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Electrochemotherapy with cisplatin increases survival and induces immunogenic responses in murine models of lung cancer and colorectal cancer

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ABSTRACT

Electrochemotherapy is an emerging treatment modality for cancer patients which can effectively reduce tumour burden and induce immunogenic cell death. Electrochemotherapy is most commonly used with bleomycin as the drug of choice, here we examine the efficacy of electrochemotherapy with cisplatin. Electrochemotherapy with cisplatin was found to effectively reduce tumour growth in a range of murine models and induce significant intratumoural recruitment of myeloid and humoral immune cells. Following the observations of immune system mobilisation, we have shown an ability of electrochemotherapy to reduce metastatic potential as determined by tumour burden in the lung, and to exert an abscopal effect by reducing growth at distal untreated secondary tumours.

1. Introduction

With increasing numbers of cancer survivors and the long term effects from anti-cancer therapies becoming a burden on an already stretched healthcare sector, there has been a focus to develop localized therapies that reduce side effects while maintaining or improving the efficacy of current therapeutics.

Electrochemotherapy (ECT) is an emerging therapy that has been trialled over the last two decades composed of local delivery of non-ablative electrical pulses to the tumour tissue, termed electroporation (EP) in combination with chemotherapy, which is most commonly administered systemically [1,2]. This therapy offers a cost effective option with minimal side-effects that is proving effective in treating a range of tumour types.

The cell membrane is a physical barrier that prevents the entry of hydrophilic drugs and other molecules into the cell. EP renders this barrier 'permeable', allowing for the influx of otherwise non- or poorly permeable anti-cancer molecules into the cytosol of cancer cells. A series of brief pulses are applied which exceed the dielectric strength of the cell membrane. This results in the reversible formation of pores in the cell membrane, a phenomenon that is dependent on the re-orientation of the lipid structures that constitute part of the membrane, made possible by the electric dipoles they possess. The applied pulses are optimized to minimise heat generation and thus prevent thermal ablation while the pores reseal within minutes leaving the cells intact. This process results in the passive transport of materials through the transiently formed pores, which has been shown to enhance the uptake of chemotherapeutics.

ECT is a relatively new method of tumour reduction and one which has a number of benefits over other modalities. Unlike other physical methods, ECT is non-destructive to healthy tissues surrounding tumour sites making it safe to use in proximity to fragile tissues where radiotherapy may not be advised and because it requires much lower doses of chemotherapy drugs it has a low side effect profile, as reported in a multicentre, European wide study [3], making it a very attractive option for the first line treatment of localized cancer. Its current use is largely restricted to palliative care for the removal of troublesome lesions, but is currently in clinical trials in combination with other treatments.

The increased uptake of drug is dependent on the nature of the chemotherapeutic administered, a 1.5–5-fold increase is seen with lipophilic drugs and up to 1000–5000 fold for lipophobic drugs such as bleomycin. For this reason, bleomycin has been often the drug of choice in ECT studies. However, systemic administration of bleomycin is highly toxic and its dose limiting toxicity is most often a result of intolerable lung injury. It is therefore hypothesized that ECT with cisplatin may offer superior therapeutic benefits, especially for lung cancer where local delivery of bleomycin to the lung is not advisable.

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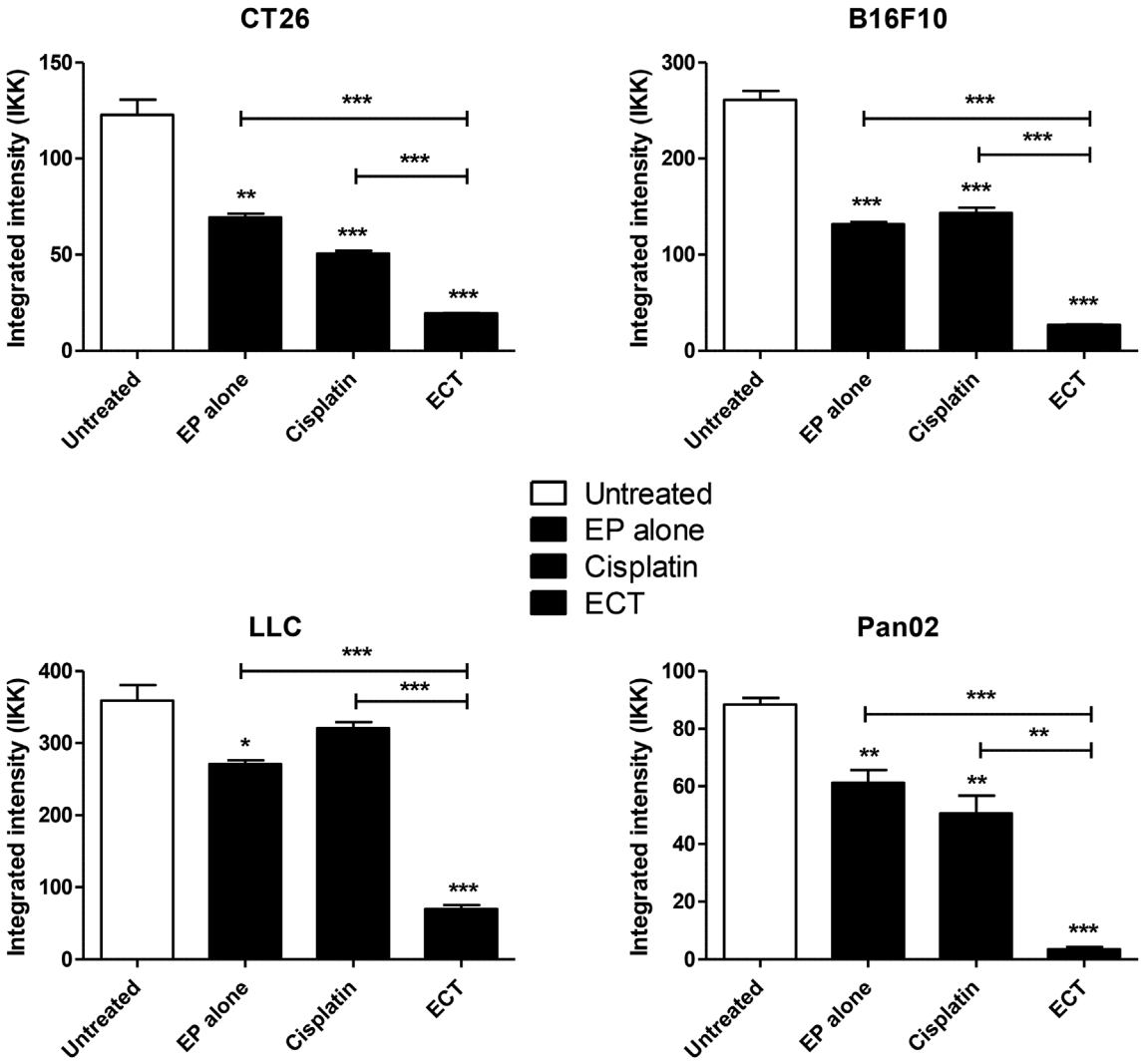

Fig. 1. Recovery of CT26, B16F10, LLC and Pan02 cells following treatment ECT. Cells were subjected to either EP, incubation with cisplatin or ECT. After 24 hrs, viable cells were selected by isolation of adherent cells only. A standardized number of cells were seeded in complete media and incubated for 10–15 days. Colonies were then fixed and stained, and the staining intensity was measured on an infrared imaging system. The graphs show signal intensity of the wells reflecting the number and size of colonies formed. Data represents the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared to untreated unless specified.

Cisplatin is a commonly used first line chemotherapy, especially for lung cancer, and ECT with cisplatin has been shown to increase survival in other tumour models, including melanoma [4,5]. EP effectively enhances the cytotoxicity of platinum based chemotherapeutics, such as cisplatin, 70 fold when used as part of ECT [6].

In addition to enhanced chemotherapy uptake, recent evidence has emerged that ECT can stimulate immune responses, the source of which is not well understood. In addition to the uptake of extracellular proteins seen during EP, intracellular proteins can also escape into the extracellular milieu and can act as a source of damage-associated molecular patterns (DAMPs) which may potentially generate immunogenic responses. Platinum based drugs, like cisplatin have been shown to cause the release of many DAMPs, including; ecto-calreticulin (CRT), High mobility group box 1 protein (HMGB1) and heat shock protein 90 (HSP90) [7–12]. The rapid uptake of chemotherapy can lead to a high concentration of apoptotic and necrotic cell death, which can also act as a supply of DAMPS in the generation of an immune response. As mentioned, ECT is currently restricted to patients in palliative care but has been shown to be successful in the local control of a wide range of cutaneous and subcutaneous malignancies such as melanoma [13–15], as well as internal cancers such as liver metastases [16], brain metastases [17] colorectal tumours [18,19] and devices are currently being developed to allow ECT to be carried out on lung tumours [20,21].

Due to the apparently immunogenic nature of ECT, there has been significant interest in the possibly synergistic combination of ECT with other treatments, such as checkpoint inhibitors, which could lead to the generation of systemic anti-tumoural immune responses and improved patient outcomes. Previous studies have outlined the positive effect of ECT using bleomycin by reducing tumour growth in a range of murine models and inducing an immunogenic form of cell death.

In this study we sought to investigate the efficacy of ECT with cisplatin at reducing tumour growth in a range of murine models, the immunogenicity of treatment and the effects of ECT on metastatic potential and distal tumour growth.

2. Materials and methods

2.1. Cell culturing

Lewis Lung Carcinoma (LLC), B16F10, and CT26 cells were obtained from the European Collection of Authenticated Cell Cultures, Pan02 cells were obtained from the National Cancer Institute. LLC cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine,

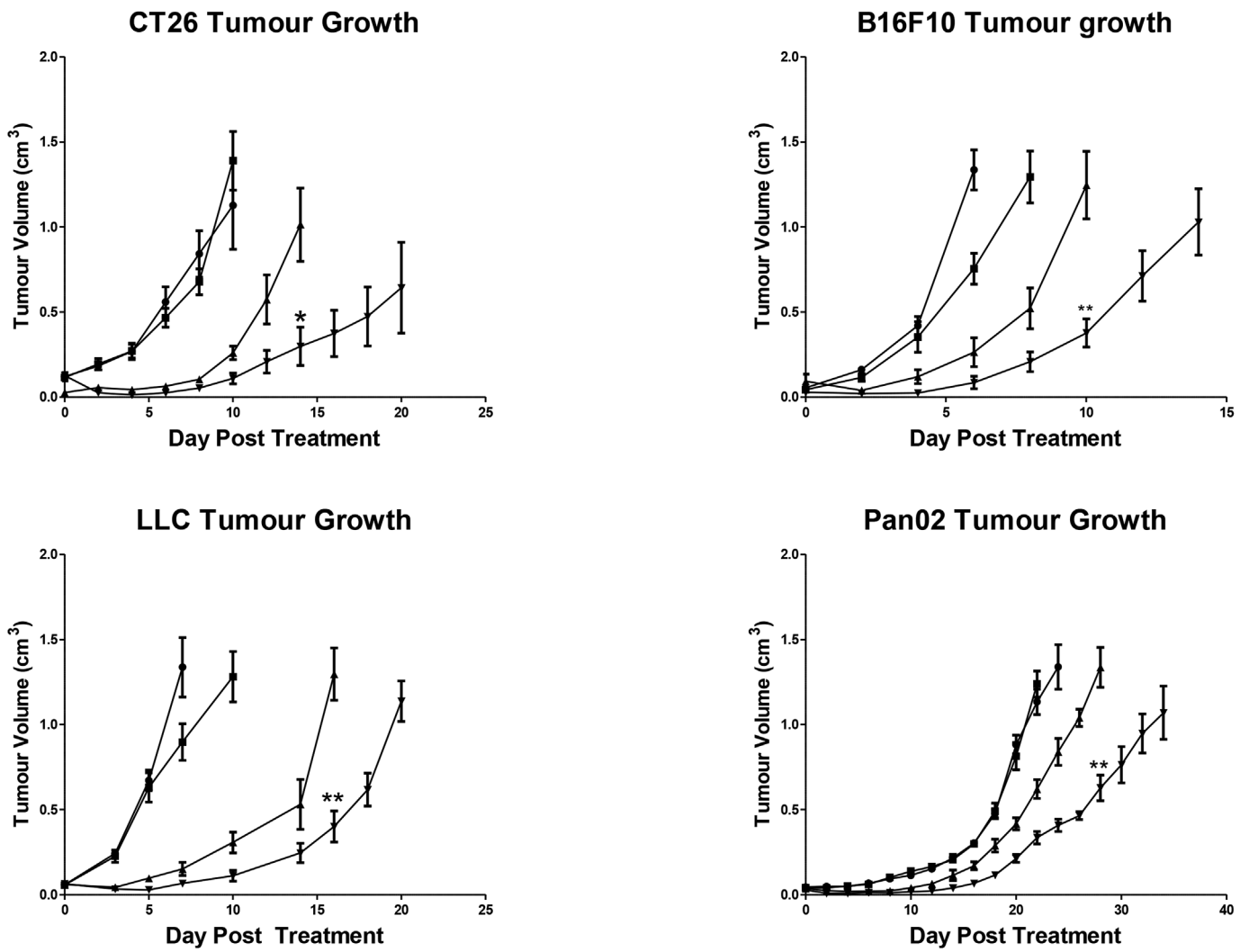

Fig. 2. Response of subcutaneous CT26, B16F10, LLC and Pan02 tumours to ECT. Subcutaneous tumours were treated with cisplatin, EP alone or ECT once they reached a size of approximately 64 mm^3. Untreated tumours received an equivalent intratumoural injection of 200 µl PBS. Data represents the mean ± SEM where n = 6. $*p < 0.05$, $**p < 0.01$ compared to cisplatin alone.

50 I.U/mL penicillin and 50 µg/mL streptomycin. Pan02, CT26 and B16F10 cells were maintained in RMPI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine, 50 I.U/mL penicillin and 50 µg/mL streptomycin. Cell lines were incubated in a 37 °C incubator with 5% CO_2.

2.2. Ethics statement

All animal husbandry and experimental procedures were approved and licensed by the Health Products Regulatory Authority (HPRA).

2.3. Tumour induction & monitoring

Subcutaneous tumours were generated by injecting 1×10^6 CT26 cells, 2×10^5 B16F10 cells, 2×10^5 LLC cells or 1×10^5 Pan02 cells. For the dual flank colorectal (CT26) model the same procedure was performed on the left flank four days after injection into the opposite (right) flank. Tumour volume was determined from two dimensional measurements of the tumour using the formula; $V = ab^2\pi/6$, where 'a' is the longest diameter of the tumour and 'b' is the longest tumour diameter perpendicular to 'a'. For the LLC model lung weights were used as a proxy of metastatic burden.

2.4. In vivo ECT parameters

Tumours were treated once they reached an approximate size of 64 mm^3 [22]. 1 mg/kg cisplatin was made up to a volume of 200 µl in PBS and injected intratumourally. To maximize penetration of cisplatin throughout the tumour, while removing the needle slowly, a fan pattern was made and cisplatin was gradually released, taking care to prevent significant bleeding. Electrical parameters were applied to the tumour using an in-house built electrode with 8 pulses of 1300 V/cm and 100µs duration, delivered at 1 Hz using a Cliniporator™ device (IGEA S.R.L, Carpi, Italy) and a unidirectional arrangement of needle electrodes with 0.3 cm inter-needle distance. The process was repeated as many times as necessary to cover the entire surface area of the tumour including all margins of at least 0.2 cm and covering the tumour in a circular fashion starting at the perimeter and working towards the centre of the tumour.

2.5. In vitro ECT parameters

1×10^6 cells were suspended in 400 µL of electroporation buffer with or without cisplatin and aliquoted into sterile EP cuvettes (Cell Projects, UK). Cuvettes were exposed to eight electric pulses at cell line-specific electric field intensities, with pulse duration of 100 µs and 1 Hz frequency using a BTX ECM 2001 square wave electroporator (BTX, San

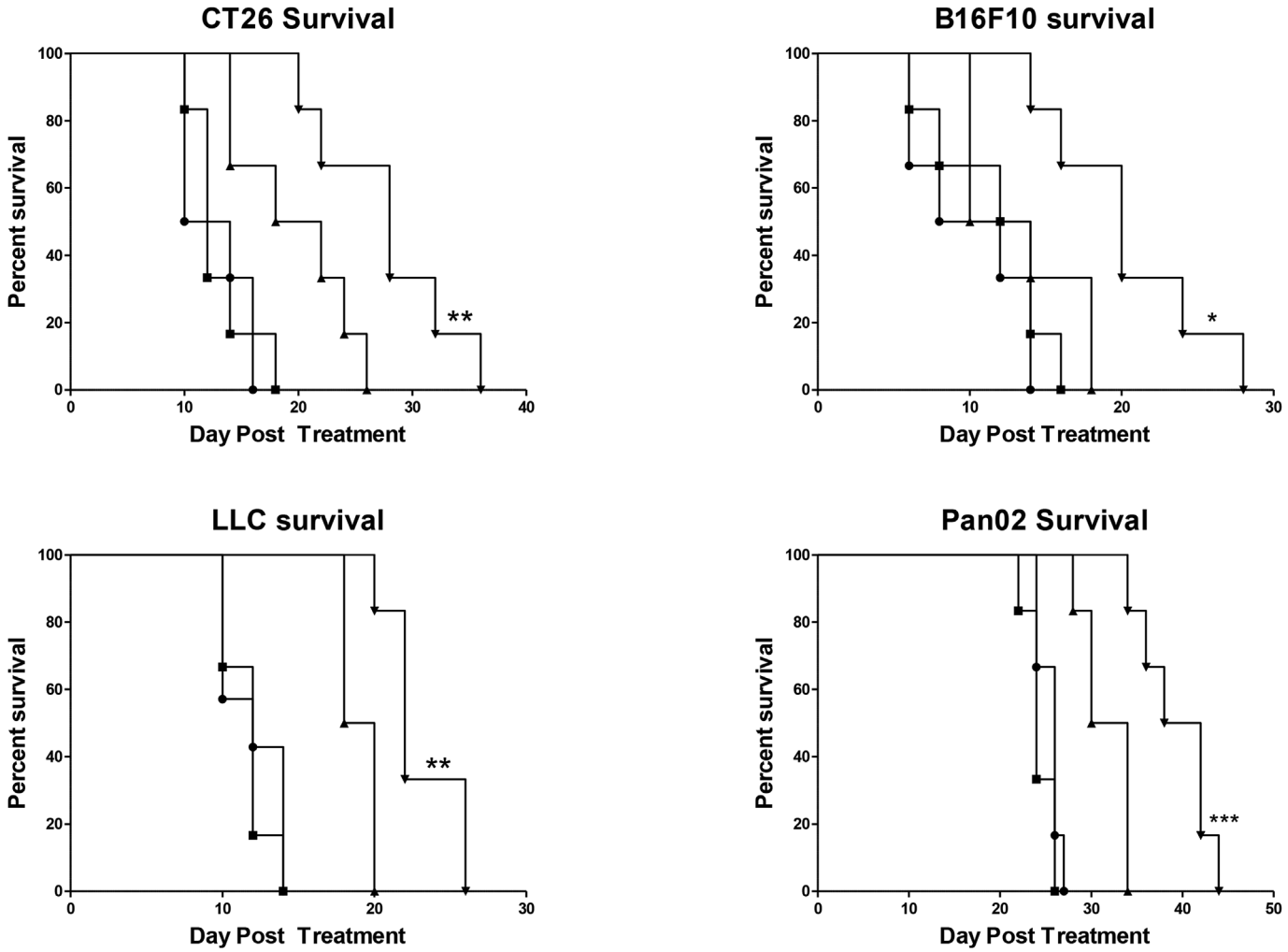

Fig. 3. Survival of mice with subcutaneous CT26, B16F10, LLC and Pan02 tumours treated with ECT. Subcutaneous tumours were treated with cisplatin, EP alone or ECT once they reached a size of approximately 64 mm^3. Untreated tumours received an equivalent intratumoural injection of 200 µl PBS. The survival curves shown are associated with the tumour volume data shown in Fig. 2. Data depicts the number of days survival post treatment. Data represents the mean ± SEM where n = 6. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to cisplatin alone.

Diego, USA). Cisplatin concentrations used were 1 µM for LLC cells and 10 µM for CT26, B16F10 and Pan02 cells, the concentrations were calculated independently for each cell line and were chosen to minimise the effect of chemotherapeutic alone while also minimising the recovery of cells after ECT. Electric field intensities were set at 0.7 kV/cm for B16F10 cells, 1 kV/cm for Pan02 and CT26 cells and 1.3 kV/cm for LLC cells. CT26 and LLC cells were electroporated in PBS, B16F10 cells were electroporated in 125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4 and 2 mM $MgCl_2$ in ddH_20 and Pan02 cells were electroporated in 10 mM HEPES, 250 mM Sucrose and 1 mM MgCl2 in ddH_20. Following electroporation, cells were rested at 37 °C in situ and without any further manipulation for 20min. Cells not subjected to electroporation were left in FACS tubes at room temperature while cuvettes were electroporated and incubated for 20min in tandem with cuvettes.

2.6. Cell recovery assay

Following treatment, cells were seeded in 6 well plates at a high concentration to allow for 60–80% confluency after 24hrs incubation at 37 °C. Following incubation live cells were selected for by discarding the supernatant and washing non-adherent cells away with PBS, adherent cells were trypsinised, counted and resuspended in complete media. Cells were seeded in triplicate in 6 well plates at a cell line specific concentration (400–1500 cells per well) and incubated for 10–15 days until the untreated control contained visible colonies. For visualization of the colonies the supernatant was discarded, cells were fixed in methanol for 10min and then stained in Prodiff stain II for 10min. Excess Prodiff stain II was removed by rinsing under a running tap. Once fully dry the plates were analysed on an Odyssey® CLx system. The signal intensities graphed are representative of Integrated Intensities (I.I. K Counts).

2.7. Processing tumour tissue for flow cytometry

Tumours were recovered 72 hrs after treatment with ECT and divided into three equal portions for flow cytometric analysis. The samples were processed into single cell suspensions and digested using a 3 mg/ml solution of collagenase and dispase at 37 °C for 10 min s. The sample was then mixed further by pipetting through 10 ml and 5 ml pipettes, before being passed twice through 70 µm nylon cell strainers. The cells were washed twice with PBS and re-suspended in 2 mls of ACT red blood cell lysis buffer for 5 min at room temperature. Lysis was stopped by adding 30 mls complete media. Cells were washed with PBS and counted using a NC-100 NucleoCounter (Chemometec) to establish the non-viable and viable population. Cells were aliquoted, at a concentration of 1×10^6 viable cells per 200 µl and fixed in 2% paraformaldehyde for a minimum of 1hr on ice. Cells were incubated in the dark for 30min in staining solution (PBS, 1% BSA, 0.1% sodium azide,

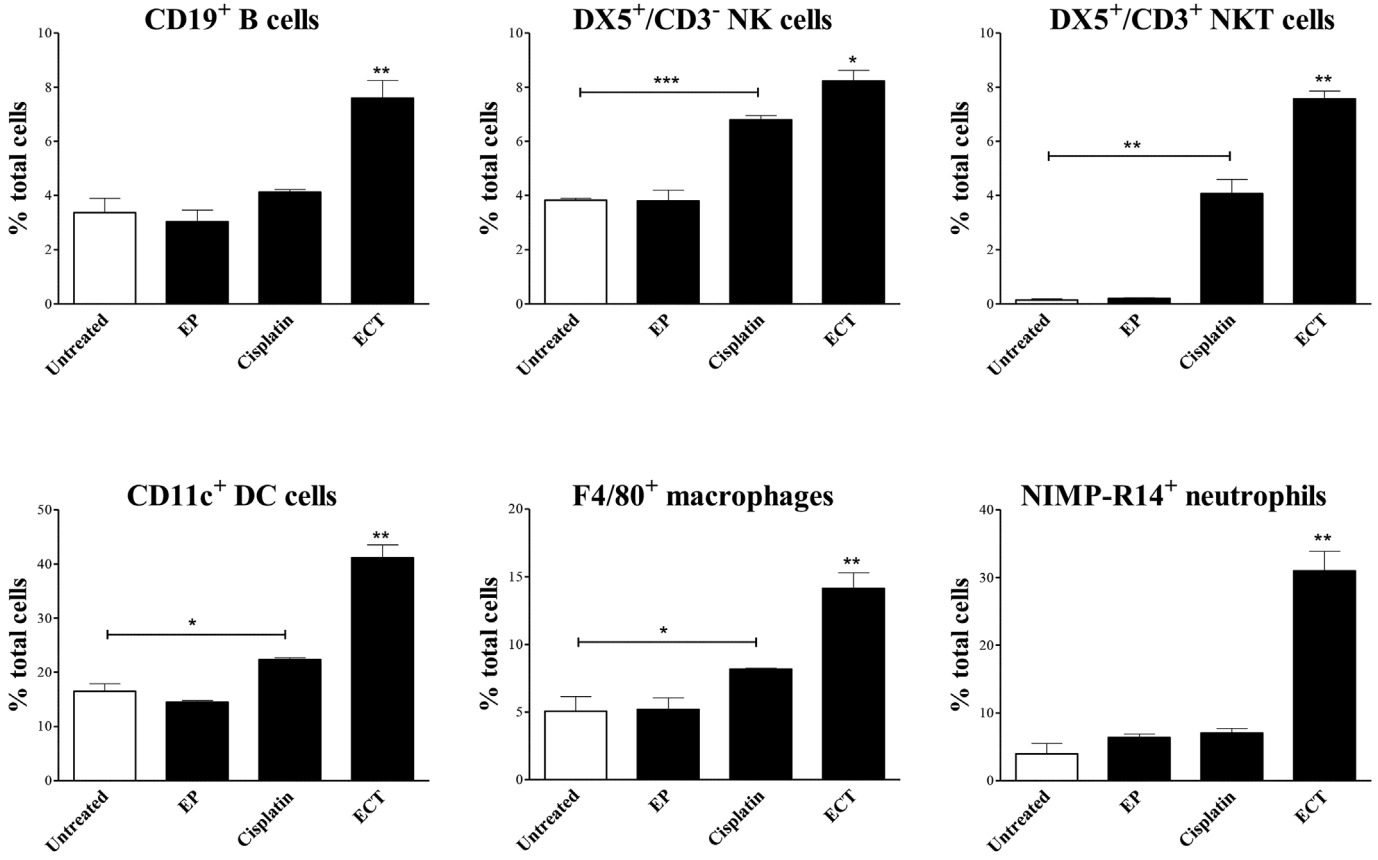

Fig. 4. Intratumoural immune cell infiltration of CT26 tumours 72 hrs post treatment. Subcutaneous CT26 tumours were treated with cisplatin, EP alone or ECT once they reached a size of approximately 64 mm^3. Untreated tumours received an equivalent intratumoural injection of 200 µl PBS. Tumours were excised and analysed for the relative abundance of different tumour infiltrating immune cells by flow cytometry. Cells were gated appropriately to distinguish $CD19^+$ B cells, $DX5^+/CD3^-$ NK cells, $DX5^+/CD3^-$ NKT cells, $CD11c^+$ DCs, $F4/80^+$ macrophages and NIMP-$R14^+$ PMN neutrophils. Levels are expressed as mean ± SEM where n = 3. $^*p \leq 0.05$, $^{**}p \leq 0.01$ compared to all other groups unless specified.

1% mouse serum, 1% rat serum, 3% buffered formalin, amine-reactive viability UV dye (Invitrogen), and fluorescently labelled antibodies). The antibodies used were DX5-APC, CD3-PE, F4/80-FITC (all Abcam), CD19-AlexaFlour488 and CD11c-FITC (both BD biosciences). Cells were washed once and resuspended in 3% formalin. Samples were read on a 5 laser BD LSR II flow cytometer.

2.8. Statistical analysis

In vivo experiments measuring subcutaneous tumour growth and animal survival were performed with a minimum of 5 mice per group. Tumour growth results were tested for significance using an unpaired Student's t-test, while survival results were tested for significance using the log-rank (Mantel-Cox) test. *In vivo* experiments to determine the levels of tumour infiltrating immune cells and experiments to measure the cytokine profile of various tissues were performed with a minimum of 3 mice per group. Graphpad Prism Version 5.03 software was used for all statistical analysis.

3. Results

3.1. Effect of ECT with cisplatin on tumour cell recovery and proliferation

Cell recovery assays were performed to determine the effects of ECT with cisplatin on tumour cell survival and the ability of surviving tumour cells to recover following treatment (Fig. 1). Cisplatin in the absence of electroporation was found to significantly decrease cell recovery in comparison to untreated cells ($p < 0.01$) in CT26, B16F10 and Pan02 cells. No effect was seen on LLC cells ($p = 0.1778$), as a lower concentration of cisplatin was used on this cell line this discrepancy was not unexpected. Electroporation showed a decrease in viability across all cell lines ($p < 0.05$). The parameters of electroporation and cisplatin concentrations vary between *in vitro* and *in vivo* models, the reduction in viability is a commonly seen artefact in *in vitro* models but is considered to have limited *in vivo* effect, the relevance of EP alone is therefore to show that ECT can induce significant loss in viability and that this effect is not caused by EP alone. ECT resulted in reduced recovery of all cell lines ($p < 0.001$).

3.2. Effect of ECT with cisplatin on growth and survival on in vivo subcutaneous tumour models

To examine if the *in vitro* effects would be recapitulated in an *in vivo* model we used a number of subcutaneous tumour models (colorectal cancer, metastatic melanoma, metastatic pancreatic cancer and metastatic lung cancer) to determine the effects of ECT with cisplatin on tumour growth (Fig. 2) and survival (Fig. 3). Treatment with ECT significantly inhibited the rate of tumour growth across all models tested. In the CT26 model, the decrease in tumour growth with ECT proved to be significant when compared to all other groups (untreated $p < 0.01$, EP alone $p < 0.001$ and cisplatin alone $p < 0.05$). In the metastatic B16F10 model the effectiveness of ECT in reducing the rate of tumour growth was also significant compared to all other groups (untreated $p < 0.001$, EP alone $p < 0.001$ and cisplatin alone $p < 0.01$). In the Pan02 model ECT significantly inhibited the rate of tumour growth (untreated $p < 0.001$, EP alone $p < 0.001$ and cisplatin alone $p < 0.01$). Similarly, in the LLC model ECT significantly inhibited growth compared to all other groups (untreated $p < 0.001$, EP alone

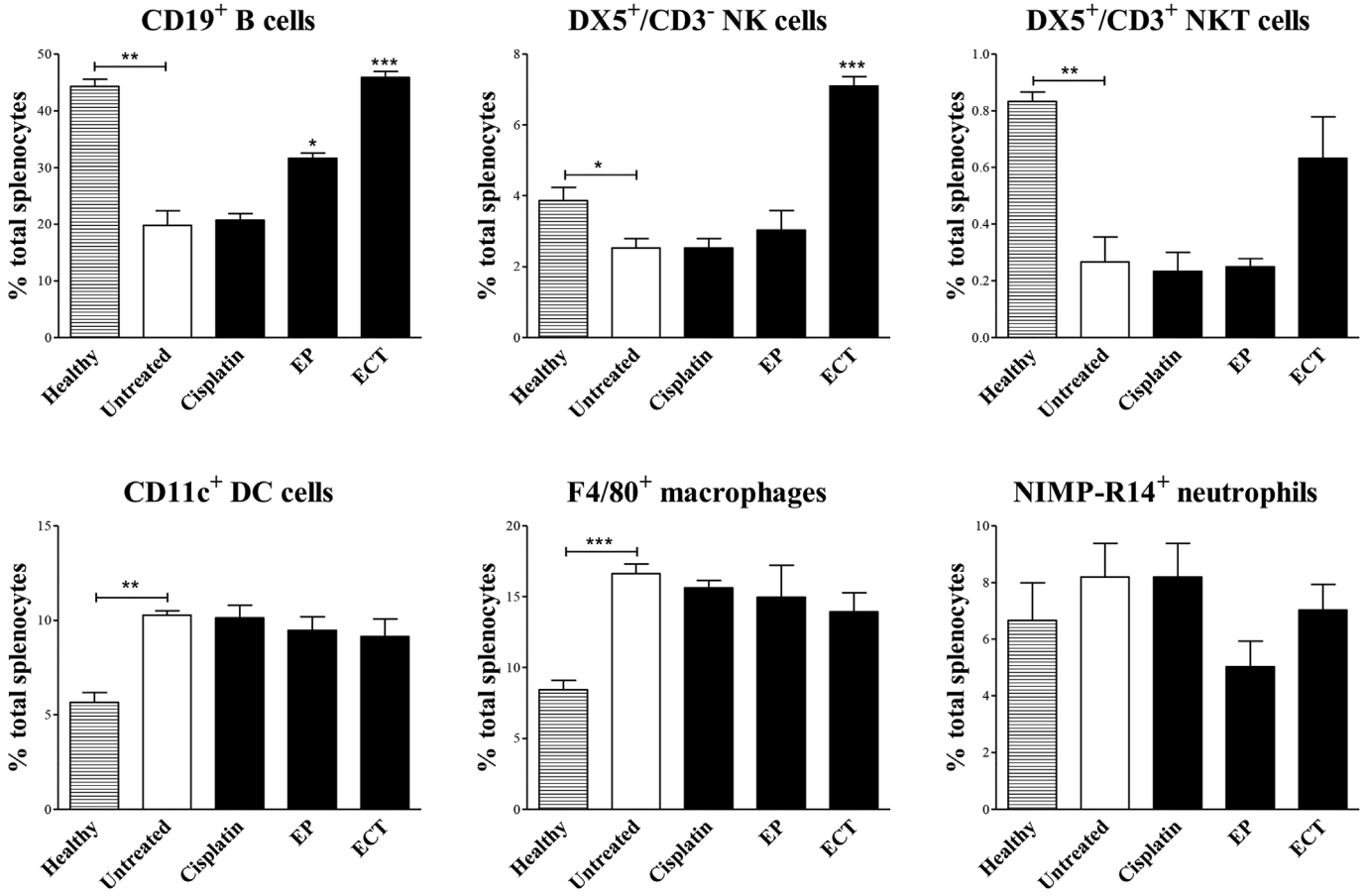

Fig. 5. Splenic immune cell levels in CT26 tumour bearing mice 72 hr s following treatment. Subcutaneous CT26 tumours were treated with cisplatin, EP alone or ECT once they reached a size of approximately 64 mm^3. Untreated tumours received an equivalent intratumoural injection of 200 µl PBS. Spleens were excised 72 h s following treatment and analysed for the relative abundance of different tumour infiltrating immune cells by flow cytometry. Cells were gated appropriately to distinguish $CD19^+$ B cells, $DX5^+/CD3$- NK cells, $DX5^+/CD3^-$ NKT cells, $CD11c^+$ DCs, $F4/80^+$ macrophages and NIMP-$R14^+$ neutrophils. Levels are expressed as mean ± SEM where n = 3. ***$p < 0.001$ compared to untreated unless specified.

$p < 0.001$ and cisplatin alone $p < 0.01$). Animals treated with ECT survived significantly longer than any other group across all models (CT26 $p < 0.01$, B16F10 $p < 0.01$, Pan02 $p < 0.001$ and LLC $p < 0.001$).

3.3. Effect of ECT with cisplatin on immune cell infiltrates

ECT with bleomycin has been recently shown to produce an immunogenic form of cell death [23], to determine if the reduction in tumour growth seen following ECT was inducing an immune response we next examined the effect of ECT on intratumoural immune cell infiltration (Fig. 4) and on intrasplenic immune cells (Fig. 5). CT26 tumours and matched spleens were excised 72 hrs following treatment and analysed by flow cytometry. The immune markers stained for were; $CD19^+$ B cells, $DX5^+/CD3^-$ natural killer (NK) cells, $DX5^+/CD3^+$ NKT cells, $CD11c^+$ dendritic cells (DCs), $F4/80^+$ macrophages and NIMP-$R14^+$ neutrophils [24]. EP had no effect on intratumoural immune cell recruitment, however cisplatin resulted in an increase in NK cells ($p < 0.001$), NKT cells ($p < 0.01$), DCs ($p < 0.05$) and macrophages ($p < 0.05$). ECT resulted in significantly enhanced recruitment of all cells examined in comparison to all other treatment groups; B cells ($p < 0.01$), NK cells ($p < 0.05$), NKT cells ($p < 0.01$), dendritic cells ($p < 0.01$), macrophages ($p < 0.01$) and neutrophils ($p < 0.01$).

Tumour growth reduced intrasplenic levels of B cells ($p < 0.01$), NK cells ($p < 0.05$) and NKT cells ($p < 0.01$) and increased the levels of DCs ($p < 0.01$) and macrophages ($p < 0.001$). Cisplatin had no impact on intrasplenic immune cells and EP resulted in a marginal increase in recruitment of B cells ($p < 0.05$), but failed to restore B cell numbers to the level seen in healthy individuals ($p < 0.01$). Treatment with ECT had no effect on intrasplenic levels of DCs, macrophages or neutrophils but did result in an increase in NK cell numbers (< 0.01) and B cells ($p < 0.001$) to levels seen in healthy mice (difference in means non-significant). ECT also resulted in a notable non-significant increase in NKT cells ($p = 0.0972$).

3.4. Generation of tumour specific response following ECT treatment

Following the documentation of both local and systemic effects on immune cell recruitment in response to ECT we next sought to examine if local delivery of ECT could affect tumour growth at distal untreated sites. A dual flank CT26 tumour model was used which mimics secondary disease. The primary tumour, inoculated 4 days before the secondary tumour, was treated with no treatment to the secondary tumour. As expected tumour growth of the treated tumour following ECT (Fig. 6a) was significantly decreased ($p < 0.01$), however tumour growth at the secondary site (Fig. 6b) was also significantly decreased following ECT ($p < 0.05$).

Further to this effect on distal tumours we examined the anti-cancer effect of ECT on spontaneous metastases by examining lung weight as a proxy for metastatic burden in the LLC tumour model (Fig. 6c). Lung weights significantly increased in untreated tumour bearing mice compared to healthy mice ($p < 0.001$) indicating metastatic spread. EP alone showed no effect on lung weight however ECT induced a significant decrease in lung volume following treatment ($p < 0.001$),

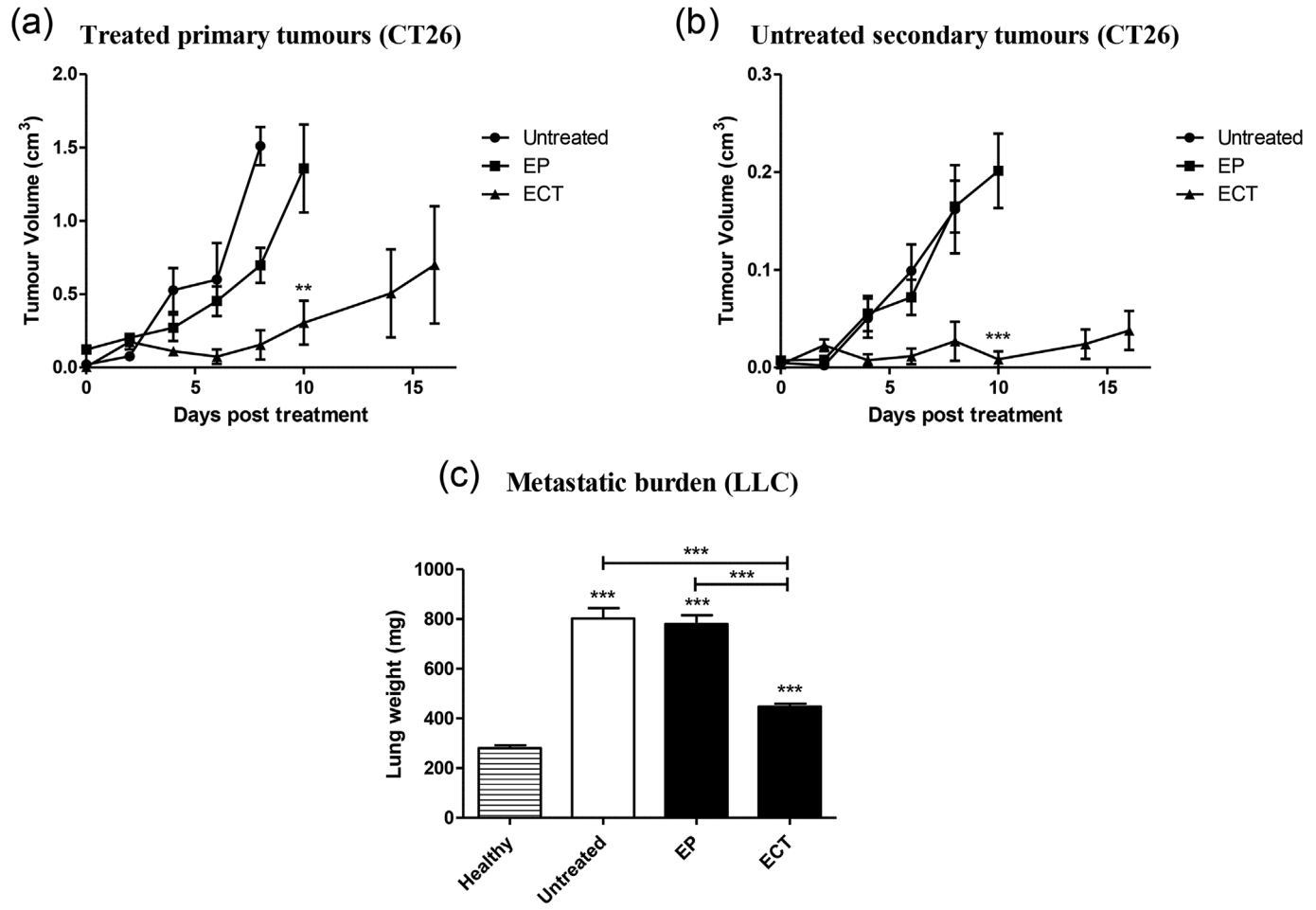

Fig. 6. Effect of ECT on distal tumour growth and metastatic potential. The primary tumours of dual flank subcutaneous CT26 tumour models were treated with cisplatin, EP alone or ECT once they reached a size of approximately 64 mm^3. Untreated tumours received an equivalent intratumoural inject of 200 µl PBS. Tumour growth curves showing the effects of the treatments on (a) directly treated primary tumours and (b) untreated distal tumours on the contra-lateral flank. Separately subcutaneous LLC tumours treated in the same manner. Lungs were excised 7 days following treatment and weighed immediately as a measure of metastatic burden (c). Data represent the mean ± SEM where n = 5 for tumour growth rate (a–b) and (n = 3) for lung weight analysis (c). ***$p < 0.001$ compared to untreated unless specified.

however tumour burden was still apparent in comparison to healthy mice ($p < 0.001$).

While every precaution is taken to prevent its occurrence, during *in vivo* electroporation with needle electrodes it is possible that a level of cisplatin may leak from the tumour into the peritumoural space, which may contribute to the reduced systemic growth.

4. Discussion

ECT as a monotherapy has already been used successfully in the treatment of a wide range of cutaneous and subcutaneous malignancies such as melanoma [13–15], as well as internal cancers such as liver metastases [16], brain metastases [17] and colorectal tumours [18,19]. However both refinement of current use and translation to further treatment regimens are primary goals of the research community.

Here we have shown that ECT with cisplatin retards tumour growth *in vivo* and results in enhanced overall survival in a range of tumour models.

The form of cell death and the nature of the immune response induced have increasingly come under the spot light, in an effort to design systemically efficacious therapies. A recent paper by Calvet et al. showed that CT26 tumour cell death induced by ECT with bleomycin displayed hallmarks of immunogenic cell death and that ECT could act as a therapeutic vaccine, preventing regrowth of CT26 tumours [23].

In agreement with the immunogenic cell death following ECT with bleomycin, we observed the intratumoural infiltration of a broad range of immune cells including B cells, NK cells, NKT cells, neutrophils, macrophages and DCs. While an anti-cancer effect was seen, it is yet to be elucidated whether the intratumoural influx of various immune cells are contributing to disease clearance or therapeutic resistance [25]. These results do however indicate that ECT with cisplatin has significant intratumoural effects beyond cell death resulting in the mobilisation of a robust immune response.

In a pilot study to examine if the effects of ECT transcended beyond the local tumour environment, a dual flank colorectal model was used. The second tumour is inoculated 4 days after the primary tumour and is a primitive model used to study the effect of treatment on distal tumour growths. ECT was found to significantly inhibit tumour growth at the distal tumour indicating an abscopal effect following therapy. This effect is not entirely unsurprising, while other groups have not documented such an effect, a case report examining ECT with calcium has shown a local and distant anti-tumour effect following treatment [26].

Immune escape is one of the major hallmarks of tumour biology and it is a central tenet to the development of anti-cancer immunotherapeutics [27]. Research is now also focused on identifying the immunogenicity of current therapeutics, with an aim to harnessing it effectively to develop more efficacious treatment regimens. The clinical efficacy of ECT with T cell checkpoint inhibitors has been investigated

in the clinic with positive outcomes seen in both case-reports and larger trials [28–30].

Here, we have shown that ECT with cisplatin displays an intrinsic ability to generate systemic anti-tumour effects and is a logical candidate for further examination in combination with immunotherapy.

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Declarations of interest

None.

Conflicts of interest

None.

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