

Gemcitabine in the Era of Cancer Immunotherapy

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Abstract

Gemcitabine has been used as a chemotherapeutic to treat solid cancers for more than 20 years. Whilst its use as chemotherapy has been declining due to the development of more potent chemotherapeutic combinations further studies of gemcitabine have identified varied immune modulatory effects on both tumour cells and leukocyte subsets. Despite these observations' gemcitabine has rarely been used clinically on the basis of its immune modulatory properties. The recent successes of immunotherapy using checkpoint inhibition are not yet realised for many solid tumours, particularly those to which gemcitabine has proven efficacious. Novel combination immunotherapy involving gemcitabine, checkpoint inhibition and additional immune modulation has the potential to increase the efficacy of checkpoint inhibition whilst expanding the number of tumours susceptible to anti-tumour immunity. This commentary discusses gemcitabine's immune modulatory properties and its potential role in combination immunotherapy.

Gemcitabine

Gemcitabine is a synthetic pyrimidine nucleoside analogue which is administered intravenously as a chemotherapeutic to treat numerous cancers. Gemcitabine requires transport into cells and activation by phosphorylation, the resulting gemcitabine triphosphate is incorporated into newly synthesized DNA during cell division, inhibiting further DNA synthesis and causing cell death. Gemcitabine is used to treat cancers including those of the pancreas, lung, breast, colon, and ovary either as first or second line treatments as a single agent or in combination.

Gemcitabine is often used when other treatment options have either failed, are unavailable or exhibit severe toxicity for frail patients. Notably, the cancers for which gemcitabine is used are amongst the least responsive to cancer immunotherapies. Cancer Immunotherapy has proven a revolutionary new treatment modality when used to treat immunogenic tumours such as Melanoma or B cell lymphoma in which checkpoint inhibition (CPI) [1] and CAR-T-cell therapy [2] are now first line treatments respectively. Yet cancers such as those of the pancreas or ovary are largely unresponsive. This unresponsiveness can be due to numerous different factors and will likely require the development of sophisticated therapies combining

multiple agents in defined sequence in order to prove efficacious. In this commentary we shall discuss the role gemcitabine may have in checkpoint immunotherapy regimens to tumours currently unresponsive to this immunotherapy.

Gemcitabine and Anti-tumour Immune Responses

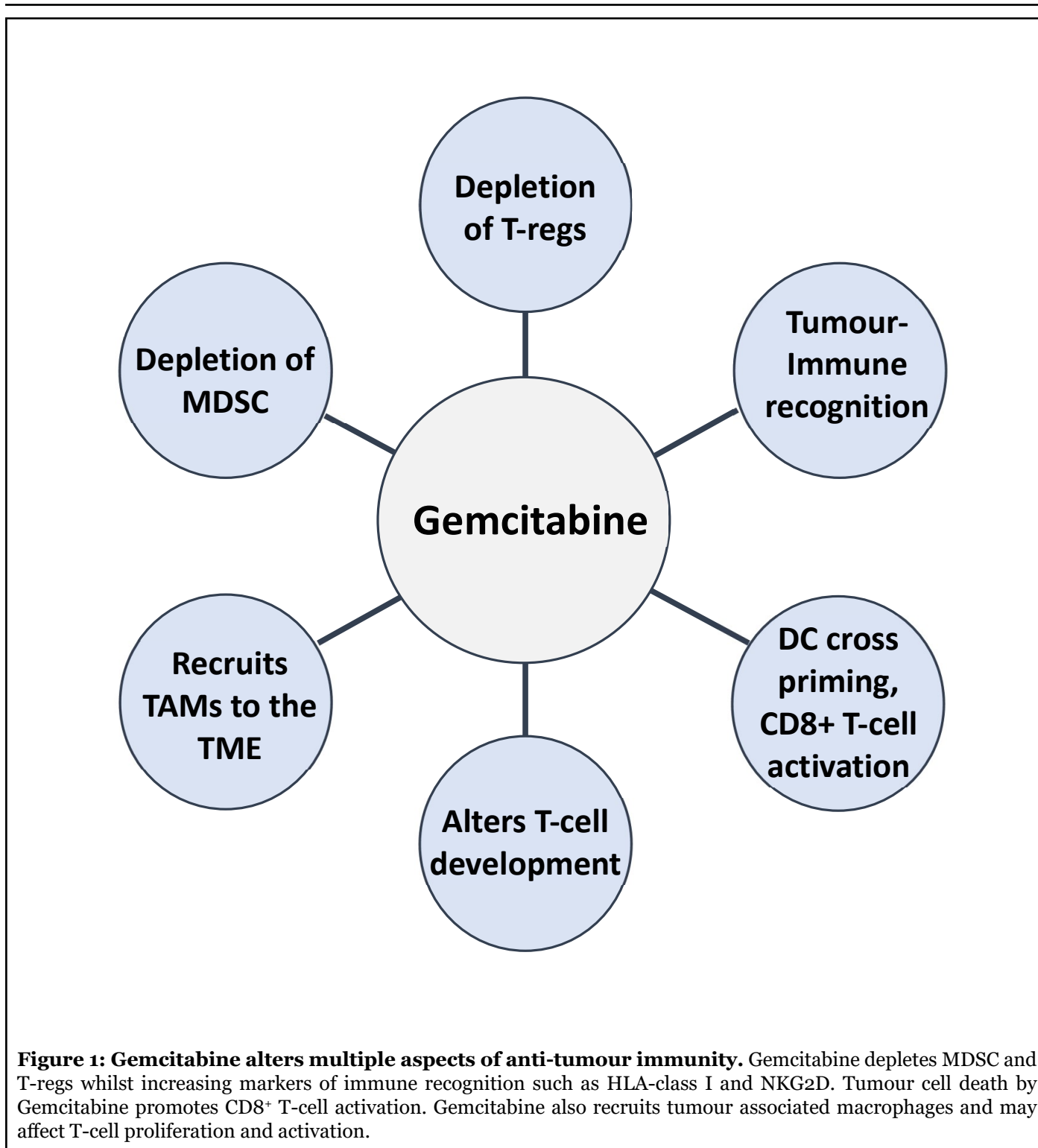
Gemcitabine has varied effects on tumour cells independent of its ability to induce tumour cell death by acting as a pyrimidine nucleoside analogue. For example, Gemcitabine has demonstrated various immune modulatory effects on cells within the tumour microenvironment (TME). We have shown in both colon and pancreatic tumour cell lines that gemcitabine can upregulate the expression of Human leukocyte class I alleles, and NKG2D ligands MIC1 A/B and ULBP proteins [3,4]. Gemcitabine also upregulates the expression of check point ligands including Programmed cell death ligand-1 (PDL-1_ and CD47 on the surface of pancreatic tumour cell lines [4]. These properties seem to be independent of gemcitabine's ability to kill these cells, occurring at time points and concentrations below which gemcitabine can kill tumour cells *in vitro* through the inhibition of cell division [4].

Gemcitabine also effects leukocytes involved in anti-tumour immune suppression. T-regulatory (T-reg) cells are important mediators of immunosuppression in the PDAC TME and PDAC patients have elevated levels of T-regs at diagnosis [5]. Therapeutic concentrations of gemcitabine are capable of selectively suppressing T-reg induction *in vitro* possibly through inhibiting T-reg proliferation [6] whilst gemcitabine administered at low doses in an orthotopic Panco2 murine model of pancreatic cancer has been shown to deplete T-regs. These T-regs are thought to proliferate within pancreatic tumours and exacerbate tumour progression, and their depletion by gemcitabine improved survival [7]. The reductions in T-regs by relatively low doses of gemcitabine can enhance the efficacy of CIK cells *in vitro* due to the reduction on IL-10 and Tumour growth factor (TGF)- β in these culture systems. Transfusion of gemcitabine treated CIK cells in tumour bearing nude mice resulted in higher levels of interferon (IFN)- γ compared to CIK cells without gemcitabine pre-treatment [8] indicating the inhibition of T-reg by gemcitabine is sufficient to activate immune responses. In humans Gemcitabine reduces T-reg numbers and the immune inhibitory cytokine TGF β -1 while restoring the T-effector/T-reg ratio in patients with pancreatic cancer [5].

Myeloid suppressor cells (MDSC) are another type of immune inhibitory cell found within the TME of tumours resistant to immunotherapy. These cells are also elevated in PDAC patients at diagnosis [5]. A single therapeutic dose of gemcitabine was able to reduce the number of myeloid suppressor cells found in the spleens of multiple murine models bearing large tumours with no significant reductions in CD4⁺ T-cells, CD8⁺ T-cells or Natural killer (NK)-cells. Importantly the reduction in splenic MDSC was accompanied by an increase in the anti-tumour activity of CD8⁺ T-cells and activated NK-cells whilst inducing significant anti-tumour effects in combination with IFN- β based immunotherapy [9] despite minimal effects as single agents. The mechanism responsible is thought to involve selective apoptosis of MDSC by gemcitabine. In another study Gemcitabine directly inhibited myeloid derived suppressor cells which led to the increased expansion of T-cells in mammary carcinoma bearing mice [10]. A murine model of pancreatic cancer demonstrated increases in both *T-regs* and MDSC by gemcitabine. In this study elimination of *T-regs* alone or in combination with DC-based vaccination had no effect on pancreatic tumour growth or survival. Treatment with gemcitabine in this model led to a significant decrease in MDSC percentages in the spleens of tumour-bearing mice but also without enhancing survival. However, combination therapy with DC vaccination followed by Gemcitabine treatment led to a significant delay in tumour growth and improved survival in pancreatic cancer-bearing mice [11]. In line with this, the addition of gemcitabine to DC vaccination

in a subcutaneous and orthotopic PDAC murine model demonstrated improved survival of mice due to a pronounced reduction of tumour-infiltrating MDSC and a decrease in migrating and metastasizing tumour cells. When combined with DC vaccination, a higher number of activated tumours infiltrating lymphocytes was recovered from tumour tissue and increased survival was observed compared to mice given the DC vaccine alone [12]. The timing and dose of gemcitabine treatment is thought to factor into its ability to therapeutically reduce MDSC [13]. The ability of gemcitabine to inhibit MDSC and improve the expansion of T-cells in T41 mammary tumour bearing mice was shown to be dependent upon the timing of the administration of gemcitabine relative to the size of the tumour [10]. In human's gemcitabine reduces MDSCs in patients with pancreatic cancer [5]. In addition to MDSC present in the TME, Increased MDSC have also been identified in the peripheral blood of patients with pancreatic cancer. Treatment with Gem also led to a decrease of this population in pancreatic patients [11].

Tumour associated macrophages (TAMs) represent third immune inhibitory cell type in the TME capable of mediating immune suppression. In contrast to Gemcitabine's ability to inhibit T-regs and MDSC studies show that gemcitabine treatment recruits TAM's to the TME. Gemcitabine increased presence of macrophages in orthotopic human pancreatic tumour xenografts from mice treated with gemcitabine as compared to those from vehicle only-treated mice [14]. Supernatant from gemcitabine treated pancreatic tumour cells polarises RAW264.7 macrophage cell line towards a pro tumour phenotype, with increased expression of arginase-1 and TGF- β 1, by stimulating the production of IL-8 [14]. In a murine model using patient-derived pancreatic tumour xenografts gemcitabine recruits M2-Type Tumour-Associated Macrophages into the Stroma of the tumour [15]. The ability of gemcitabine to promote the recruitment and function of TAM's might be mitigated by combination with TAM suppressing agents. The chemotherapeutic bisphosphonate Zoledronic acid (ZA) is capable of inhibiting the generation of TAM's [16]. This decrease in TAM's has been associated with an increase in MDSC indicating that ZA's effect on these immune suppressive cell types is the opposite to that of Gem [17]. We have shown that gemcitabine and ZA in combination improves the inhibition of tumour growth and in combination with Pomalidomide increases the activation of DC and CD8⁺ T-cells *in vitro*. A murine model of pancreatic cancer demonstrated that gemcitabine plus ZA in combination significantly inhibited tumour growth and the development of liver metastasis [18]. Despite the putative role of gemcitabine in recruiting TAM's to the TME, the weight of evidence is suggestive of a role for gemcitabine in enhancing anti-tumour immune responses prompting the use of gemcitabine as an immune modulator in combination with CPI therapy (Figure 1).



Gemcitabine and Checkpoint Inhibition

The immune modulatory properties of gemcitabine make it a good candidate for combination with checkpoint inhibition immunotherapy for cancer such as pancreatic cancer with which immunotherapy has had limited success [19]. Most of the clinical studies to date are based on the treatment of pancreatic cancer, typically thought to be a

non-immunologic tumour, and thus a good model with which to test agents intended to reverse immune inhibition and increase the infiltration of anti-tumour T-cell immune responses of the kind associated with checkpoint inhibitor responsiveness. However, combinations of gemcitabine and checkpoint inhibition have for the most part proven ineffective when used to treat cancer patients (Table 1).

Clinical phase	Cancer	Drug	Mechanism of action	No of patients	Median OS (Months)	References
Chemotherapy						
Phase II/ III	Metastatic pancreatic cancer	FOLFIRINOX, Gemcitabine	Cytotoxic drugs	342	11.1 (95% CI, 9.0 - 13.1) in FOLFIRINOX therapy vs 6.8 (95% CI, 5.5 - 7.6) in Gemcitabine therapy	68
Phase III	Advanced pancreatic cancer	Nab-paclitaxel, Gemcitabine	Cytotoxic drugs	861	8.5 (95% CI, 7.89 - 9.53) in combination therapy vs 6.7 (95% CI, 6.01 - 7.23) in Gemcitabine	24
Checkpoint inhibition						
Phase II	Locally advanced/ metastatic pancreatic cancer	Ipilimumab	CTLA-4 inhibitor	27	Ineffective for the treatment, no improvement in the survival rate	21
Phase II	Metastatic Pancreatic Ductal Adenocarcinoma	Tremelimumab and Durvalumab	CTLA-4 inhibitor, PD-1 inhibitor	65	3.1 (95% CI, 0.08-16.22) in combination therapy vs 3.6 months (95% CI, 2.7-6.1 months) in Durvalumab monotherapy	69
Phase I	Metastatic pancreatic cancer	Tremelimumab and Gemcitabine	CTLA-4 inhibitor, Cytotoxic drug	34	7.4 (95 CI 5.8-9.4)	70
Phase II	Metastatic pancreatic ductal adenocarcinoma	Tremelimumab	CTLA-4 inhibitor	20	4 (95% CI 2.83-5.42)	71

Chemotherapy versus single agent checkpoint inhibition						
Phase III	Stage IV/ Recurrent non-small-cell lung cancer	Nivolumab, Gemcitabine, Cisplatin, Carboplatin, Paclitaxel, Pemetrexed	PD-L1 inhibitor, platinum-based chemotherapy	423	14.4 (95% CI, 11.7-17.4) in Nivolumab alone vs 13.2 (95% CI, 10.7-17.1) in chemotherapy	20
Phase II	Metastatic pancreatic ductal adenocarcinoma	GVAX, Ipilimumab, FOLFIRINOX	vaccine, CTLA-4 inhibitor, cytotoxic drugs	82	9.38 (95% CI, 5.0-12.2) in Ipilimumab + GVAX therapy vs 14.7 (95% CI, 11.6-20.0) in FOLFIRINOX therapy	72
Phase III	Metastatic Triple Negative Breast Cancer	Pembrolizumab, capecitabine, eribulin, gemcitabine, vinorelbine	PD-1 inhibitor, Cytotoxic drugs	622	10.7 (95% CI, 9.3- 12.5) in Pembrolizumab therapy vs 10.2 (95% CI, 7.9 - 12.6) in chemotherapy	73
Gemcitabine based chemotherapy plus Checkpoint inhibition						
Phase Ib/ II	Metastatic pancreatic cancer	Pembrolizumab, Gemcitabine, Nab-Paclitaxel	PD-1 inhibitor, Cytotoxic drug	17	15.0 (95 CI, 6.8-22.6)	26
Phase Ib	Advanced pancreatic cancer	Ipilimumab and Gemcitabine	CTLA-4 inhibitor, Cytotoxic drug	16	MOS: 8.5 months (95 CI, 2.2-10.3)	74

Table 1: Clinical trials of Gemcitabine and other chemotherapies, single agent checkpoint inhibition and combinations of chemotherapy plus checkpoint inhibition.

Single agent CPI has shown little promise in typically non-immunological tumours. In a phase III study Nivolumab was not associated with significantly longer progression-free survival than gemcitabine-based doublet chemotherapy among patients with previously untreated stage IV or recurrent squamous NSCLC [20]. A Phase 2 trial of single agent Ipilimumab for pancreatic adenocarcinoma was ineffective for the treatment of advanced pancreas cancer [21]. Monotherapy with CPI for triple negative breast cancer [22] and ovarian cancer [23] has also been largely unsuccessful.

Combination treatment involving gemcitabine in combination with anti-cytotoxic T-lymphocyte-associated (CTLA)-4 antibodies has not demonstrated notable clinical efficacy. A phase I dose escalation trial of the anti-CTLA-4 antibody tremelimumab in combination with gemcitabine in chemotherapy-naïve patients with metastatic pancreatic cancer demonstrated a median overall survival was 7.4 months a slight increase compared to previously reported results for gemcitabine alone [24] but lower than the gemcitabine plus Nab-paclitaxel combination [24]. A phase Ib dose finding study of ipilimumab and gemcitabine for advanced pancreatic cancer reported an OS of 8.5 months for the combination [25].

A Phase Ib/II study of gemcitabine, nab-paclitaxel, and pembrolizumab in metastatic pancreatic adenocarcinoma, including 11 chemotherapy naïve treated patients, showed that the combination can be safely given to chemotherapy naïve PDAC patients and reported an improved median overall survival of 15 months compared previously reported results for standard weekly $\times 3$ every 28-day gemcitabine and nab-paclitaxel dosing [26]. This study demonstrated the safety of the combination and suggests that gemcitabine, nab-paclitaxel plus checkpoint inhibition may represent a promising chem-immunotherapeutic combination in the setting of pancreatic cancer. This is consistent with the use of Nab-Paclitaxel in combination with the anti-PDL-1 antibody Atezolizumab in pre-selected triple negative breast cancer [27], a combination that has been granted approval. A phase I trial of Nivolumab alone or in combination with cisplatin plus gemcitabine in patients with unresectable or recurrent biliary tract cancer demonstrated a median overall survival was 5.2 months (90% CI 4.5–8.7) for the Nivolumab alone group and 15.4 months (90% CI 11.8–not estimable) in the combination group providing supportive evidence for future larger randomised studies of nivolumab plus gemcitabine in this cancer [28]. In a preclinical study of mesothelioma, a cancer in which CPI has already demonstrated encouraging results, has shown that the combination of gemcitabine with anti-PD-1 has synergistic effects, whilst in two mesothelioma patients the gemcitabine (1,000 mg/m² weekly) plus pembrolizumab (200 mg every 3 weeks) combination could overcome

previous resistance to single agent treatment. This provides important proof of principle evidence that gemcitabine plus CPI combinations can prove efficacious [29]. Taken together the studies conducted thus far indicate that CPI to the PD-1/PDL-1 pathway holds greater promise in combination with gemcitabine and other immune modulatory chemotherapies than that of CPI targeting the CTLA-4 – CD28 pathway.

Recent research indicates that the efficacy of both chemotherapy and immunotherapy, including that of checkpoint inhibition, is dependent on the composition of gut microbiome [30,31]. Some microbiomes permit patients to respond to CPI whilst others are associated with CPI resistance. The mechanisms for this effect are currently not well understood but likely involve the translocation of microbes or products of microbial metabolism across the gut epithelium. Chemotherapeutics have demonstrated an ability to alter the composition of the gut microbiome with important implications for anti-tumour immunity [32] although little data exists regarding whether gemcitabine effects the gut microbiome. A gemcitabine-treated xenografted mouse models of PDAC has revealed proinflammatory alterations in the faecal microbiota, with decreases in Firmicutes and Bacteroidetes bacteria associated with CPI efficacy, as well as activation of the NF- κ B inflammatory pathway in tumour tissues [33]. Thus, the immune modulatory effects of gemcitabine which support CPI immunotherapy may be held in check by microbial changes mediating CPI resistance, although studies are needed in human subjects to measure both changes in microbial species and the effects on anti-tumour immunity. We have observed that gemcitabine plus short chain fatty acids, microbial metabolites with diverse immune modulatory properties, synergise to inhibit the growth of pancreatic, lung and colon cancer cell lines whilst improving the expression of markers of immune recognition such as HLA-class I (unpublished observation).

Gemcitabine can reduce immune suppression whilst anti-PD-1 checkpoint inhibition can rescue anti-tumour T-cells from exhaustion. However, despite indications that gemcitabine plus anti-PD-1 CPI combinations hold promise they've yet to prove sufficiently efficacious in combination. This is likely due to defects in T-cell priming and activation in cancer patients and understanding how gemcitabine effects anti-tumour T-cell responses will be important in determining how best to combine therapies and improve responses to non-immunogenic cancers.

Gemcitabine and T-cell Responses

The effect of gemcitabine on T-cell responses has generated conflicting data. Gemcitabine has been shown to cause reductions in Lymphocyte counts were over the

first 21 days of administration with subsequent recovery by day 28. Notably this decrease was not reflected in a change in any single subset. Percentages of CD3⁺ T-cells and CD4/CD8 ratios were relatively steady throughout this treatment period. Activation of T-cells with anti-CD3 plus anti-CD28 also demonstrated intracellular IFN- γ production that was upregulated following gemcitabine treatment [34] suggesting that whilst some T-cell depletion occurs existing T-cell function is unaffected. This reduction in lymphocytes was identified in a subsequent study showing an initial reduction in lymphocyte counts in pancreatic cancer patients, including CD3⁺ lymphocytes, prior to stabilization and a return to pre-treatment levels. There was an indication that gemcitabine may decrease the activation of memory T-cells and promote the activation of naïve T-cells. The authors conclude that gemcitabine is not immunosuppressive [35]. A later study determined that gemcitabine increases the effector T-cell: *T-reg* ratio and that effector T-cells demonstrated undiminished proliferative capacity throughout the cycle of gemcitabine therapy [5]. Gemcitabine has enhanced efficacy of the OVA-DC vaccine whilst simultaneously reducing the frequency of OVA specific CD8⁺ T-cells. DC migration to draining lymph nodes and antigen cross-presentation were unaffected and the mechanism of action is thought to be an increase in CD8⁺ T-cells into tumours and possibly sensitisation of the tumour to cytotoxic T-cells [36], in line with our observation that gemcitabine increases the expression of class I HLA on pancreatic tumour cell lines [4]. Importantly the suppression of T-cells could be avoided by starting chemotherapeutic treatment after two cycles of vaccination, indicating that gemcitabine inhibits early but not established stages of effector T-cell responses [36]. Despite the immune modulatory properties of gemcitabine, it has typically prescribed for its chemotherapeutic effect on tumour cells. Yet apoptosis induced by gemcitabine has been shown to result in the induction of cross priming of DC's, activation of CD8⁺ T-cells and subsequent tumour cytotoxicity demonstrating gemcitabine-based tumour cytotoxicity is, in principle, sufficient to induce anti-tumour T-cell immune responses [37,38]. In support of the view that gemcitabine is best regarded as an immune modulator, the anti-tumour effects of gemcitabine were lost in immune deficient, nude mice. Anti-tumour effects of gemcitabine are also detected in mice bearing tumours resistant to the *in vitro* cytotoxic effects of gemcitabine further supporting the view that gemcitabine's mechanism of action is, at least in part, based upon inducing anti-tumour immune responses in addition to its well characterised ability to diminish both MDSC and T-regs.

Taken together these studies indicate that gemcitabine may promote T-cell activation through improving immune recognition of tumours whilst directly inhibiting at least some aspects of T-cell development. The effects of gemcitabine

on T-cell responses warrant further study in order to improve its role in immunotherapeutic combinations such as with anti-PD-1 antibodies. This is particularly important considering that non-immunogenic tumours against which gemcitabine is used are characterised by a marked CD8⁺ T-cell dysfunction including pancreatic [39], breast [40], Ovarian [41] and Lung [42] cancers in which T-cell responses are either ineffective or excluded from the TME. This T-cell dysfunction contributes to the lack of efficacy of checkpoint inhibition. Non-immunogenic tumours are also characterised by the presence of immune inhibiting leukocytes including MDSC, TAMs and T-regs within the tumour microenvironment (TME). T-cell dysfunction is due to chronic exposure to antigen and dysfunctional T-cell subsets are defined by the expression of various checkpoints such as PD-1, TIM-3 or LAG-3 and markers of activation such as CD38. The expression of PD-1 and CD38 are associated with poor prognosis in gemcitabine treated PDAC patients, particularly on CD101⁺ expressing T-cells which represent an exhausted phenotype that cannot be salvaged by anti-PD-1 therapy [43] whereas CD101⁻ cells are associated with CPI responses [44]. A recent study using a murine model of PDAC demonstrated that CD38^{hi}, PD-1^{hi} T-cells are susceptible to apoptosis upon interaction with anti-PD-1 antibody due to poor T-cell priming [45]. A number of transcription factors have been implicated in controlling the progression of T-cells towards the exhausted or dysfunctional phenotypes characteristic of PDAC, including Thymocyte selection-associated high mobility group box protein (Tox) and T-cell factor (TCF)-1. These transcription factors act to drive exhausted CD8⁺ T-cell phenotypes with different functional capacities and ability to respond to CPI [46] as the anti-tumour effector functions of dysfunctional T-cells may or may not be rescued by CPI, depending upon the extent to which their transcriptional programs have been affected [45]. Gemcitabine demonstrates both epigenetic [47] and miRNA [48] modulating properties but it is currently unknown whether gemcitabine may interact with dysfunctional T-cell subsets or effect T-cell transcriptional and epigenetic regulation or the consequences of these putative interactions.

Studies describing the effects of gemcitabine on T-cell function indicate that the immune potentiating properties of gemcitabine, alone or in combination with CPI, are likely to be squandered unless adequate T-cell priming is addressed. Combination with innate immune modulators, immunogenic chemotherapy or cytokines provide numerous opportunities to improve upon existing gemcitabine and CPI combination immunotherapy.

Gemcitabine Combination Therapy

Studies involving the combination of gemcitabine and innate immune agonists may offer a method with which

to effectively prime T-cell responses to CPI whilst tackling immune suppression with gemcitabine. A number of studies have been conducted on the role of TLRs and their agonists on tumours such as pancreatic cancer [49]. The synthetic TL2/6 agonist macrophage activating lipopeptide (MALP)-2 was combined with gemcitabine in a phase I/II trial during which intra-tumourally injected MALP-2 during surgery was followed by gemcitabine chemotherapy. Mean survival was 17.1 months [50]. Our Randomised, open-label, phase II study combining gemcitabine and innate immune modulation using heat killed mycobacterium called IMM-101 in the setting of pancreatic cancer demonstrated a significant increase in a pre-defined metastatic subgroup of 7.0 months for the combination compared to 4.4 months for single agent gemcitabine. A remarkably sustained responses in a subset of responding patients [51]. IMM-101 acts through stimulation of TLR2 and 6 resulting in increased expression of IFN- γ from T-cells and NK cells. The durable response in a subset of patients is indicative of responses seen in cancer immunotherapy patients despite the lack of checkpoint inhibition and the setting of pancreatic cancer.

Combination treatment with gemcitabine and Poly I:C in murine models of lung and breast cancer synergistically delayed the tumour growth and prolonged the survival of the mice, increasing tumour cell apoptosis and anti-tumour immune responses. The combination treatment also synergistically inhibited tumour cell growth and *in vitro* [52]. Some studies indicate that the inhibition of TLR4 signalling improves the anti-tumour efficacy of gemcitabine whilst another study shows that the expression of TLR4 on breast cancer cell lines reduces the efficacy of the chemotherapeutic paclitaxel which is capable of binding to TLR4 [53]. The use of a TLR4 dependent agonistic polysaccharide derived from *Strongylocentrotus nudus* eggs in combination with gemcitabine in BxPC-3 pancreatic tumour-bearing mice improved tumour suppression through the activation of NK-cells due to increased expression of NKG2D [54] in line with our observations that gemcitabine can increase the expression of the NKG2D ligand MICA on pancreatic tumour cell lines [4].

The use of TLR7/8 agonist R848 treatment of a murine model of pancreatic ductal adenocarcinoma, in the absence of gemcitabine, resulted in smaller tumour mass, increased CD8⁺ T-cell infiltration and activity, decreased T-reg frequency and greater survival duration [55]. These effects are thought to be due, in part, through the expression of TLR7 in tumour associated stromal cells. A study of TLR7 agonist Resiquimod plus anti-PD-1 immunotherapy failed to regress tumours in a murine model of squamous cell carcinoma characterised by the recruitment of immune suppressive CD11b⁺ myeloid cells. The addition of low does Gemcitabine to remove these cells resulted in improved

efficacy of the Resiquimod plus anti-PD-1 immunotherapy combination [56].

Gemcitabine in combination with CpG DNA oligonucleotides demonstrated synergy in pancreatic [57] and lung [58] tumour murine models whilst the synthetic oligonucleotide TLR9 agonist IMO-2055 in combination with gemcitabine and carboplatin induced anti-tumour immune responses [59]. A TLR2 + 9 agonist, rIipo-E7m/CpG, in combination with gemcitabine was capable of clearing large tumours in murine models with the combination proving superior to single agents in reducing the number of immunosuppressive cells whilst increasing the number of tumours infiltrating CD8⁺ cytotoxic T-cells. Gemcitabine enhanced tumour eradication through the inhibition of a broad range of immunosuppressive cells [60]. In humans, an open-label phase III clinical study assessed the addition of Toll-like receptor 9-activating oligodeoxynucleotide PF-3512676 to gemcitabine/cisplatin chemotherapy in patients with non-small-cell lung cancer (NSCLC) did not demonstrate improved PFS or OS over gemcitabine/cisplatin chemotherapy but with increased toxicity including increased influenza-like symptoms among patients receiving PF-3512676, suggestive of the onset of immune responses [61].

These studies highlight the potential of combining gemcitabine treatment with innate immune modulation, particularly via activation of TLR pathways. The majority of studies combining gemcitabine and immune modulation have taken place in the absence of checkpoint inhibition using blocking antibodies to the PD-1/PDL-1 pathway. For immunogenic tumours such as melanoma or lung cancer CPI plus immune modulatory combinations are being investigated [62]. It will be intriguing to determine whether combinations of gemcitabine plus innate immune stimulation and anti-PD-1 therapy improve responses further.

Gemcitabine has also been combined with other immune modulatory agents. Combination treatment with gemcitabine plus PX-478, an inhibitor of hypoxia-inducible factor-1 α , significantly enhanced Gem's anti-tumour effects and increased the proportion of tumour infiltrating T-lymphocytes in Pancreatic tumour bearing immune-competent but not in immune-deficient mice. Only co-treated cells induced DC maturation, phagocytosis and IFN- γ secretion by cytotoxic T lymphocytes in *in vitro* assays [63]. Combination therapy with the PPAR γ Agonist Rosiglitazone and gemcitabine modulated T-cell populations by enhancing circulating CD8⁺ T-cells and intra-tumoural CD4⁺ and CD8⁺ T-cells while reducing the presence of T-regs suggesting that the combination decreases immune suppressive mechanisms in immunocompetent mice [64]. The Immune modulatory drug Lenalidomide was paired with gemcitabine in phase I

dose escalation trial of advanced pancreatic cancer. In this trial, patients demonstrated impaired immune function prior to therapy. Treatment with Lenalidomide augmented T-cell reactivities, which were abrogated by gemcitabine. The addition of lenalidomide to gemcitabine seemed to have no therapeutic impact compared to gemcitabine alone [65]. In C26 colon carcinoma bearing mice the low dose, metronomic administration of cyclophosphamide and gemcitabine demonstrated largely T-cell dependent reductions in tumour growth, curing one third of the mice demonstrating anti-tumour immunity. This combination therapy decreased Foxp3 mRNA, a phenotypic marker of T-regs, whilst increasing Th1 associated IFN- γ mRNA expression within tumour tissue coupled with a reduction in the tumour infiltrating MDSC [66]. Combinations of gemcitabine with other modalities has shown promise. For example, P21 inhibitors in combination with Gemcitabine reduced tumour size and increased the number of intra-tumoral T-cells [67]. Numerous gemcitabine based chemotherapeutic combinations, despite originally studied for their ability to kill tumour cells, demonstrate immune modulatory properties and warrant further study in the context of CPI immunotherapy.

Conclusions

Gemcitabine was first licensed to treat tumours in 1997 and is still used as first or second line chemotherapy, typically in tumours which are often non-responsive to

immunotherapy such as checkpoint inhibition and are in urgent need of more efficacious anti-cancer therapies. These will require the development of innovative therapeutic regimens characterised by combinations of agents administered in a rationally defined dose and sequence. These combinations will likely need to address innate immune priming, tumour cell immunogenicity, the immune suppression of the TME and T-cell priming and activation. It remains to be seen whether gemcitabine is best thought of as a chemotherapeutic of limited clinical use or a potent immune modulator of the tumour microenvironment, however gemcitabine has demonstrated varied immune modulatory effects and may prove useful beyond its ability to induce tumour cell death. Gemcitabine plus CPI combinations have not yet proven consistent clinical efficacy however Gemcitabine paired with innate immune modulators such as IMM-101 or Poly I:C or with additional agents such as Nab-Paclitaxel have shown promise. Clinical trials involving combinations of gemcitabine, checkpoint inhibition and immune modulation are underway (Table 2) and have the potential to address many of the factors limiting the efficacy of immunotherapy in cancers such as pancreatic cancer (Figure 2). We have previously reported on a complete response (>2 years) in a case study of metastatic pancreatic cancer involving treatment with gemcitabine, lenalidomide and a heat killed preparation of *Mycobacterium vaccae*. In a more recent case study, a complete response was observed involving gemcitabine, heat killed *Mycobacterium Obuense* and the CPI

Cancer	Chemotherapy	Checkpoint inhibition	Immune modulation	NCT number
Metastatic pancreatic cancer	Gemcitabine and Nab-paclitaxel	Pembrolizumab (anti-PD-1 antibody)	GB1275, a CD11b antagonist	NCT04060342
Solid tumours including cervical cancer	Gemcitabine and Vinorelbine	Atezolizumab (anti-PDL-1 antibody)	Ro6874281 is a monoclonal antibody linked with IL-2 and targeting FAP on cancer associated fibroblasts	NCT03386721
Pancreatic cancer	Gemcitabine and Nab-paclitaxel	Atezolizumab (anti-PDL-1 antibody)	Intra-tumoural injection with oncolytic virus LOAd703.	NCT02705196
Triple negative breast cancer	Gemcitabine and Nab-paclitaxel	Durvalumab (anti-PDL-1 antibody)	Neoantigen peptide vaccine plus Poly I:C as an adjuvant	NCT03606967

Table 2: New clinical trials involving gemcitabine, checkpoint inhibition and immune modulation.

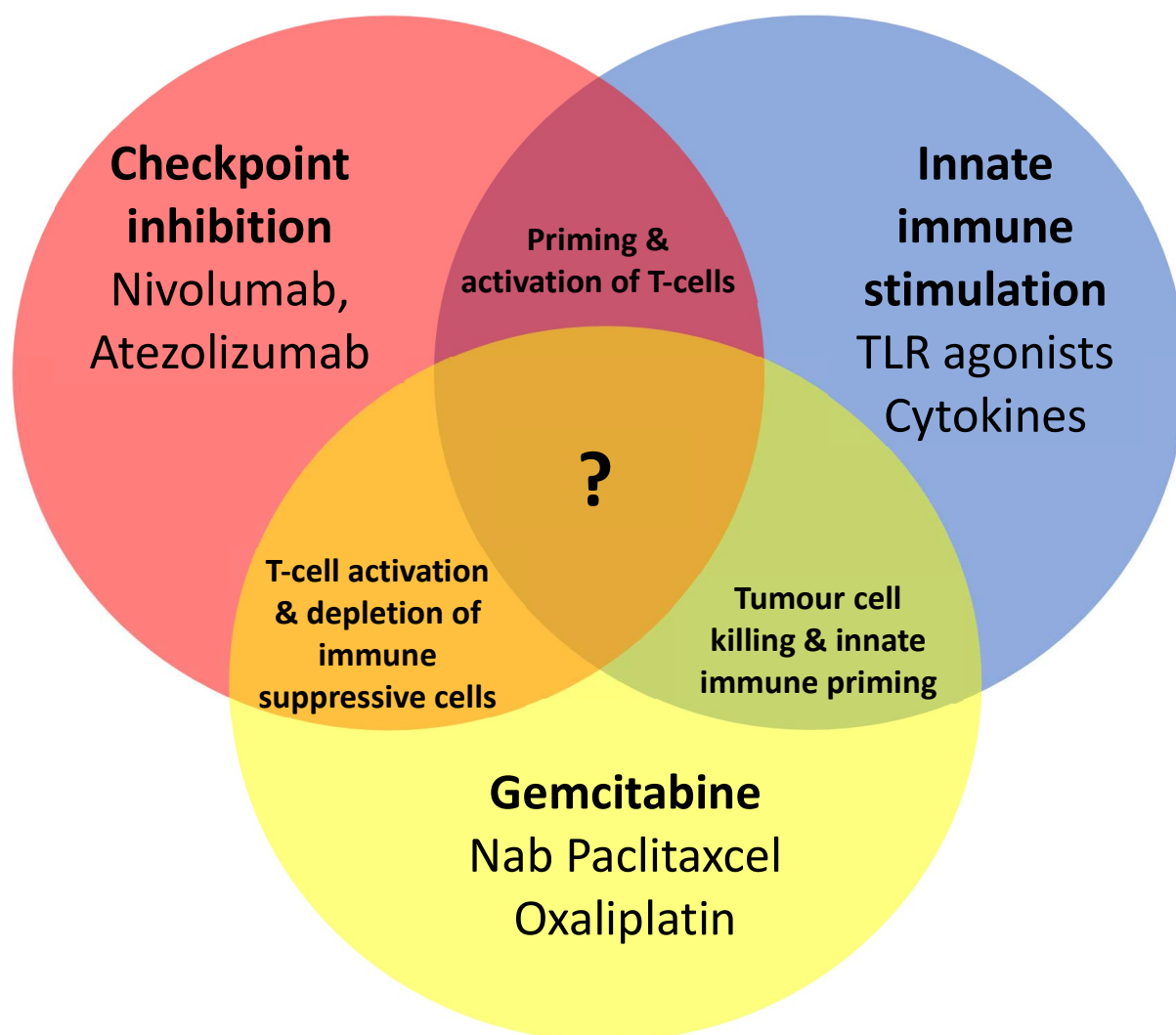


Figure 2: Rationale for gemcitabine-based combination therapy. Multiple components of the anti-tumour immune response need to function in concert for effective checkpoint inhibitor immunotherapy. Many of these components are dysfunctional in patients who are non-responsive to CPI. Single agent immunotherapy does not target enough of these components to activate anti-tumour immune responses. This will need to be addressed with novel immunotherapeutic combinations in order to improve CPI efficacy against non-immunogenic cancers.

Pembrolizumab (unpublished observation). Future trials with gemcitabine, CPI and an innate immune-modulators will prove whether gemcitabine may be a useful component of combination immunotherapy regimens against multiple cancers in the future.

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