseline CRP ($P = 0.006$) were associated with survival. In a multivariate model, logged CA19-9 and CRP both at baseline were each independently predictive of survival with hazard ratios (95% CI) for baseline CA19-9 levels of 1.30 (1.07–1.59), ($P = 0.009$) and CRP levels of 1.55 (1.00–2.39) ($P = 0.049$).

The results of the M30 Apoptosense assay before and 48 h after chemotherapy, along with percentage change and immune response status, is shown for 42 patients in Table 4. Three out of 18 (16.7%) patients in the sequential chemoimmunotherapy arm and 7 out of 24 (29.2%) patients in the concurrent chemoimmunotherapy had $>30\%$ change in the M30 assay (two-tailed Fisher's exact $P = 0.473$). If a more conservative 10% cutoff is used to infer induction of apoptosis, this increased to 9 out of 18 (50%) and 17 out of 24 (70.8%) respectively (two-tailed Fisher's exact $P = 0.209$). The logged M30 data for mean matched differences between pre- and post-chemotherapy for the sequential and concurrent chemoimmunotherapy treatment arms showed evidence of apoptosis ($P = 0.058$ and $P = 0.0018$, respectively).

The median (95% CI) survival for the 10 patients with apoptosis defined as an M30 $>30\%$ rise was 253 (92–304) days compared with 344 (216–443) days for those 32 patients with an M30 $<30\%$ rise (log-rank $\chi^2 = 3.4015$, $P = 0.065$) with a hazard ratio (95% CI) of 0.50 (0.23–1.06) ($\chi^2 = 3.26$, $P = 0.071$). The median (95% CI) survival for the 26 patients with apoptosis defined as an M30 $>10\%$ rise was 295 (216–399) days compared with 344 (187–527) days for those with an M30 $<10\%$ rise (log-rank $\chi^2 = 0.707$, $P = 0.401$) with a hazard ratio (95% CI) of 0.75 (0.38–1.47) ($\chi^2 = 0.70$, $P = 0.403$).

In all, 5 of 10 patients in the sequential chemoimmunotherapy arm and 9 of 20 patients in the concurrent chemoimmunotherapy had a positive immune response (Table 5). There was no association between an apoptotic response 48 h following chemotherapy and a positive immune response irrespective of whether a cutoff of $>30\%$ or $>10\%$ increase in M30 levels was used (Table 5). Thus, 7 out of 9 immune responders in the concurrent chemoimmunotherapy arm and 4 out of 5 responders in the sequential chemoimmunotherapy arm had no evidence of apoptosis induction using a 30% cutoff on the M30 Apoptosense assay.

DISCUSSION

This study has shown that combination gemcitabine and capecitabine therapy did not reduce CRP, IL-6 or GM-CSF levels in patients with advanced pancreatic cancer. Moreover, apoptosis secondary to chemotherapy did not correlate with enhanced immunogenicity of GV1001. We have previously shown that a combination of gemcitabine and the oral fluoropyrimidine capecitabine (GemCap) failed to reduce the levels of circulating MDSCs in patients with advanced pancreatic cancer independent of response (Annels *et al*, 2014). We now show that serum levels of the two main cytokines that drive the production of MDSCs in pancreatic cancer, GM-CSF and IL-6, did not significantly fall during treatment with GemCap. The accompanying lack of a fall in

Table 2. Serum CRP, IL-6 and GM-CSF levels in advanced pancreatic cancer patients pre- and post-chemotherapy treatment and radiological response ($n = 38$)

	CRP (mg l^{-1})		CRP	IL-6 (pg ml^{-1})		IL-6	GM-CSF (pg ml^{-1})		GM-CSF
Radiological response	Pre	Post	% Change	Pre	Post	% Change	Pre	Post	% Change
PD	6	38	533.33	23.52	33.07	40.6	0	0	0
PD	27	16	−40.74	25.59	14.98	−41.46	0	0	0
PD	5	5	0	20.98	21.91	4.43	0	0	0
PD	38	31	−18.42	22.88	56.91	148.73	0	0	0
PD	60	48	−20	10.52	13.95	32.6	0	0	0
PD	8	11	37.5	36.53	17.91	−50.97	4.51	0	−100
PD	16	5	−68.75	21.15	7.96	−62.36	72.43	34.12	−52.89
PD	5	24	380	30.49	50.11	64.35	0	34.96	0
PD	238	175	−26.47	34.19	33.27	−2.69	0	0	0
PR	8	25	212.5	9.44	2.88	−69.49	0	0	0
SD	5	20	300	28.91	19.03	−34.18	0	0	0
SD	11	7	−36.36	23.84	22.63	−5.08	0	51.61	100
SD	5	9	80	41.9	23.39	−44.18	0	0	0
SD	5	5	0	20.06	17.87	−10.92	0	0	0
SD	7	60	757.14	37.56	57.93	54.23	0	0	0
SD	7	27	285.71	21.56	403.25	1770.36	0	0	0
SD	34	21	−38.24	1163.43	914.06	−21.43	549.41	426.57	−22.36
SD	5	10	100	9.49	13.05	37.51	2.88	0	−100
SD	13	29	123.08	36.55	17.85	−51.16	30.4	0	−100
SD	5	17	240	35.82	50.34	40.54	0	0	0
SD	10	8	−20	24.17	20.11	−16.8	0	0	0
SD	25	6	−76	29.31	11.81	−59.71	0	0	0
SD	92	37	−59.78	49.65	24.72	−50.21	0	0	0
SD	5	5	0	10.12	10.28	1.58	0	0	0
SD	5	5	0	50.89	125.54	146.69	0	109.01	0
SD	5	5	0	10.28	15.74	53.11	0	0	0
SD	41	5	−87.8	19.63	12.29	−37.39	0	0	0
SD	27	33	22.22	24.3	38.6	58.85	0	0	0
SD	5	5	0	20.39	12.45	−38.94	0	0	0
SD	13	13	0	24.91	12.61	−49.38	11.08	2.41	−78.25
SD	6	9	50	10.73	14.38	34.02	0	0	0
SD	5	28	460	14.01	19.4	38.47	6.28	10.27	63.54
SD	5	5	0	21.08	19.73	−6.4	24.34	7.92	−67.46
SD	11	10	−9.09	19.4	7.23	−62.73	1.68	0.28	−83.33
SD	5	5	0	22.9	11.4	−50.22	0	0	0
SD	5	26	420	15.36	17.75	15.56	0	17.73	0
SD	9	5	−44.44	23.45	18.71	−20.21	0	0	0
SD	5	5	0	14.09	9.37	−33.5	0	0	0
PD median (IQR)	16 (5.5 to 49)	24 (8 to 43)	−18.4 (−33.6 to 209)	23.5 (21 to 32.3)	21.9 (14.5 to 41.7)	4.4 (−46 to 52.5)	Yes = 2 No = 7	Yes = 2 No = 7	Yes = 2 No = 7
SD median (IQR)	5.5 (5 to 12)	9 (5 to 23.5)	0 (−14.5 to 111)	23.2 (17.4 to 32.6)	18.3 (12.5 to 24.1)	−13.9 (−41.6 to 38)	Yes = 7 No = 21	Yes = 8 No = 20	Yes = 8 No = 20
All median (IQR)	7 (5 to 16)	10.5 (5 to 27)	0 (−20 to 123)	23.2 (19.4 to 30.4)	18.3 (12.6 to 33.1)	−8.66 (−8.7 to 38.5)	Yes = 9 No = 29	Yes = 10 No = 28	Yes = 10 No = 28

Abbreviations: CRP = C-reactive protein; GM-CSF = granulocyte macrophage-colony-stimulating factor; IL-6 = interleukin 6; IQR = interquartile range; PD = progressive disease; PR = partial response; SD = stable disease.

CRP is to be expected given that CRP is a transcriptional target of GM-CSF and IL-6 signalling (Deng *et al*, 2006; Nishikawa *et al*, 2008). Thus, the use of gemcitabine and fluoropyrimidines as positive immunomodulators alongside immunotherapies in pancreatic cancer must take into account the failure of these chemotherapy agents to affect these key immunosuppressive cytokines, although caution is needed in interpreting our results, as we do not know whether circulating IL-6 and GM-CSF levels

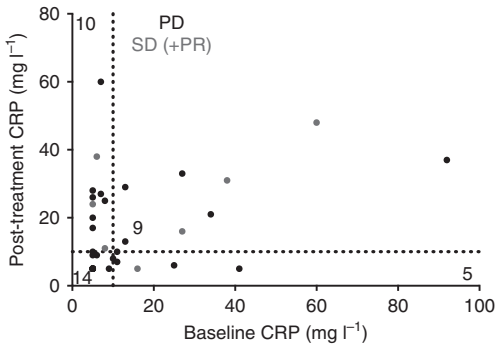

Figure 1. The CRP baseline levels plotted against the post-GemCap CPR levels. Indicated are the number of patients with $>10\,mg\,l^{-1}$ or $<10\,mg\,l^{-1}$ levels pre- and post-GemCap treatment. PD = progressive disease; PR = partial response; SD = stable disease.

Table 3. Univariate and multivariate analyses of the impact on survival of clinical features along with logged CRP, IL-6 and GM-CSF levels

Univariate	Hazard ratio (95% CI)	*P*-value
Response: PD v SD/PR	**8.61 (2.97–24.98)**	**<0.0001**
Age	0.98 (0.95–1.02)	0.40
Stage: locally advanced *vs* metastatic	0.66 (0.32–1.37)	0.26
ECOG 0 v 1	1.02 (0.45–2.28)	
ECOG 1 v 2	0.42 (0.14–1.26)	0.22
ECOG 0 v 2	0.41 (0.15–1.17)	
Log CA19-9: baseline	**1.33 (1.10–1.61)**	**0.004**
Log CRP: baseline	**1.76 (1.18–2.65)**	**0.006**
Log CRP: post treatment	1.38 (0.89–2.13)	0.16
Log CRP: difference	0.75 (0.46–1.23)	0.25
Log IL-6: baseline	0.73 (0.47–1.14)	0.16
Log IL-6: post treatment	0.84 (0.61–1.15)	0.27
Log Il-6: difference	1.05 (0.64–1.75)	0.84
GM-CSF: baseline	0.75 (0.33–1.71)	0.50
GM-CSF: post treatment	0.81 (0.36–1.81)	0.60
Multivariate		
Log CA19-9: baseline	**1.30 (1.07–1.59)**	**0.009**
Log CRP: baseline	**1.55 (1.00–2.39)**	**0.049**

Abbreviations: CA19-9 = carbohydrate antigen-19-9; CI = confidence interval; CRP = C-reactive protein; ECOG = Eastern Cooperative Oncology Group; GM-CSF = granulocyte macrophage-colony-stimulating factor; IL-6 = interleukin 6; PD = progressive disease; PR = partial response; SD = stable disease. The bold values are statistically significant.

reflect levels in the tumor microenvironment. We examined the effect of combined gemcitabine and capecitabine on IL-6, GM-CSF and CRP. Two other regimens are now commonly used in advanced pancreatic cancer: gemcitabine and nab-paclitaxel and FOLFIRINOX. We are not aware of any data looking at the sequential effects of cytokines with these regimens. However, the cytokines we investigated were selected based on their importance to MDSC biology in pancreatic cancer, and there are data on the impact of the other chemotherapy agents used in these regimens on MDSCs. Unlike gemcitabine and 5-FU, paclitaxel and oxaliplatin had no effect on tumoural MDSC numbers in preclinical models (Vincent *et al*, 2010). Indeed, there was a numerical increase in MDSCs with oxaliplatin. Irinotecan increased MDSC number, increased MDSC NO^- and ROS production and blocked the apoptotic effect of 5-FU on MDSCs in a colorectal cancer model (Kanterman *et al*, 2014). These data suggest that these other combination regimens are unlikely to have a beneficial effect on MDSCs independent of any significant reduction in tumour volume and in the case of FOLFIRINOX may be detrimental. We plan to prospectively examine the effects of FOLFIRINOX on MDSC number and function to test this hypothesis.

Table 4. Serum levels of M30 (apoptosense) in patients pre- and post-chemotherapy along with the percentage change and immune response status: sequential chemoimmunotherapy *n* = 18, concurrent chemo-immunotherapy (*n* = 24)

Pre-chemotherapy M30 ($U\,l^{-1}$)	Post-chemotherapy M30 ($U\,l^{-1}$)	M30 % change	Immune response
Sequential chemoimmunotherapy			
Median (IQR) = 230.12 (185.3–406.1)	**Median (IQR) = 236.88 (188.3–481.5)**	**Median (IQR) = 11.77 (−6.7–23.5)**	
406.09	373.59	−8	Negative
159.42	188.32	18.13	NA
185.27	219.15	18.29	Negative
133.71	131.53	−1.63	Positive
176.79	186.36	5.41	Negative
182.35	218.93	20.06	Positive
249.66	232.95	−6.69	Positive
236.27	240.81	1.92	Positive
264.74	660.32	149.42	Positive
451.13	388.79	−13.82	NA
505.93	745.08	47.27	Negative
337.78	417.17	23.50	Negative
411.02	504.14	22.66	NA
193.31	481.53	149.10	NA
188.39	158.97	−15.62	NA
219.87	214.43	−2.47	NA
223.96	184.02	−17.83	NA
651.78	832.69	27.76	NA
Concurrent chemoimmunotherapy			
Median (IQR) = 235.81 (184.7–352.0)	**Median (IQR) = 336.66 (517.3–228.1)**	**Median (IQR) = 14.34 (5.4–36.0)**	
214.54	243.00	13.26	Negative
1046.50	1199.07	14.58	NA
340.53	326.21	−4.21	Positive
284.29	312.52	9.93	Positive
239.17	240.03	0.36	Negative
293.62	347.11	18.22	Negative
568.56	788.15	38.62	Negative
346.60	411.60	18.75	Positive
166.10	487.64	193.58	Positive
184.64	209.04	13.22	Negative
148.43	164.12	10.57	Negative
539.93	525.25	−2.72	NA
357.34	360.64	0.92	Negative
184.70	223.56	21.04	Negative
171.26	509.29	197.37	Positive
176.86	226.19	27.89	Positive
180.47	634.77	251.73	NA
203.97	195.89	−3.96	Positive
232.44	265.19	14.09	Positive
205.12	229.96	12.11	Positive
650.12	1072.13	64.91	Negative
281.48	375.23	33.31	NA
806.24	1193.86	48.08	Negative
191.15	177.42	−7.18	Negative

Abbreviations: IQR = interquartile range; NA = not available.

Table 5. Number of immune responders in sequential and concurrent chemoimmunotherapy split by apoptosis

	Sequential chemoimmunotherapy arm			Concurrent chemoimmunotherapy arm	
	Immune response[a]			Immune response[a]	
Apoptosis[b]	**Yes ($n=5$)**	**No ($n=5$)**	**Apoptosis[b]**	**Yes ($n=9$)**	**No ($n=11$)**
Yes ($n=2$)	1	1	Yes ($n=5$)	2	3
No ($n=8$)	4	4	No ($n=15$)	7	8
Apoptosis[c]	**Yes ($n=5$)**	**No ($n=5$)**	**Apoptosis[c]**	**Yes ($n=9$)**	**No ($n=11$)**
Yes ($n=5$)	2	3	Yes ($n=14$)	6	8
No ($n=5$)	3	2	No ($n=6$)	3	3
Two-tailed Fisher's exact test	$P=1.00$			$P=1.00$	

[a]Defined as T-cell proliferative response or positive delayed-type hypersensitivity (DTH).
[b]Defined as ⩾30% increase in M30 value at 48 h.
[c]Defined as ⩾10% increase in M30 value at 48 h.

The number of patients included was relatively small but the demographics and outcomes of this subset was similar to the entire group of patients treated with initial chemotherapy alone before vaccination and there was no suggestion of a possible tendency towards reduction in the analytes during chemotherapy. The median CRP levels were numerically higher after chemotherapy even in patients with stable disease and there were more patients with elevated CRP after treatment than before. Although there was no control group to investigate changes independent of chemotherapy, obtaining sufficient sequential samples from patients with advanced pancreatic cancer is clearly problematic.

The biological basis of our clinical observation that gemcitabine and fluoropyrimidines do not reduce IL-6 and GM-CSF has been recently demonstrated (Takeuchi *et al*, 2015). Gemcitabine and 5-FU treatment of pancreatic cancer cells significantly increased the production of IL-6 and GM-CSF by these cells. Human monocytes differentiated into monocytoid MDSCs when conditioned media from pancreatic cancer cells were added to the culture medium and conditioned media from pancreatic cancer cells treated with either gemcitabine or 5-FU reduced HLA-DR expression and enhanced Arginase1 and NOS2 expression even further. The MDSCs derived from chemotherapy-treated cell conditioned media were even more suppressive than those derived using untreated conditioned media. The expression of HLA-DR on infiltrating CD14+ cells was much lower in the pancreatic cancers of patients receiving preoperative chemotherapy as compared with those going straight to surgery.

Immunomonitoring in the TeloVac trial involved both DTH responses and T-cell proliferation. In all, 19 (12%) of 154 patients who had sequential chemoimmunotherapy were positive to DTH, and 47 (20%) of 233 patients who had concurrent chemoimmunotherapy (Middleton *et al*, 2014). T-cell proliferation was positive in 10 (31%) of 32 patients given sequential chemoimmunotherapy and 10 (15%) of 68 patients given concurrent chemoimmunotherapy. Both DTH and T-cell responses were not predictive of survival. Chemotherapy-mediated apoptosis induction was not significantly associated with the immune responsiveness to the GV1001 peptide vaccine. It may not be possible to enhance immunogenicity through apoptosis-mediated increased antigen cross-presentation if the microenvironment remains unfavourable with high levels of immunosuppressive cytokines such as IL-6 and GM-CSF.

Caspase-mediated apoptosis will cause cleavage of cytokeratin 18 (released following necrosis of malignant and normal epithelial cells) producing the M30 fragment. This cleavage product is not specific to cancer cells undergoing apoptosis either as part of tumor biology or induced by chemotherapy (Dive *et al*, 2010). Previously, we found no association with survival and circulating M30 levels in a range of patients with early, locally advanced and metastatic pancreatic cancer and similar findings were made in the present study (Dive *et al*, 2010). A two-fold increase over the baseline spread of M30 assay values has been proposed (Cummings *et al*, 2006) but we only had a single baseline sample. Others have proposed lower level changes as important given the association between changes and objective response based on ROC characteristics (Brandt *et al*, 2010).

The majority of the patients for whom we had samples for the M30 assay were in the group receiving concomitant chemotherapy at the time of vaccination, and this may have reduced the level of circulating antigen-specific T cells as has been described with gemcitabine therapy preclinically (Bauer *et al*, 2014). In addition, any intratumoral release of telomerase peptides secondary to apoptosis induction may also have been insufficient to immunologically synergise with the exogenously administered antigen in the vaccine.

Using a variety of chemotherapies and tumour antigens (both peptides and proteins), Kang *et al* (2013) demonstrated therapeutic synergy and enhanced numbers of intratumoural and systemic antigen-specific CD8+ T cells when chemotherapy and vaccination were combined in comparison with single-agent therapy. However, antigen density within the tumour was critical: synergy only occurred when antigen was delivered directly into the tumour and did not occur when antigen was delivered by the standard subcutaneous route. Chemotherapy alone enhanced intratumoural dendritic cell density but this effect was only therapeutically and immunologically relevant if there were very high levels of antigen available for uptake and this could only be achieved by direct intratumoural delivery. Chemotherapy-induced apoptosis and the development of an immunologically more favourable microenvironment were insufficient to synergise with antigen delivery by a standard route.

In conclusion, we have shown that combination gemcitabine and capecitabine chemotherapy did not reduce the levels of the immunosuppressive cytokines IL-6 and GM-CSF or the inflammatory marker CRP. Furthermore, there was no evidence that apoptosis induction secondary to this chemotherapy significantly enhanced the immunogenicity of an intradermally administered peptide vaccine. These observations may have implications for the use of gemcitabine and fluoropyrimidines as immunomodulatory agents in pancreatic cancer. Alternative methods to block the action of immunosuppressive cytokines are required. Both IL-6 and GM-CSF function via activation of JAK2/STAT3, and the direct inhibition of JAK2 using ruxolitinib has improved outcome in pancreatic cancer patients treated with gemcitabine precisely in those with an elevated CRP reflecting STAT3 activation (Hurwitz *et al*, 2015). The phase III confirmation of these data is eagerly awaited.

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CONFLICT OF INTEREST

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