The Effects of Gemcitabine and Capecitabine combination chemotherapy on myeloid derived suppressor cells in patients with advanced pancreatic cancer.

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ABSTRACT

In pre-clinical models the only two chemotherapy drugs which have been demonstrated to directly reduce the number of myeloid derived suppressor cells (MDSCs) are gemcitabine and 5-fluorouracil (5-FU). Here we analyse the dynamics of MDSCs, phenotyped as Lin-DR-CD11b+, in patients with advanced pancreatic cancer receiving the combination of gemcitabine and capecitabine, a 5-FU pro-drug. Gemcitabine and capecitabine reduced MDSCs in 58% of patients (n = 19). MDSC% fell in 3/9 patients with above-median baseline MDSCs, conversely in 5/8 patients with minimal tumour volume change on treatment the MDSC% went up. Increases in MDSC% in patients with unchanged tumour volume appeared to correlate with sustained cancer-related inflammatory cytokine up-regulation. Thus, we found no evidence that gemcitabine and capecitabine directly reduces MDSC% in patients: increased tumour burden or sustained cancer-related inflammation during chemotherapy are likely to be associated with increased MDSC levels. In a separate cohort of 21 patients treated with gemcitabine and capecitabine together with a concurrently administered telomerase vaccine, the MDSC level fell in 8 of the 9 patients who developed an immune response to the vaccine. 6 of these patients had above-median pre-vaccination MDSC levels. Thus, a high pre-vaccination MDSC% does not preclude development of immunity to a tumour-associated antigen. This data suggests that antigen-immunity is more likely to develop when a cancer vaccine is administered in the context of adjunctive treatment which actively checks the progress of increasing immunosuppression which occurs with increasing disease volume and/or sustained levels of cancer-related inflammation.

Keywords: Myeloid derived suppressor cell, Chemotherapy, Gemcitabine, Capecitabine, Pancreatic Cancer
This paper demonstrates no evidence that Gemcitabine and Capecitabine directly reduce MDSC% in patients. Any decrease in MDSC levels is likely to reflect predominantly the effect of the resultant disease control.
INTRODUCTION

MDSCs are a heterogeneous family of immature myeloid cells arrested in their differentiation program by a variety of tumor-secreted factors. MDSCs inhibit the activity of CTLs in a variety of ways: high levels of intracellular arginase in MDSCs depletes the cellular micro-environment of arginine, an essential amino acid for T-cell activation [1]; uptake and depletion of cystine by MDSCs depletes T cells of a further amino acid required for T-cell activation [2]; MDSC-mediated down-regulation of L-selectin, a molecule that targets T-cells to lymph nodes [3]; the production by MDSCs of the free radical peroxynitrite, which causes the nitration/nitrosylation of the T-cell receptors and CD8 molecules of CTLs, thus preventing their recognition of the peptide-MHC complex on tumour cells [4]. This latter suppressive mechanism has been recently described to operate at the level of the cancer cell where MDSC production of peroxynitrite causes tyrosine nitrosylation of the MHC class I molecules on tumor cells thus preventing the binding of peptide epitopes [5].

Chemotherapy remains the standard treatment for inoperable pancreatic cancer, and various data suggest that two of the main chemotherapeutic agents used in this disease may impact beneficially on MDSCs. In a pivotal paper, mice harboring a variety of different tumors were treated with a single dose of Gemcitabine and the impact on the percentage of splenic Gr-1+/CD11b+ cells was assessed [6]. Gemcitabine significantly reduced the number of splenic MDSCs at 48 hours. This effect was specific: the numbers of CD4+, CD8+ and B cells were not affected. This quantitative effect was functionally important. When splenocytes from animals bearing large tumors were added to a mixture of tumour cells and CTLs in a modified Winn assay the growth inhibitory effects of the CTLs was lost. The addition of an equal number of splenocytes from tumour bearing animals treated 48 hours earlier with Gemcitabine had no such effect and the added CTLs maintained their suppressive activity on tumour growth. Vincent and colleagues confirmed this effect: 5 days following the administration of Gemcitabine there was significant reduction in the percentage of CD11b+ MDSCs in the spleens and tumour beds of tumour-bearing mice [7]. Whilst Cyclophosphamide, Doxorubicin, Oxaliplatin and Paclitaxel had no such effect, 5-FU also significantly reduced the percentage of
MDSCs and to a greater degree than Gemcitabine. Again this cytoreductive effect was MDSC-specific. The tumour growth suppressive effects of Gemcitabine and 5-FU both depended on the presence of T cells and the implication from this work was that the chemotherapy effect on MDSCs allowed the restoration of T-cell dependent anti-tumour responses.

These pre-clinical data raise the critical question as to whether chemotherapy, and in particular Gemcitabine and 5-FU, also results in a reduction in MDSCs in patients? We are aware of two clinical studies which address the effects of chemotherapy on MDSC numbers [8, 9]. In the first where the chemotherapy was not specified, MDSC numbers tracked with tumor volume in 6 patients, with numbers falling with response and increasing with progression. Similarly, in 25 patients with advanced colorectal cancer, MDSCs were significantly higher (and increased from baseline) in patients with progressive disease compared with those with a response to chemotherapy. Again, the regimen was not specified. These studies suggested that any effects on MDSCs from chemotherapy were likely to be related to changes in tumour volume rather than specific effects of chemotherapy on MDSCs per se.

We have recently demonstrated significant elevations of MDSCs in patients with pancreatic cancer, and in multivariate analysis MDSC levels were shown to be an independent prognostic factor for survival [10]. We here prospectively analyze the effect of the administration of the combination of Gemcitabine and Capecitabine, a 5-FU pro-drug, on MDSC levels in pancreatic cancer to try to answer two questions.

1. Does the combination of gemcitabine and capecitabine, the only two chemotherapy agents which pre-clinically have been shown to reduce MDSC levels, have an effect on MDSC levels in patients with cancer, and if so does this effect appear to be independent of tumour response?

2. Does any quantitative effect of gemcitabine and capecitabine on MDSCs have any impact at a functional level?

Pre-clinical data demonstrates that the reduction of MDSCs enhances the efficacy of
cancer immunotherapy [11]. Furthermore, pre-vaccination levels of MDSCs appear to have a significant impact on the immunogenicity of cancer vaccines in humans [12]. Thus, we examined the relation between changes in MDSC level and the development of an immune response to a cancer vaccine (GV1001) administered concomitantly with gemcitabine and capecitabine. GV1001 is a promiscuous class II peptide vaccine against human telomerase. In the phase I/II study of GV1001 in pancreatic cancer using the doses utilized in the current study the overall immune response to vaccine alone (proliferation positive and/or DTH positive) was 75% [13].

We demonstrate firstly that there does not appear to be a consistent effect of gemcitabine and capecitabine on MDSC levels independent of the effect of treatment on tumour bulk. Secondly, a high pre-vaccine MDSC level does not preclude an immune response to a cancer vaccine. However, in patients with high baseline MDSC levels who mount an immune response to a cancer vaccine delivered concomitantly with chemotherapy, there is generally a fall in MDSC levels but again this fall appears to be mainly as a result of disease stabilization or response secondary to chemotherapy.
METHODS

Patients

20-30 mL of venous blood was collected from pancreatic cancer patients (n=40) participating in the TeloVac study (ISRCTN 43482138) and age/sex matched normal healthy controls (n=24). Age and sex matched healthy controls were recruited from surgical minor operation clinics at the Royal Surrey County Hospital, UK. The final results of TeloVac will be reported separately: the patients described herein represent a small sample of patients randomized and for whom suitable samples were available for sequential MDSC analysis. No subject had a history of autoimmune disease or of recent steroid therapy and no control donor had a prior history of cancer. All subjects provided written informed consent approved by the local human investigators committee. All patients were treated with combination gemcitabine and capecitabine chemotherapy: gemcitabine was given intravenously at 1,000 mg/m$^2$ weekly × 3 every 4 weeks together with capecitabine administered orally at 1,660 mg/m$^2$/d (830 mg/m$^2$ twice daily) for 3 weeks followed by 1 week's rest. 19 patients received combination chemotherapy alone. 21 patients received the class II hTERT vaccine GV1001 concurrently with the same chemotherapy, given according at the previously published dose and schedule [13].

Peripheral blood samples were taken prior to and following chemotherapy. For patients receiving gemcitabine and capecitabine alone blood was drawn after seven weeks of treatment, one week following the fifth gemcitabine infusion, immediately prior to the sixth gemcitabine infusion and whilst the patient was taking capecitabine. For patients receiving gemcitabine and capecitabine concomitantly with GV1001, blood was drawn after 10 weeks of treatment to co-incide with an immunomonitoring time-point, one week after the seventh gemcitabine infusion immediately prior to the eighth gemcitabine infusion and whilst the patient was taking capecitabine. Blood was drawn into li-heparin tubes (BD Biosciences, Europe) or CPT tubes for shipment to the biomarker repository. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque gradients. PBMC were counted, frozen at -80°C and stored in liquid N2 for subsequent batch-analysis.
Immunophenotypic analysis of cells

PBMC were recovered and washed in 0.15M phosphate buffered saline, Dulbecco’s A (Oxoid, UK). Cells were aliquoted for MDSC analysis. The LIVE/DEAD Cell Stain Kit (Invitrogen, UK) was used to differentiate viable and dead cells. After washing in binding buffer (BD Biosciences, Europe) the following anti-human monoclonal antibodies were used for flow cytometry: anti-HLA-DR-APC-Cy7, anti-Lin1(CD3,14,16,19,20,56)-FITC and anti-CD11b-PECy7 (BD Biosciences, Europe). Following immunostaining cells were washed in binding buffer and analysed using a MACSQuant flow cytometer with MACSQuantify software (Miltenyi Biotec).

Delayed-type hypersensitivity (DTH) skin tests

GV1001 (100µg) was administered intradermally in the lower abdomen, contralaterally to the vaccination site. Patients were asked to record the size of the DTH reaction 48 hours after administration and report to the clinician. A positive DTH reaction was defined as erythema and induration with an average diameter of 5mm.

In vitro proliferation assays

Thawed PBMCs were seeded in 48 well plates (ThermoFisher Scientific, USA) at 2 x 10^6 cells/well in X-VIVO 15 (Lonza, UK) with 10% pooled human serum (Innovative Research, USA) and 20µg/ml GV1001 peptide. Following 3 days of culture, 10units/ml IL-2 (Peprotech, UK) was added to the media. On day 11, the GV1001 enriched cells were harvested and plated at 1 x 10^5 cells/well (50µl) in a round bottom 96 well plate. To the pre-stimulated cells, irradiated (45Gy) autologous PBMCs (1 x 10^5 cells/well 50µl) were added to act as antigen presenting cells (APCs). GV1001 specific proliferation was tested for by the addition of 100µl of either control media, GV1001 (20µg/ml) or positive control PHA (5µg/ml). After incubation for a further 2 days, 1µCi/well of ^3H-thymidine was added for 16 hours before counting. A positive proliferative response to GV1001 was defined as a stimulation index (SI) above 1.8 with a significant difference in counts per minute from four replicates.
Cytokine Analysis

Cytokine levels were assayed on patient serum collected at the same time as PBMCs using the Bio Rad Bio Plex 27 Pro Cytokine, Chemokine and Growth Factor Assay (Bio Rad Laboratories, USA) using the Bio Rad Bio Plex Instrument following manufacturer’s instructions.

Tumor Burden Assessment

Independent assessment of tumor burden pre- and post-chemotherapy was performed by a radiologist blinded to MDSC results, using RECIST v1.1 criteria for measurement of evaluable lesions on CT imaging [14]. The sum of the long axis measurement of tumor lesions and short axis measurement of pathological lymph nodes was used to measure tumor burden in millimeters.

Statistical Analysis

Median levels of Lin-DR-CD11b+ cells were compared in pancreatic cancer patients versus controls using an unpaired t test with Welch’s correction. The correlation between baseline MDSC and cytokine was analysed with Spearmans Rank test and the non-parametric Mann-Whitney test was used to identify significant differences in cytokines with dichotomised at median MDSC levels. Paired Wilcoxon test was used to compare pre and post chemotherapy cytokine levels.
RESULTS

Levels of pro-inflammatory cytokines do not correlate with baseline levels of Lin-DR-CD11b+ cells in pancreatic cancer patients

Cryopreserved PBMCs from 40 patients with advanced pancreatic cancer treated with gemcitabine and capecitabine chemotherapy were analysed. 21 of the patients received concomitant therapy with the telomerase vaccine GV1001. Their baseline pre-treatment values are included in the calculation of the pre-treatment medians and the correlations of baseline levels of pro-inflammatory cytokines with MDSC levels. In the analysis of the effect of gemcitabine and capecitabine on Lin-DR-CD11b+ cells only sequential samples in the 19 patients who received gemcitabine and capecitabine alone were analysed. This phenotype was used to mark MDSCs based on the recent work of Kotsakis and colleagues [15].

There was a significant increase in Lin-DR-CD11b+ cells in pancreatic cancer patients (n=40) compared with controls (p<0.0001). The median baseline Lin-DR-CD11b+ cells in patients (expressed as a % of live PBMCs) was 1.85 (range 0.62 – 8.45): corresponding values in 24 controls were median 0.82 (range 0.16 – 2.2). There was no correlation between baseline levels of pro-inflammatory cytokines and baseline MDSCs in the 33 pancreatic cancer patients for whom we had full cytokine data (Spearman’s coefficient: IL-6 = 0.153, IL-1β = 0.22, VEGF = -0.0389, TNFα = 0.0587, MCP-1 = -0.226). When MDSC levels were dichotomized at the median, there were no significant differences in the baseline level of these cytokines in patients with high MDSCs compared with those with low MDSCs (Figure 1).

Gemcitabine and capecitabine therapy does not consistently reduce Lin-DR-CD11b+ cell numbers – the contribution of disease control and the degree of cancer-related inflammation

In the patients receiving chemotherapy alone (n=19), gemcitabine and capecitabine therapy resulted in a fall in Lin-DR-CD11b+ cells in 58% of the patients. Figure 2 shows the percentage change in MDSCs colour coded for response (red for progressive disease,
green for stable disease and violet for partial response) from the greatest increase to the greatest decrease during treatment with gemcitabine and capecitabine. In the 7 patients with progressive disease (PD) the Lin-DR-CD11b+ level went up in 5 and down in 2 (range -60% to +662%). In the 10 patients with stable disease (SD) Lin-DR-CD11b+ levels increased in 6 and fell in 4 (range -68% to +604%). Both patients achieving a partial response had falls in the Lin-DR-CD11b+ %. We obtained accurate tumour measurements and in 8/10 of the patients with stable disease there was no significant increase or decrease in the sum of the longest diameters. In these patients where the relative contribution of a change in Lin-DR-CD11b+ % secondary to any significant change in tumour bulk was minimal, Lin-DR-CD11b+ % went up in 5 patients and down in 3. These data suggest that there is no consistent reduction in Lin-DR-CD11b+ % secondary to gemcitabine and capecitabine chemotherapy per se. Changes in Lin-DR-CD11b+ % had a tendency to track relatively closely with tumour response. This is well demonstrated in the patients with baseline Lin-DR-CD11b+ greater than the median, the group where a fall in MDSCs would be most likely to be of the greatest immunological benefit (Table 1). In these patients the Lin-DR-CD11b+ % increased in 6 (3 with progressive disease and 3 with 0% tumour volume change) and in 3 it fell (1 of these patients obtaining a partial response and the other an 11% reduction in tumour volume). In only 3 of these patients was the Lin-DR-CD11b+ % less than the baseline median following gemcitabine and capecitabine therapy.

Given the lack of a strict association of MDSC level changes with objective response to therapy we next analysed whether the changes in MDSC level tracked with changes in the degree of cancer-related inflammation using as a surrogate changes in IL-6 and other inflammatory cytokines during treatment. IL-6 levels went up in 7/19 patients during gemcitabine and capecitabine treatment: 4 of these patients had progressive disease. The other 3 had stable disease and in these 3 patients MDSC% increased: in 2 of these MDSC levels above median at baseline increased even further. Table 2 shows the changes in MDSC% against the changes in inflammatory cytokines during chemotherapy in the 10/19 patients with stable disease. In the four patients in whom there was a fall in MDSC%, IL-6 fell in all 4 and in one of these patients (patient 7) a fall in IL-6 level from 152.72 pg/ml to 8.66 pg/ml over 7 weeks of chemotherapy was associated with a fall in
MDSC% from 2.54 to 1.59. In the six patients with stable disease in whom the MDSC% increased, in 3 the baseline MDSC level was below the median and remained so following chemotherapy. In two patients where an above median baseline MDSC level continued to rise on treatment there was a significant increase in IL-6 (patients 1 and 3 – VEGF levels also increased from 37.59pg/ml to 70.69pg/ml in patient 3, the only patient without progressive disease to show such an increase). In the remaining patient a 9% increase in MDSC level was associated with a 17% decrease in IL-6 level.

*High baseline MDSC levels do not preclude an immune response to a cancer vaccine administered concomitantly with gemcitabine and capecitabine*

As cryopreserved PBMCs were used no functional in vitro assays were performed [13]. As an alternative we analysed the development of an immune response to GV1001 in the 21 patients receiving concomitant gemcitabine and capecitabine and vaccine (positive proliferation assay, and/or the development of a positive DTH, to GV1001). This was felt to be a clinically pertinent readout of the functional effect of any gemcitabine and capecitabine-mediated quantitative impact on MDSCs. Table 3 details the 9 patients in this cohort who developed an immune response. In 8/9 of these patients the MDSC% fell during chemo-immunotherapy. 6/9 had baseline Lin-DR-CD11b+ % greater than the patient median and in all of these the MDSC level fell. All immune responders had radiological disease control (either PR or SD) at the time that blood was drawn for the analysis of proliferation response, which coincided with the timing of the sequential MDSC assay.
DISCUSSION

This study demonstrates that there appears to be no consistent reduction in MDSC level in pancreatic cancer patients treated with gemcitabine and capecitabine. We analysed a homogeneously treated group of patients obtaining samples at the same time point in all, immediately prior to a gemcitabine infusion, one week following the last gemcitabine infusion and whilst the patient was taking daily capecitabine. This time point correlated with the first radiological assessment of tumour response. MDSC changes tended to track with tumour volume: most patients with progressive disease demonstrated an increased MDSC level during chemotherapy and those who obtained a partial response a decreased level. In patients with no significant change in tumour volume during therapy, gemcitabine and capecitabine did not consistently reduce MDSC levels as would be expected if the chemotherapy reduced MDSC levels independently of response, through a direct effect on MDSCs: MDSC level was just as likely to go up as to go down. Suzuki and colleagues demonstrated that a single dose of Gemcitabine significantly reduced the number of Gr-1+/CD11b+ cells in the spleens of mice harboring a variety of different tumors, with no effect on the number of CD4+, CD8+ or B cells [6]. Vincent and colleagues demonstrated that 5-FU triggered the dose-dependent apoptosis of MDSCs: thymidylate synthase levels in MDSCs were lower than in splenocytes or tumor cells [7].

In spite of these pre-clinical data we cannot recommend the use of gemcitabine and fluropyrimidine treatment in cancer patients specifically with the expectation that this will result in a reduction of MDSC level. Any decrease is likely to reflect predominantly the effect of the resultant disease control, although even in patients with no change at all in disease volume the MDSC level may actually go up. These data support previously published observations [8,9] but are important because they are from patients homogeneously treated with a combination of the only two chemotherapy drugs which pre-clinically have been shown to reduce MDSC levels directly.

We hypothesized that longitudinal changes in MDSCs levels during gemcitabine and capecitabine chemotherapy might in part be related to temporal changes in the degree of inflammation within tumours. A reduction in tumoural inflammation might be more pronounced where chemotherapy was slowing rapid tumour growth which would account
for the modest tracking of MDSC levels with tumour response, but the degree of inflammation in any tumour is likely to be somewhat independent of size and growth dynamics and this might account for the lack of a strict association between objective response to chemotherapy and the trajectory of MDSC levels. This might also explain the differential effects on MDSC levels in patients with stable disease. We used sequential changes in IL-6 and other inflammatory cytokines as a surrogate for the status of cancer-related inflammation and correlated changes in MDSC levels with changes in the levels of IL-6 during treatment with gemcitabine and capecitabine. We demonstrated that in patients with stable disease the direction of MDSC change tracked well with the direction of IL-6 change. These data support a model whereby significant increases in tumour burden during chemotherapy are likely to be associated with MDSC increases and significant decreases in tumour burden with decreased MDSC level whilst in those with little change in tumour bulk the direction of MDSC change will be determined by the longitudinal change in the degree of cancer-related inflammation. Continued significant tumoural inflammation, in spite of stabilization of tumour volume, is likely to support the continued production of MDSCs: reduced inflammation concomitant with disease stabilization is likely to limit MDSC accumulation. These two factors appear to override any specific quantitative effect of gemcitabine and capecitabine chemotherapy per se on MDSC levels.

Given the above we were initially surprised to see no association between baseline levels of pro-inflammatory cytokines and baseline MDSCs. Using a 4T1 mammary carcinoma model Bunt and colleagues demonstrated significant elevations of the pro-inflammatory cytokines IL-6, MCP-1, TGFβ and IL1β in tumour tissue showing that tumour growth is associated with an inflammatory milieu [16]. Intratumoral inflammatory cytokine levels were significantly reduced and accumulation of CD11b+Gr1+ MDSCs in the blood was delayed in IL-1R−/− mice. Overexpression of IL-6 in 4T1 cells compensated for the loss of IL-1R with no delay in MDSC accumulation. However, it is important to note that the delay in accumulation of MDSCs in the IL-1R−/− mice was short-lived and by day 26 from inoculation MDSC levels were at the same high level as in BALBc mice. This could explain a lack of correlation between baseline IL-6 (and other inflammatory cytokines including IL1β) and MDSC levels at a time when the cancer is already well established.
GM-CSF appears to have more profound effects on MDSC biology than IL-1β and IL-6 in the 4T1 model [17], and this is supported by data demonstrating the critical role of GM-CSF in the induction of human CD33+ MDSCs from normal PBMCs [18]. GM-CSF plays a key role in the generation of MDSCs in KPC mice where oncogenic KRAS and mutant p53 is targeted to the pancreas [19]. Levels of IL-6 were generally low in the KPC cell lines and tended to be higher in the normal ductal cells. GM-CSF drove GR-1+CD11b+ cell generation whereas IL-6 was minimally effective: neutralization of GM-CSF completely abrogated MDSC generation from c-kit+ precursors by conditioned media whereas anti-IL-6 had no effect. 19/20 human pancreatic ductal adenocarcinoma samples expressed GM-CSF by immunocytochemistry. Serum GM-CSF levels were generally below the level of detection with our assay precluding any useful correlation of GM-CSF levels and MDSC dynamics. Very low levels of GM-CSF relative to other haematopoietic cytokines in patients with pancreatic cancer have been previously reported [20].

One limitation of this study is the number of patient samples analysed. However, it is well appreciated that obtaining sequential PBMC samples for MDSC evaluation is difficult. As we point out in the introduction, we are aware of only two studies that address the effect of chemotherapy on sequential MDSCs; one analyses only six patients and in the second analysing 25 patients, the regimen was not specified [8, 9].

A further limitation of this study is that it utilized cryopreserved cells. This reflects the reality of obtaining PBMC samples from multiple centres in the context of a clinical trial and the advantages of batch testing stored samples. An important recent study demonstrated that cryopreservation led to a significant reduction in the percentage of DR-Lin-CD33+ and DR-Lin-CD15+ cells compared to fresh cells, whereas the percentages of the monocytic cell populations, DR-Lin-CD11b+ and DR-/low CD14+ cells were not significantly affected by cryopreservation [15]. Hence, we restricted our quantitative analysis to the Lin-DR-CD11b+ monocytic MDSC subset, which is little effected in terms of recovery, viability and phenotype by cryopreservation. It is worth noting that the medians and ranges which we derived in the current study are very similar to those in our previous work where we phenotyped MDSCs as Lin-/low DR- CD11b+ CD33+ [10].
Kotsakis and colleagues also demonstrated that freezing had a profound effect on the function of recovered MDSCs [13]. Freezing and re-thawing abolished the inhibitory effects of MDSCs on the proliferation of stimulated autologous CD4+ cells, MDSC arginase positivity and PMA/ionomycin- stimulated ROS production by MDSCs. Thus, we did not perform any functional assays on our cryopreserved cells as this would not have provided any useful additional information. Instead, we sought to understand the functional effect of any gemcitabine and capecitabine-mediated quantitative effects on MDSCs by correlating the effects of chemotherapy on MDSC levels with the development of an immune response to the GV1001 vaccine.

The MDSC level fell in 8 of the 9 patients who developed an immune response to GV1001 administered concomitantly with gemcitabine and capecitabine. In 6 of these patients the initial pre-vaccination MDSC level was above the median for the cancer patient cohort. A direct causal relationship cannot be claimed on the basis of these data - that disease control secondary to chemotherapy reduces circulating MDSC levels and this permits the development of an immune response to a concomitantly administered cancer vaccine. However, high pre-vaccination MDSC levels are clearly not an absolute bar to the development of immunity to a tumour associated antigen. It is reasonable to hypothesise that this is more likely to occur with any concomitantly administered treatment which checks the progress of increasing immunosuppression which will occur with increasing volumes of disease and/or increasing levels of cancer-related inflammation either in untreated patients or in those who fail to gain control of their disease on therapy.
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Conflict of Interest

The authors declare that they have no conflict of interest.
REFERENCES


Figure Legends

**Fig. 1** No significant differences in the level of pro-inflammatory cytokines in patients with high MDSCs compared with those with low MDSCs

Peripheral blood mononuclear cells from 33 pancreatic cancer patients obtained pre-treatment (at baseline) were immunostained for HLA-DR-APC-Cy7, Lin1(CD3,14,16,19,20,56)-FITC and CD11b-PECy7 and with ViViD to discriminate live and dead cells. Following immunostaining cells were analysed using a MACSQuant flow cytometer with MACSQuantify software. In addition serum cytokines TNFα, MCP-1, IL-1b, IL-6 and VEGF were determined using the Bio Rad Bio Plex 27 Pro Cytokine, Chemokine and Growth Factor assay using the Bio Rad Bio Plex Instrument. Baseline MDSCs dichotomized at median (1.85) plotted with baseline IL-6, IL-1b, VEGF, TNFα, and MCP-1 (pg/ml). P value generated for Mann-Whitney test shows no significant differences.

**Fig. 2** Gemcitabine and Capecitabine therapy does not consistently reduce Lin-DR-CD11b+ MDSCs

In patients receiving chemotherapy alone (n=19) PBMCs were obtained pre and post-treatment and immunostained for HLA-DR-APC-Cy7, Lin1(CD3,14,16,19,20,56)-FITC and CD11b-PECy7 as well as ViViD to discriminate live and dead cells. Following immunostaining cells were analysed using a MACSQuant flow cytometer with MACSQuantify software. The graph depicts the percentage change in Lin-DR-CD11b+ MDSCs from baseline in patients during treatment with Gemcitabine and Capecitabine. Red denotes patients with progressive disease on treatment, green stable disease and violet partial response by RECISTv1.1.

**Table 1. Changes in Lin-DR-CD11b+ cells during chemotherapy in patients with baseline values > median.**

Cryopreserved PBMCs obtained from patients with advanced pancreatic cancer before (baseline) and after treatment with gemcitabine and capecitabine chemotherapy were immunostained for HLA-DR-APC-Cy7, Lin1(CD3,14,16,19,20,56)-FITC and CD11b-PECy7 and ViViD to discriminate live and dead cells. Following immunostaining cells
were analysed using a MACSQuant flow cytometer with MACSQuantify software. Numbers represent % change in the Lin-DR-CD11b+ cells only in those patients with baseline Lin-DR-CD11b+ greater than the median. There was no consistent reduction in Lin-DR-CD11b+ % secondary to gemcitabine and capecitabine chemotherapy. SD = stable disease and PR = partial response and PD = progressive disease

Table 2. Changes in MDSC% during gemcitabine and capecitabine chemotherapy correlated with changes in serum TNFα, MCP-1, IL-1b, IL-6 and VEGF in patients with stable disease on therapy.

PBMC from ten patients with stable disease during gemcitabine and capecitabine treatment were analysed both pre and post treatment by flow cytometry for changes in Lin-DR-CD11b+ cells and changes in serum cytokines TNFα, MCP-1, IL-1b, IL-6 and VEGF by the Bio Rad Bio Plex 27 Pro Cytokine, Chemokine and Growth Factor assay.

Table 3. Trajectory of Lin-DR-CD11b+ percentage in immune responders in patients receiving concomitant GV1001 and gemcitabine/capecitabine chemotherapy.

PBMC from nine patients who developed an immune response (positive proliferation assay, and/or the development of a positive DTH) to the vaccine GV1001 whilst also receiving concomitant gemcitabine and capecitabine were immunostained for Lin-DR-CD11b+ MDSCs and analysed by flow cytometry both at baseline and post therapy. DTH = delayed type hypersensitivity testing to GV1001, NR = not reported, ORR = objective radiological response, SD = stable disease and PR = partial response.