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Supernatants of Tumours Treated with Chemotherapy Can Alter Tumour Growth and Development *In Vivo*

WAI MAN LIU, KATHERINE ANN SCOTT and ANGUS GEORGE DALGLEISH

Department of Oncology, Institute for Infection and Immunity, St George's, University of London, London, U.K.

Abstract. *Background: Tumour-derived supernatants are comprised of bioactive substances that have the capacity to transform host systems rendering them more supportive of tumour growth. Certain chemotherapies are able to alter the make-up of these supernatants. Materials and Methods: We explored the effects that vaccination with supernatants derived from tumours may have on tumour growth in a BALB/c model. Results: A number of cytokines were detected in the supernatants capable of increasing B-cell lymphoma 2 (BCL2) protein expression in cancer cells; of note, significantly higher levels of granulocyte-macrophage colony stimulating factor (GM-CSF) were detected in chemotherapy-treated supernatants compared to controls. Vaccinating mice with supernatants from untreated tumours significantly impeded the growth of sub-cutaneous-implanted tumours. However, this anticancer effect was significantly diminished if the supernatants used were from cancer cells treated with gemcitabine. Conclusion: The study lends in vivo support to the idea that tumours produce bioactive components that can influence host biology and that certain chemotherapies can negate these.*

We have previously shown that supernatants derived from tumours are bioactive and exhibit the ability to behave as primitive forms of communications between cancer cells, immune cells and their microenvironment (1-3). Consequently, this suggests that tumours are capable of altering elements of the microenvironment to promote their survival *in situ*. Disrupting this interaction could, thus, serve to reverse this cancer-supporting nature of tumour supernatants and hinder tumour progression (4). These tumour supernatants are made up of a microvesicular fraction comprising RNA transcripts and a cytokine fraction that is tumour-specific. However, some elements are present in a number of tumours and other cells. Importantly, the composition of these supernatants can be altered by treating cells with certain chemotherapies, which ultimately neutralises/negates their cancer-supporting character.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic growth factor that was initially characterised and studied as a cytokine that supported the differentiation of leucocytes down the myeloblastic lineage (5). Clinically, it is used in a way to ameliorate therapy-related neutropaenia in patients with cancer. It has also, due to its activating effect on cell-mediated immunity, been incorporated into therapeutic cancer vaccines as an immunological adjuvant (6). Mechanistically, it can enhance the antigen-processing of professional antigen presenting cells and drive differentiation of immune precursors towards a dendritic cell phenotype (7, 8). However, the majority of clinical trials involving its use as such have been modest at best and commentators have highlighted a duality in its immunological effects that is dependent upon doses that may underlie poor results. Specifically, if used at low doses, GM-CSF was more likely to result in an enhanced immune response. Conversely, at higher doses, it would instead activate myeloid-derived suppressor cells, which could render the immune system less likely to exert suitable anticancer effects (9, 10).

In addition to these effects, tumour-derived supernatants can indirectly alter tumour growth; there are also direct effects caused by GM-CSF and other cytokines found in them. Tumour cells express a range of receptors to which cytokines can bind leading to the activation of intracellular signalling cascades that, in turn, determine the number of cellular processes, as well as cellular fates, such as proliferation and apoptosis. Indeed, a number of tumours produce GM-CSF, thus offering a way for tumour survival that can be supported in an autocrine manner (11, 12).

The effects of supernatants on tumour growth, be it direct or indirect, have been explored in a number of models; however, their impact has not been studied *in vivo*. For this

Correspondence to: Wai Liu, Ph.D., Department of Oncology, Institute for Infection and Immunity, St George's, University of London, 2nd Floor, Jenner Wing, London, SW17 0RE, U.K. Tel: +44 02087255037, e-mail: w.liu@sgul.ac.uk

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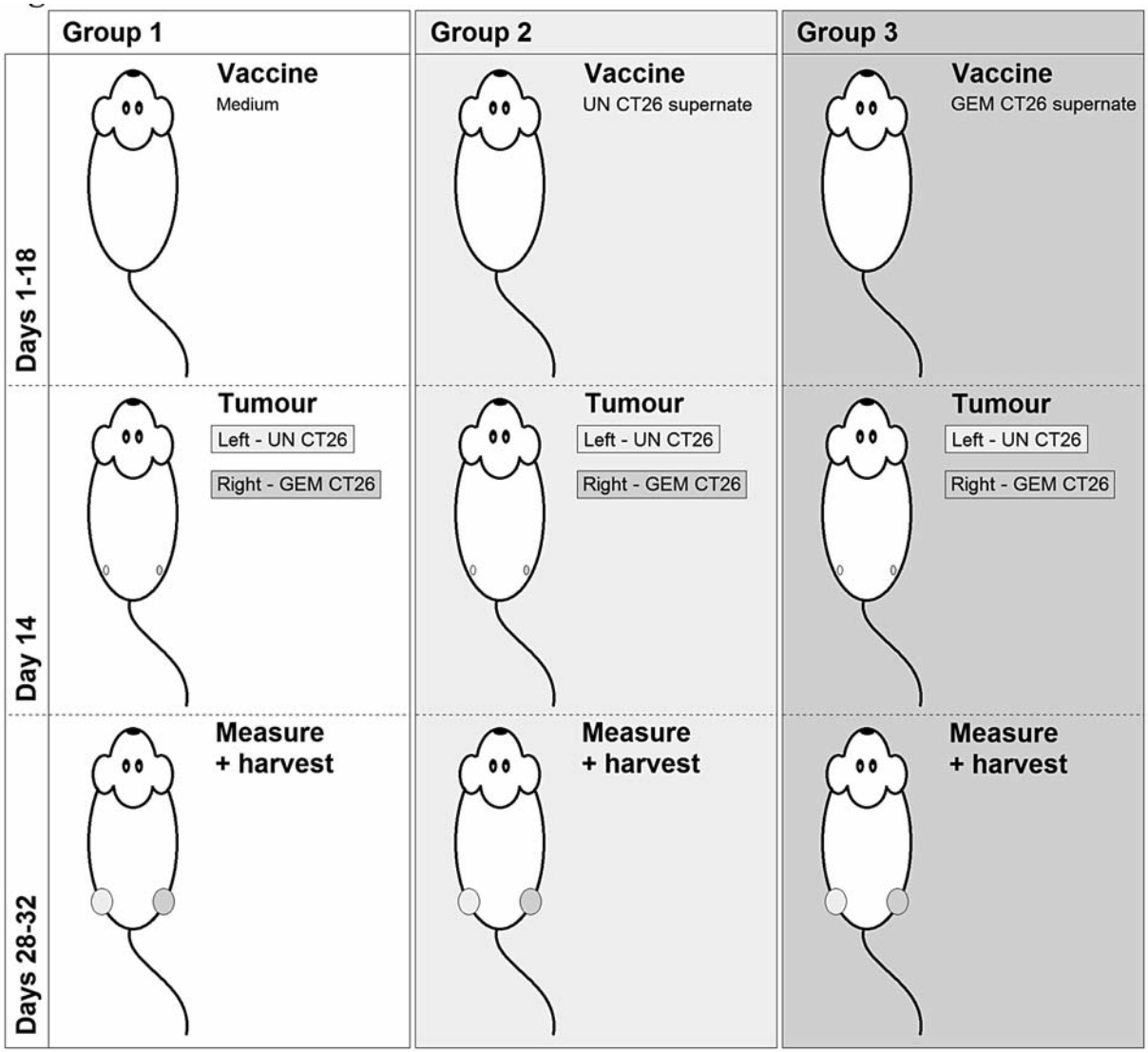

Figure 1. *In vivo treatment schedule. BALB/c mice were separated into three groups of at least five animals and pre-treated with basal medium, supernatant from untreated (UN) CT26 tumour or supernatant from GEM-treated tumours. On day 14, untreated CT26 cells were injected into the left flank of all the mice and tumours challenged with a sub-optimal dose of GEM was injected into the right flank. Tumour growth was measured at regular intervals and mice were sacrificed on day 32.*

reason, we have developed a model in mice with a competent immune system and assessed the effects of supernatants on tumour establishment and development. Throughout our investigation, we have worked on the hypothesis that supernatants from untreated tumours contain bioactive substances that promote/support tumour growth, whilst supernatants from treated tumours will exhibit different characteristics. As part of this approach, we have also explored the idea that chemotherapy can stress tumour cells in a way that leads to changes in the quality and/or quantity of the substances that they exude.

Materials and Methods

Animals, cell line and chemotherapy drugs. Female BALB/c mice were purchased from and maintained by the Biological Research Facility of St. George's University of London. Animals were used at >8 weeks of age and after acclimatisation for at least one week. All procedures were performed according to local rules of the facility and under the project licence approved by the Home Office of the United Kingdom. The CT26 colorectal cancer cell line that was syngeneic to the BALB/c mouse was obtained from the Cancer Research UK Cell Production Unit (London, UK) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine

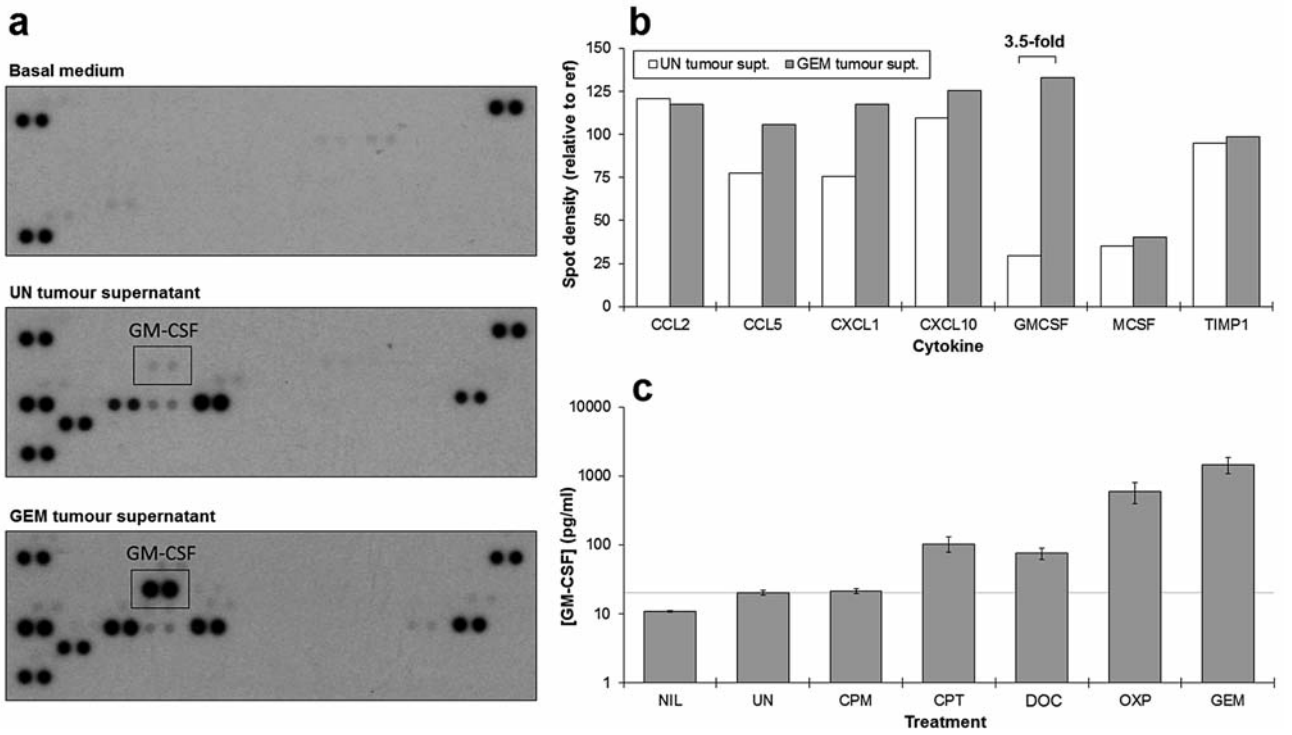

Figure 2. *Cytokine profiles of supernatants from tumours after treatment with chemotherapy. CT26 cells were cultured for 72 h with sub-optimal concentrations of (~IC_{25}: see Materials and Methods). Supernatants were collected and cellular debris removed by centrifugation before assessment of cytokines by a spotted array (a, b). The concentrations of GM-CSF in the different supernatants were further quantified by ELISA (c). Each column represents the means and standard deviations (SDs) of at least four separate experiments.*

serum and 2 mM glutamine (basal medium). Cells were grown at 37˚C in a humidified atmosphere with 5% CO_2 and the number of passages were kept to a minimum and discarded after reaching ~12.

Camptothecin (CPT), cyclophosphamide (CPM), docetaxel (DOC), oxaliplatin (OXP) and gemcitabine (GEM) (Sigma-Aldrich Ltd., Dorset, UK) were dissolved in phosphate-buffered saline at a stock concentration of 10 mM and stored at –20˚C until required.

Cytotoxicity assays. The activity of the chemotherapy agents on CT26 cells were assessed principally by using a standard methyl thiazoletetrazolium (MTT) assay as described previously (1).

Tumour-derived supernatants. Exponentially-growing CT26 cells (0.5×10^5 /ml) were reset in culture flasks and allowed to adhere overnight. CPM (5 μM), CPT (0.1 μM), DOC (0.5 μM), GEM (0.1 μM) or OXP (0.1 μM) were added to the flasks, which represented ~ inhibitory concentration $(IC)_{20}$ for each drug. After 72 h incubation, exhausted media were gently aspirated and cellular debris removed by ultra-centrifugation at 12,000 rpm for 10 min. The cytokine content of each supernatant was then measured by using a proprietary multi-analyte spotted-array product (Proteome ProfilerArray; R&D Systems Europe Ltd., Abingdon, UK) according to manufacturer's instructions. GM-CSF levels in these samples were also quantified using an ELISA kit (Peprotech EC Ltd., London, UK). The supernatants were stored at –80˚C until required and freeze-thaw cycles minimized by aliquoting.

Combination assays. The effect of supernatants on tumour cells and their sensitivity to chemotherapy was assessed by the MTT assay and by cell counting. CT26 cells (3×10^4 /well) were reset onto 96-well plates and allowed to adhere overnight before medium was gently aspirated and replaced with tumour-derived supernatants. Supernatants from tumours treated with CPM, GEM or OXP were also used. Following culture for 48 h, a cytotoxic concentration of CPT (3 μM) was added to each of the well and cell numbers assessed after a further 48 h. In addition, CT26 cells were also plated onto 6-well plates (5×10^4 /well) under the same treatment regimen. Cell numbers were then assessed daily by cell counting under a light microscope with the aid of trypan blue staining to discriminate living/dead cells.

Immunoblot analysis. Cells were harvested and solubilised for immunoblotting analysis as described previously (1). Probing was performed using anti-BCL2 and anti-GAPDH (both from New England Biolabs, Hitchin, UK) at a dilution of 1:1,000 and bands were visualised by using the SuperSignal West Pico chemiluminescent substrate (Fisher Scientific UK Ltd., Loughborough, UK).

In vivo tumour model. This model involved two stages that lasted a total of 32 days (Figure 1). After acclimatisation, BALB/c mice were separated into three groups based upon the tumour supernatant with which they would be treated. This first "vaccination" stage involved an intra-peritoneal injection (100 μl)

of either i) supernatant from untreated CT26 cells; ii) supernatant from CT26 cells treated with GEM; or iii) basal medium. Injections were performed every other day for a total of 18 days. The second "tumour challenge" stage occurred on day 14, which involved CT26 cells that had been processed in 2 different ways: i) untreated cells or ii) cells that had been treated with 0.1 μM GEM for 48 h. These cells were harvested and washed twice and re-suspended in phosphate buffered saline (PBS) at a concentration of 1.5×10^7 /ml. Cell viabilities for both samples were >90%. Tumour suspensions (300 μl) were then injected into the dorso-lateral flanks of the mice. Untreated cells were injected into the left flank, whilst the GEM-treated cells were injected into the right flank. Tumour growth was then checked daily for the remaining duration of the study by taking measurements of the tumour in two dimensions (width (w) and length (l)). Tumour volume was then determined by using the equation: $V = \pi/6\times w\times l^2$. Mice were sacrificed on day 32 or when tumour size violated the conditions of the licence.

Splenocyte analysis. To understand the immune setting of mice at the end of the vaccination period, spleens were extracted from mice for flow cytometric analysis of T-effector cells. Spleens were pooled and then sliced into smaller pieces before being passed through a cell strainer with the aid of PBS. Cells were then centrifuged at 1,200 rpm for 5min prior to the removal of red blood cells by using a hypotonic ammonium chloride solution. Following on from a further washing step in a wash buffer (PBS containing 1% foetal bovine serum and 0.1% sodium azide), samples were incubated at room temperature and in the dark with an antibody cocktail of 1:1,000 phycoerythrin conjugated anti-CD3 and fluorescein isothiocyanate conjugated anti-CD4 (both from BD Biosciences Ltd., Oxford, UK). Cells were washed and fixed in 4% paraformaldehyde and analysed using a FACS Calibur (BD Biosciences Ltd, Oxford, UK). Ten thousand events were acquired from each sample and the percentage distribution of immune subsets was determined using the WinMDI program, v2.9 (http://facs.scripps.edu/software.html).

Results

Certain chemotherapy alters the amount of cytokines produced by tumours. The cytokine composition of the tumour supernatants was assessed by a proprietary spotted-array capable of assessing 40 different cytokines and chemokines. The profile of supernatants derived from untreated tumours was compared against those from tumours treated with GEM at a sub-optimal concentration of 0.1 μM (~IC_{20}). The densities of the spots on the arrays were then determined and the significant hits quantified by ELISA. The results showed that tumours exuded a handful of cytokines that were detectable by the array (Figure 2a). Treating tumours with GEM altered the levels of these proteins produced by tumours and, thus, found in the supernatant; and out of these, GM-CSF was the only one that was drastically altered (Figure 2a, b). Subsequent ELISA showed the concentration of GM-CSF in tumour-derived supernatant was 20±1.7 pg/ml *cf*. 11±0.19 pg/ml in basal medium. GEM treatment resulted in this concentration to increase significantly to 1,455±385 pg/ml (p=0.001) (Figure 2c). The impact of equi-active concentrations of other common chemotherapy drugs on GM-CSF levels was also studied and results showed distinct effects on expression (Figure 2c).

Tumour supernatants can affect responses of cells to chemotherapy. We next assessed the effects of supernatants on the expression of the pro-survival protein BCL2 in tumours. Our previous data suggested that GM-CSF can influence the levels of expression of the pro-survival protein BCL2 (13). The results showed that treating cells with supernatants derived from GEM- or OXP-treated tumours resulted in increased levels of BCL2 compared to those seen in cells treated with the other supernatants tested (Figure 3a). Next, the effect that supernatants may have on the capacity of tumour cells to respond to a cytotoxic challenge was tested by assessing the amount of cell killing caused by a ~IC_{50} concentration of CPT following pre-treatment with different supernatants. CT26 cells were pre-cultured with basal medium or with a supernatant for the first 48 h before the cells were washed and transferred into fresh medium containing CPT. Cell numbers were assessed by MTT and results showed CPT would reduce them only if cells had been pre-treated with basal medium, supernatant from untreated tumour cells or supernatant from CPM-treated tumour cells (Figure 3b-d). Conversely, the CPT-induced reduction in cell numbers was impeded significantly if cells were pre-treated with supernatants from tumours cultured with GEM or OXP (Figure 3c, d). This was most clearly highlighted when comparing the percentage change in cell numbers caused by CPT treatment in cells following pre-treatment with the supernatants (Figure 3d). Flow cytometry also revealed differences in the DNA profile of cells pre-cultured with supernatants before CPT challenge. Specifically, CPT induced a clear blockade in the G_2-phase of the cell cycle and a concomitant increase in the sub-G_1 population (apoptosis). However, these were negated when supernatants from GEM or OXP were instead used to prime the cells (Figure 3e).

Tumour supernatants inhibit tumour growth in vivo. The effect that tumour supernatants may have on tumour growth *in vivo* was examined by introducing a colorectal cancer cell line into the flanks of BALB/c mice to which it was syngeneic. Animals were pre-treated with supernatants for two weeks prior to inoculating with tumour. The supernatants were either from untreated tumours or from those treated with GEM at a sub-optimal concentration of 0.1 μM (~IC_{20}). This was used as a method of pre-exposing mice to the bioactive substances within the supernatants. Following this vaccination, the animals were injected with tumours that mimicked the supernatants that were used, *viz*. untreated and

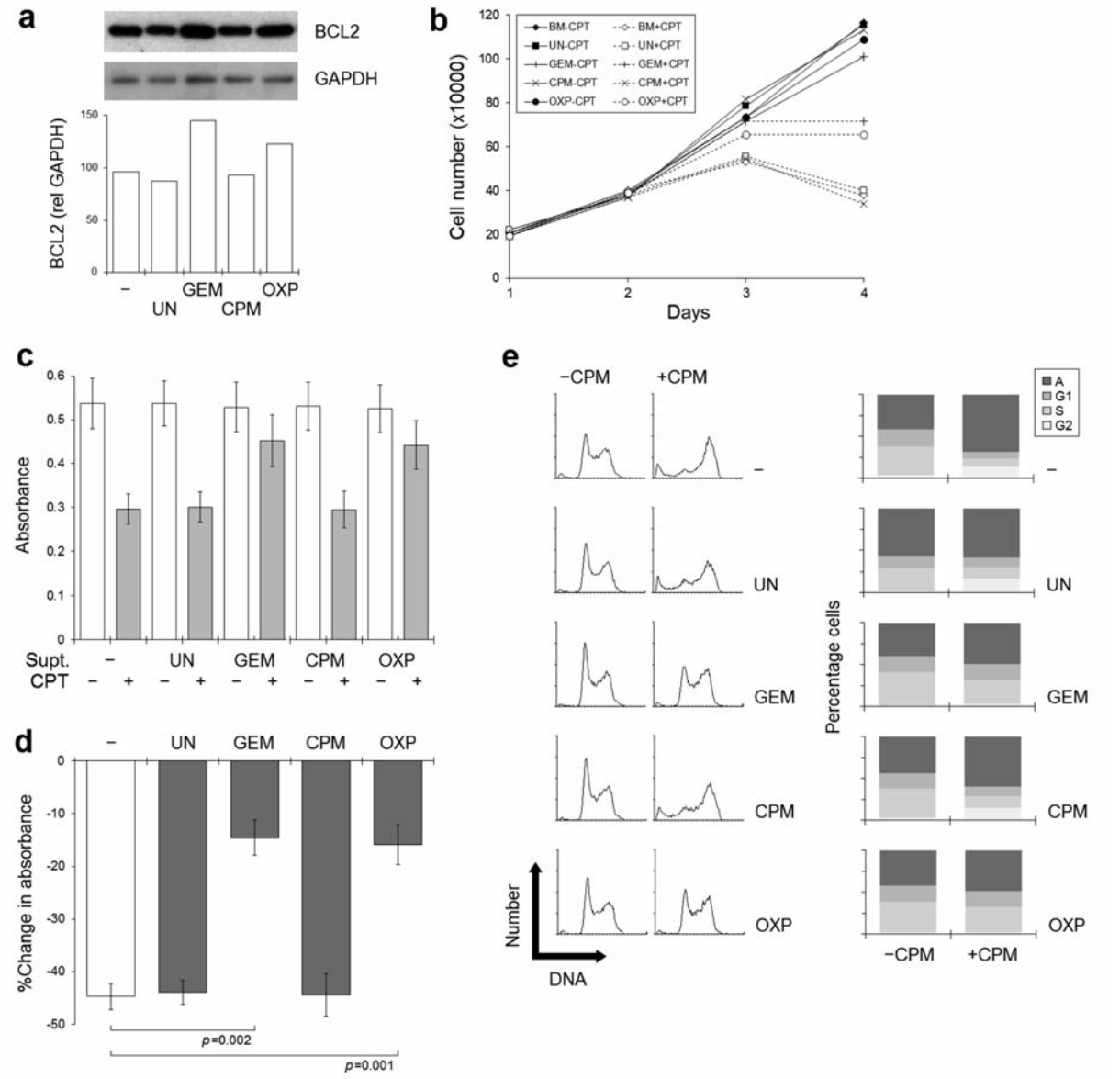

Figure 3. *The effect of supernatants on the sensitivity of CT26 cells to subsequent exposure to camptothecin (CPT). CT26 cells were cultured in vitro for two days with the supernatants from untreated tumours (UN), tumours treated with gemcitabine (GEM), with cyclophosphamide (CPT) or with oxaliplatin (OXP) prior to washing and culturing in fresh medium containing 3 μM CPT. Treatment with the supernatants alone resulted in differences in BCL2; being increased in cells treated with supernatants from tumours treated with GEM or OXP (a). These cells were treated with CPT, which resulted in significant reductions in cell numbers as assessed by cell counting (b) and the MTT assay (c, d). Flow cytometry showed that CPT caused a characteristic blockade in the G_2 phase of the cell cycle and a concomitant increase in the sub-G_1 population (apoptosis/necrosis) only in those samples where BCL2 were not increased (e). In those cells where BCL2 levels were increased, the DNA profile showed a reduced G_1 peak and a modest sub-G_1 peak (e). Columns represent the means and standard deviations (SDs) of at least three separate experiments.*

GEM pre-treated. These two kinds of tumours were placed into opposite flanks of the animal and the effect of the vaccinations on their growth *in vivo* was assessed concurrently. This allowed for the assessment of the effect of vaccination on tumours that had been pre-treated differently in the same mouse.

The results showed that in untreated animals, both "types" of tumours successfully grew and reached sizes at the top-end of the permissible range by day 16 post-inoculation with tumour (day 32 total). Specifically, tumour volumes were 1,045±62 mm^3 *vs*. 961±110 mm^3 for untreated tumours and GEM pre-treated tumours, respectively (Figure 4a). In mice

vaccinated with supernatant from untreated tumours, the growths of the tumours in both flanks were significantly impeded (243±70 mm^3 and 360±70 mm^3 for untreated and GEM pre-treated tumours, respectively) (Figure 4a). Furthermore, although not reaching statistical significance, volumes appeared smaller for the untreated tumours (p=0.087). In comparison, tumour volumes in the animals vaccinated with supernatants from GEM-treated tumours, were much larger than those in mice vaccinated with the other supernatant (748±87 mm^3 and 665±45 mm^3 for untreated and GEM pre-treated tumours, respectively) (Figure 4a). However, these tumours were also significantly smaller than those seen in mice that were not vaccinated. These differences were most noticeable when tumour volumes for each of the three cohorts of mice were consolidated and considered regardless of tumour type (Figure 4b).

The untreated tumours from the left flank of the mice were excised and BCL2 levels measured by western blotting. The results showed that BCL2 levels were higher in those cells from mice that had been pre-conditioned with supernatants from GEM-treated tumour cells (Figure 4c).

Tumour supernatants do not alter the number of CD3+ splenocytes. The spleens from vaccinated mice were processed and splenocytes harvested for further immunophenotyping by flow cytometry. The percentages of CD3+ CD8+ lymphocytes were similar in the mice, being at 5.3%, 5.6% and 5.0% in mice that were either unvaccinated, vaccinated with untreated-tumour supernatant or with GEM-treated tumour supernatant, respectively (Figure 4d). Similarly, there was no difference between the percentages of lymphocytes that were CD3+ CD4+ in untreated mice and in those vaccinated with supernatant from GEM-treated tumours (14.6% *vs*. 15.1%, respectively) (Figure 4d). Additionally, major histocompatibility complex class I H-$2K^b$ expressions on CT26 tumours harvested from the mice were also assessed by flow cytometry and results showed no significant difference in the three groups (Figure 4e).

Discussion

As part of our continuation studies into the effects that chemotherapies may have on tumour growth and survival, we explored the effects that supernatants derived from tumour cells may have on tumour growth in an animal model. The possible role immunity may play in clearing cancer was also tested as our experimental model involved mice with a competent immune system and tumour cells that were syngeneic to the animal. Our earlier studies had shown that these tumour-derived supernatants were composed of bioactive components, such as cytokines and microvesicular bodies containing RNA transcripts, which served to support tumour growth and development *in vitro*. Furthermore, treatment with certain chemotherapies could negate/neutralise these exudates leading to reductions in cancer supporting processes. Therefore, the principal aim of the current study was to assess the growth of a tumour introduced to the flanks of mice pre-cultured with supernatants from tumours that were or were not treated with a variety of common chemotherapeutic agents. Our results showed that tumour growth was inhibited when particular supernatants were used as this "vaccine" and that responses appeared to be related to the amount of GM-CSF within them. Moreover, we also showed that the presence of higher levels of GM-CSF in supernatants could exert a chemo-protective effect in tumour cells, rendering them less sensitive to subsequent exposure to chemotherapy.

Supernatants are the media in which tumours are bathed and, consequently, will collect the exudates released by the tumour. We have previously profiled tumour-derived supernatants and shown them to be rich in cytokines, RNA transcripts packaged in microvesicles and cellular debris (2). These materials are bioactive and can act as primitive routes of communication between cells and the microenvironment. We and others have described the concept that these bioactive exudates of tumours act as "fertilisers" to the seed and soil analogy of Fidler (14). These can serve to tamper with the actions of immune sentinels that are intrinsically in place to counteract tumour invasion or to alter the microenvironment to benefit the tumour (3). These exudates can support cancer-supporting processes, such as angiogenesis; thus, neutralising their influence could disrupt cancer growth. Indeed, we have shown that the composition of supernatants can be altered by culturing the tumours, from which they are derived, with certain chemotherapy rendering them less "cancerous" in nature (2, 4). This novel observation appends the current dogma that chemotherapy works by killing tumour cells directly and alludes to the notion that supplementary effects exist that are similarly anti-cancerous.

Since the constitution of tumour supernatants are cell-type specific, in the first part of the current investigation, we assessed the cytokines in our samples by using a spotted antibody array to detect proteins involved in tumourogenesis. The results showed a handful of cytokines that were detected in the supernatants of untreated tumour cells, which were primarily involved in inflammation. However, the expressions of these same cytokines were altered in the supernatants derived from tumour cells cultured with a minimally cytotoxic concentration GEM. The prominent change was seen in the level of GM-CSF, which was increased 3.5-fold in supernatants from GEM-treated cells. Subsequent investigations revealed that GM-CSF levels could be altered by other chemotherapies too.

The ordered differentiation of haematopoietic precursor cells into cells of the granulocytic and monocytic lineages

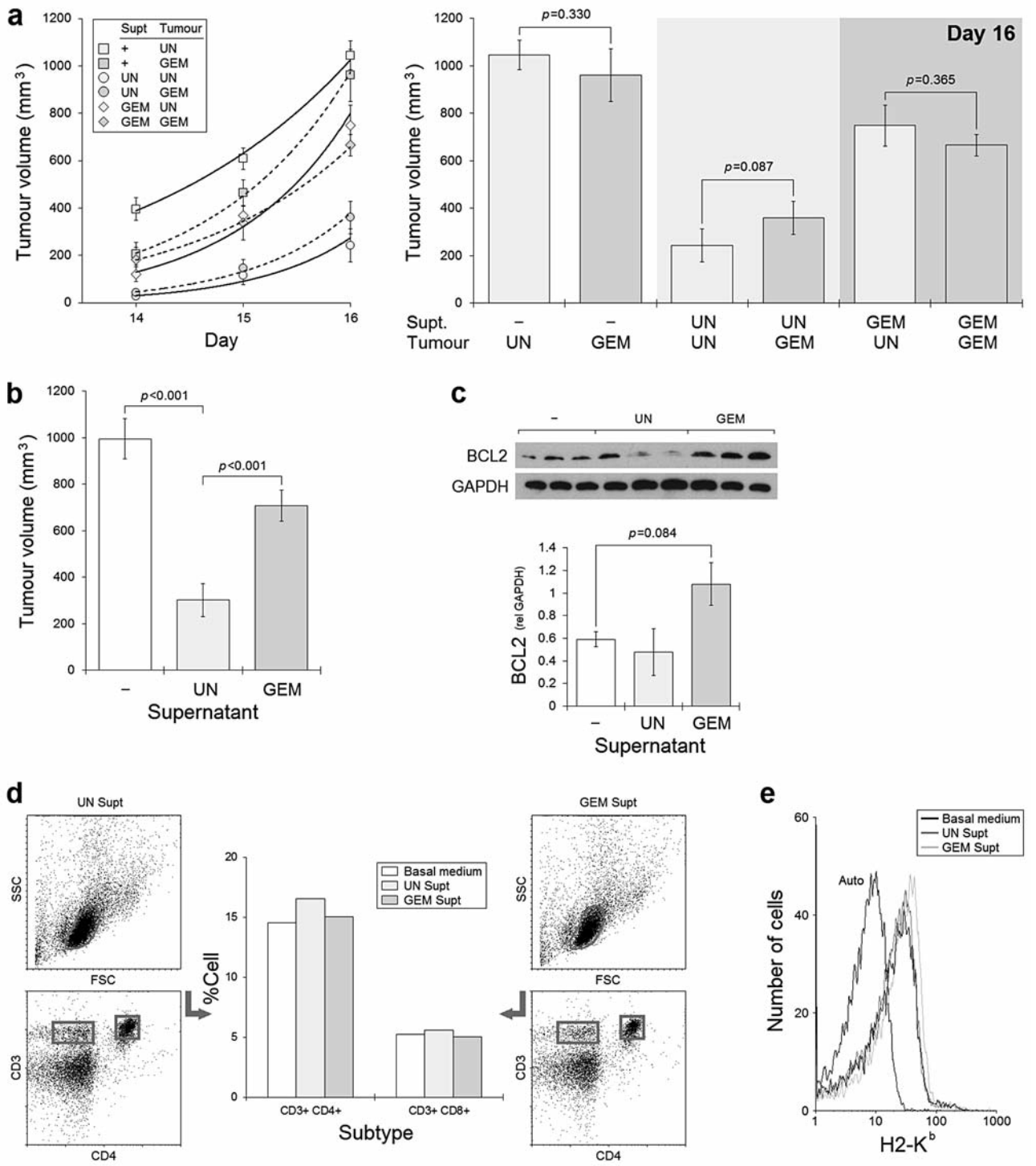

Figure 4. *The effect of supernatants on tumour growth in vivo. BALB/c mice were vaccinated every other day with supernatants from untreated tumours (UN) or from tumours treated with gemcitabine (GEM). Tumours were injected subcutaneously into the flanks and were either untreated or pre-stressed with GEM. Tumour growth was tracked for the remaining duration of the study and measured on days 14, 15 and 16 post-tumour inoculation (a). An average tumour volume, irrespective of their being untreated or treated with GEM was calculated and presented in (b), which shows that sizes were significantly different in mice vaccinated with supernatants from UN tumours or GEM-treated tumours. BCL2 protein levels in CT26 tumours excised from mice were assessed by Western blotting (representative of three) (c). Splenocytes were harvested and pooled from each mouse group and the percentages of cells expressing CD3/CD4 or CD3/CD8 were assessed by flow cytometry (d). H-2k^b expression was also examined on tumours following treatment with the two supernatants (e) (Supt=supernatant).*

are tightly regulated by a series of cytokines and growth factors. GM-CSF is a potent growth factor that was first isolated in the mid-1970s. It is a pleiotropic substance with wide ranging effects. By binding to receptors, GM-CSF can initiate a diverse range of intracellular pathways that underlie all aspects of cellular function. It primarily serves to promote the development and maturation of cells of the immune system. In this regard, it has been employed as part of therapeutic strategies (7) as a way of driving immunity towards a more Th1-biased and tumour-specific setting. However, GM-CSF action appears to be concentration-dependent as inappropriately high doses can result in a hyperactive immune state resulting in severe inflammation and the promotion of a pro-cancerous environment (15, 16). Indeed, in patients suffering inflammatory diseases, the use of GM-CSF therapeutically to ameliorate neutropaenia caused by a number of medical treatments can inadvertently worsen the symptoms of the initial disease. Furthermore, in clinical trials, testing the use of GM-CSF as an adjuvant to a vaccine in melanoma patients showed that lower doses of GM-CSF were beneficial to patients as such an approach served to improve the overall impact of dendritic cells function and did not increase the number of myeloid-derived suppressor cells (MDSCs). Conversely, increasing the doses leads to a negative impact on immune function (8) by increasing the number of MDSCs, thus causing a subsequent reduction in the intensity of specific T-cell responses that are necessary for an anti-tumour response (16-18). The source of the higher levels of GM-CSF is not limited to cells involved in the immune process but has been shown to also arise from *de novo* synthesis by the tumour cells (17), which compounds the problem causing GM-CSF concentrations to be high and, thus, pro-cancerous in nature.

In addition to these effects, GM-CSF can also directly influence the tumour cell survival through its ability to dial the "apoptosis rheostat" towards the anti-apoptosis end (19-24). This shift in the balance of BCL2 superfamily proteins towards anti-apoptotic members suggests a possible way that supernatants containing GM-CSF may interfere with tumour growth, survival and death processes (25). Therefore, we tested the hypothesis that supernatants containing GM-CSF may exert a protective effect in tumours; an effect possibly exerted *via* the up-regulation of BCL2. Our results showed that only those supernatants that contained GM-CSF, as confirmed by ELISA, were capable of increasing BCL2 protein expression in murine CT26 cells. Parenthetically, this was in agreement with our earlier reports that media spiked with GM-CSF is able to increase BCL2 levels in a number of human tumour cells (13). Since the conclusions of our earlier studies showed that exogenously applied GM-CSF had the capacity of rendering tumour cells less sensitive to common chemotherapy, we re-tested this in the current study using a mouse cell line. The effect that a pre-culture with supernatants may have on subsequent sensitivity to chemotherapy was, thus, assessed and the results revealed that pre-treating cells with supernatants derived from GEM- or OXP-treated cells rendered cells less sensitive to the effects of CPT. Specifically, treatment with CPT resulted in a reduction of cell numbers only in control cultures and in those where the pre-treatment involved supernatants from untreated or CPM-treated tumours. Furthermore, the reductions in cell numbers were also associated with reductions in the percentage of viable cells (data not shown).

Following these *in vitro* investigations, we studied the effect of tumour supernatants in mice bearing tumours. Methodologically, two types of supernatants were assessed in this model; supernatants from untreated tumours or those from tumours cultured with GEM. Supernatants were administered every other day into the peritoneum of mice for two weeks. CT26 cells were prepared in two ways; untreated or pre-treated with a sub-optimal dose of GEM and inoculated sub-cutaneously into opposing flanks of the mice. The idea was to use cells with different backgrounds, unstressed or GEM-stressed, and to examine the effects of the supernatants on their growth *in vivo* (Figure 1). In mice vaccinated with untreated tumour supernatant, both types of tumours grew more slowly resulting in final tumour sizes that were significantly smaller than to those seen in untreated mice. Final tumour sizes were also smaller in mice vaccinated with GEM-treated tumour supernatant; however, they were significantly larger than those seen in mice vaccinated with supernatant from untreated tumours. BCL2 expressions in the tumours extracted from the animals were then examined and found higher in those from mice vaccinated with supernatants from GEM-treated tumours. Additionally, there was no difference in H-2K^b expression in the tumours after treatment with either of the supernatants. This was performed as differences in tumour volumes could be due to altered visibilities of tumour to the immune system in these mice (1, 26).

An additional value of injecting two types of CT26 cells to opposing flanks of an immune-competent mouse would be the ability to assess treatment and growth potential *in vivo* using different tumours and vaccinations. Since we have shown that supernatants can influence the quality of cells involved in immunity (1, 27, 28) we tested whether vaccinating mice with the supernatant from untreated tumours could provide the suitable danger signals to inhibit/hinder the growth of untreated tumour cells subsequently introduced to the flank of the animal. We also inoculated into opposing flanks of these mice tumours that had been treated with GEM to investigate the participation of the immune cells as this mismatch of vaccine and inoculated tumour may upset anticancer action. The results showed that there was no significant difference in the volumes of tumours in the sides of the mice and that tumour

growth was dependent upon the vaccine/supernatant used. This suggested that engaging the immunity may not be a mechanism of inhibiting tumour growth in this experimental setting, which was a proposition supported by the work in splenocytes. Specifically, the percentages of CD3+/CD4+ and CD3+/CD8+ cells were assessed by flow cytometry as a crude indicator of immune state and shown to be similar in the groups of mice.

These data suggest that supernatants derived from tumours are bioactive and can influence the biology of tumours. Although we have shown that culturing cells with GEM can cause them to increase their production of GM-CSF that can subsequently influence tumour cell fate, there is no question that the direct effect of GEM is beneficial in the clinic. It is the gold-standard treatment for pancreatic cancer (29) and is also used to treat a variety of solid tumours (30). Additionally, we have shown that supernatants from GEM-treated cells can exert an anticancer effect by reducing angiogenesis (2). However, this paradox may simply be due to the relatively small volumes involved and the high concentrations of the GM-CSF that are found in the supernatants in our *in vitro* and murine models. Similarly, the sequence of administration may also have a bearing as it has been shown that GM-CSF action in cancer patients are schedule-dependent (8). These are important issues that we continue to explore, partly by studying tumour and immune effector cells isolated from the ascites of patients with cancer.

In summary, the current *in vivo* investigation was instigated to support the work of our earlier *in vitro* studies that identified an effect that certain chemotherapy drugs had on altering the profile of supernatants collected from tumours. These supernatants were rich in cytokines and their cytokine content was influenced by treatment with different chemotherapies. Some of these cytokines were known mediators of tumour growth and survival and, thus, when used as a putative vaccine, capable of inhibiting/disrupting tumour growth in mice. Furthermore, this anticancer effect was significantly reduced when using supernatants from certain chemotherapy-treated tumours that had higher levels of GM-CSF. Tumours excised from these mice generally exhibited higher levels of BCL2, which is an anti-apoptotic protein that has been linked to GM-CSF action. The possibility that chemotherapy is able to manipulate the exudates of tumours, thus, rendering them less tumorogenic is fascinating and suggests another facet of chemotherapy that can be explored and subsequently utilised when treating patients with cancer.

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Conflicts of Interest

None.

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