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Potentiation of electrochemotherapy effectiveness by immunostimulation with IL-12 gene electrotransfer in mice is dependent on tumor immune status

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ABSTRACT

Electrochemotherapy (ECT) exhibits high therapeutic effectiveness in the clinic, achieving up to 80% local tumor control but without a systemic (abscopal) effect. Therefore, we designed a combination therapy consisting of ECT *via* intratumoral application of bleomycin, oxaliplatin or cisplatin with peritumoral gene electrotransfer of a plasmid encoding interleukin-12 (p. t. IL-12 GET). Our hypothesis was that p. t. IL-12 GET potentiates the effect of ECT on local and systemic levels and that the potentiation varies depending on tumor immune status. Therefore, the combination therapy was tested in three immunologically different murine tumor models. In poorly immunogenic B16F10 melanoma, IL-12 potentiated the antitumor effect of ECT with biologically equivalent low doses of cisplatin, oxaliplatin or bleomycin. The most pronounced potentiation was observed after ECT using cisplatin, resulting in a complete response rate of 38% and an abscopal effect. Compared to B16F10 melanoma, better responsiveness to ECT was observed in more immunogenic 4 T1 mammary carcinoma and CT26 colorectal carcinoma. In both models, p. t. IL-12 GET did not significantly improve the therapeutic outcome of ECT using any of the chemotherapeutic drugs. Collectively, the effectiveness of the combination therapy depends on tumor immune status. ECT was more effective in more immunogenic tumors, but GET exhibited greater contribution in less immunogenic tumors. Thus, the selection of the therapy, namely, either ECT alone or combination therapy with p. t. IL-12, should be predominantly based on tumor immune status.

List of abbreviations

AM ± SE	arithmetic mean ± standard error
BLM	bleomycin
CDDP	cisplatin
CR	complete response
CTX	chemotherapy
ECT	electrochemotherapy
EP	electroporation
GAR	growth after rechallenge
GET	gene electrotransfer
GrB	granzyme B
H & E	hematoxylin and eosin staining
IC_{50}	drug concentration that reduces cell survival to 50%
IHC	immunohistochemistry

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IL-12	interleukin-12
MEA	multielectrode array applicator
MHC-1	major histocompatibility complex class 1
OXA	oxaliplatin
PAS	periodic acid-Schiff staining
PD-L1	programmed death-ligand 1
p. t.	peritumoral

1. Introduction

Novel treatment strategies pursue the idea of blocking the negative regulators of immune activation using immune checkpoint inhibitors in combination with ablative therapies. Ablative therapies in addition to direct cytotoxic effects can prime the immune system [1] by inducing immunogenic cell death [2] and releasing damage-associated molecular patterns [3] and tumor antigens that can induce *in situ* vaccination [4,5]. However, it is unlikely that local ablative therapies completely subvert the immunosuppressive tumor microenvironment [6]. Therefore, additional immunostimulation to boost the response is also desired.

Among immunostimulators, interleukin-12 (IL-12) is a cytokine with proven effectiveness [7] but a disputed toxicity profile. Namely, despite promising preclinical data, recombinant IL-12 caused systemic toxicity during clinical testing [8]. However, gene electrotransfer (GET) of a plasmid encoding IL-12 has been proven safe and controlled, thus representing an effective therapeutic approach [9–13]. GET can be performed intratumorally, intramuscularly or peritumorally into the skin (p. t.), which is an immunologically active tissue and thus an attractive target [14–16].

Electrochemotherapy (ECT) is an effective ablative therapy that uses electroporation to facilitate cisplatin (CDDP) and bleomycin (BLM) uptake into cells, potentiating their cytotoxicity [17,18]. To increase the armamentarium of drugs for ECT, oxaliplatin (OXA) [19] and calcium [20] were introduced. ECT also elicits an immune response that contributes to its local effectiveness [19,21,22]. Although the overall local effectiveness of ECT in clinics is 80% of local tumor control and 60–70% of complete response (CR) rate after once-only treatment [17], its abscopal effect has only been observed sporadically [23].

Here, we evaluated the combination of ECT with p. t. GET of plasmid DNA encoding murine IL-12, which was used to increase the already high local effectiveness of ECT. First, we compared the local effectiveness of ECT based on the drug used and tumor immune status. Second, we tested to what extent p. t. IL-12 GET contributes to the local effectiveness of ECT. We decided to use intratumoral ECT and p. t. GET to spatially segregate the treatments. Finally, we investigated whether adjuvant IL-12 GET therapy could also elicit an abscopal effect.

2. Materials and methods

2.1. Cell lines and animals

Briefly, 4 T1 mammary carcinoma cells (ATCC; obtained 2017) and CT26 colorectal carcinoma cells (ATCC; obtained 2017) were cultured in Advanced RPMI 1640 Medium. B16F10 (ATCC) and B16F10 tdTomato (gift from Muriel Golzio, Institute of Pharmacology and Structural Biology, Toulouse, France) malignant melanoma cells were cultured in Advanced MEM. Both media were supplemented with 5% fetal bovine serum (Thermo Fisher Scientific), 10 mM L-glutamine (GlutaMAX, Thermo Fisher Scientific), 100 U/ml penicillin (Grünenthal) and 50 mg/ml gentamicin (Krka). Cells were grown in a 5% CO_2 humidified incubator at 37 °C. All cells were mycoplasma negative (MycoAlert™, Lonza). B16F10 cells were authenticated in 2019 (IDEXX BioAnalytics).

Seven- to eight-week-old (20–22 g) female C57BL/6NHCrl, C57BL/6Nhsd, BALB/cOlaHsd, BALB/cAnCrl and Crl: SKH1-Hn^{hr} mice (Envigo RMS S.r.l. or Charles River Laboratories) were used. All procedures were performed in compliance with guidelines for animal experiments of the EU Directives, ARRIVE Guidelines and the permission of the Ministry of Agriculture Forestry and Food of the Republic of Slovenia (Permission No. U34401–1/2015/43). Mice were randomly divided into groups consisting of 6–13 animals, for each experiment, the number of animas is indicated in the graphs or/and in the figure captions. All the experiments including mice were performed at least once; however, the experiments showing tumor growth after the combination therapies on B16F10 melanoma were repeated two or three times. Animal weight and general health, which were determined through the examination of the coat and demeanor, were monitored daily.

2.2. Plasmids

Empty (pCTRL-ORT) and therapeutic plasmid encoding IL-12 (pORF-mIL-12-ORT) [24] were isolated and purified using the EndoFree Plasmid Mega Kit (Qiagen) and diluted in endotoxin free MiliQ water (625 ng/μl). Plasmid concentration and quality were determined as described previously [24].

2.3. Electrochemotherapy in vitro and clonogenic assay

Electrochemotherapy *in vitro* and clonogenic assays were performed as described previously [19]. The inhibitory concentration for each drug that reduced cell survival to 50% (IC_{50}) was determined graphically in each experiment from the survival curve. IC_{50} for CDDP/ECT and OXA/ECT in B16F10 were adopted from our previous study [19]. A Corning cell Counter (CytoSMART Technologies) was used to measure tumor cell size *in vitro*.

2.4. Tumor induction and treatment protocol

For primary tumors, a suspension of 1×10^6 B16F10 and 0.5×10^6 CT26 or 4 T1 cells (100 μl 0.9%, NaCl) was injected subcutaneously into the right flanks of mice. For the B16F10 dual-flank model, 1×10^6 (day 0) and 0.8×10^6 (day 3) B16F10 cells (100 μl, 0.9% NaCl) were injected into the right and left flanks, respectively. For the CT26 dual-flank model, 3×10^5 and 5×10^4 CT26 cells (100 μl, 0.9% NaCl) were injected into the left and right flanks, respectively.

Treatment (Fig. 1) was performed when primary tumors reached 40 mm^3 as measured by Vernier caliper and calculated as described in [19]. During treatment, mice were anesthetized with 1–3% isoflurane (Chiesi). Single p. t. GET was administered using noninvasive multielectrode array (MEA) applicator with circular distribution of electrode pins (Iskra Medical) as described previously [15]. For repetitive p. t. GET, the procedure was repeated every two days (day 0, 2, 4) or at longer intervals (day 0, 6, 10, 14). Animals in the control group were injected with endotoxin free MiliQ water. The intratumoral ECT consisted of a 40 μl injection of BLM (10 μg, 5 μg, 4 μg, 2.5 μg, 1.5 μg; Medac), OXA (170 μg, 85 μg; Teva) or CDDP (80 μg (80 μl), 40 μg, 30 μg, 20 μg, 10 μg, 2.5 μg; Fresenius Kabi AG) in 0.9% NaCl as previously described [19]. Animals from the control group were injected with 0.9% NaCl. Biologically equivalent low doses, *i.e.*, doses that lead to 25 day tumor growth delay and caused no CR, of BLM, OXA and CDDP in ECT were determined in B16F10 tumors, and these same doses were then also used in 4 T1 and CT26 tumors. Furthermore, due to the tumor model-dependent antitumor effectiveness of ECT, biologically equivalent doses of BLM and CDDP in ECT were also examined for 4 T1 and CT26 tumors. Study design is presented in Fig. 1D.

Combined treatment consisted of p. t. IL-12 GET followed by ECT after 5 min. Two different protocols of combined treatment with repetitive GET were also performed (Fig. 1). Tumor-free mice (CR) were challenged with a subcutaneous injection of 1×10^6 B16F10, 0.5×10^6 4 T1 or 0.5×10^6 CT26 cells (100 μl, 0.9% NaCl)) into the left flank 100 days after tumor remission. Tumor growth after rechallenge (GAR) was followed, and mice that remained tumor-free for 100 days were marked

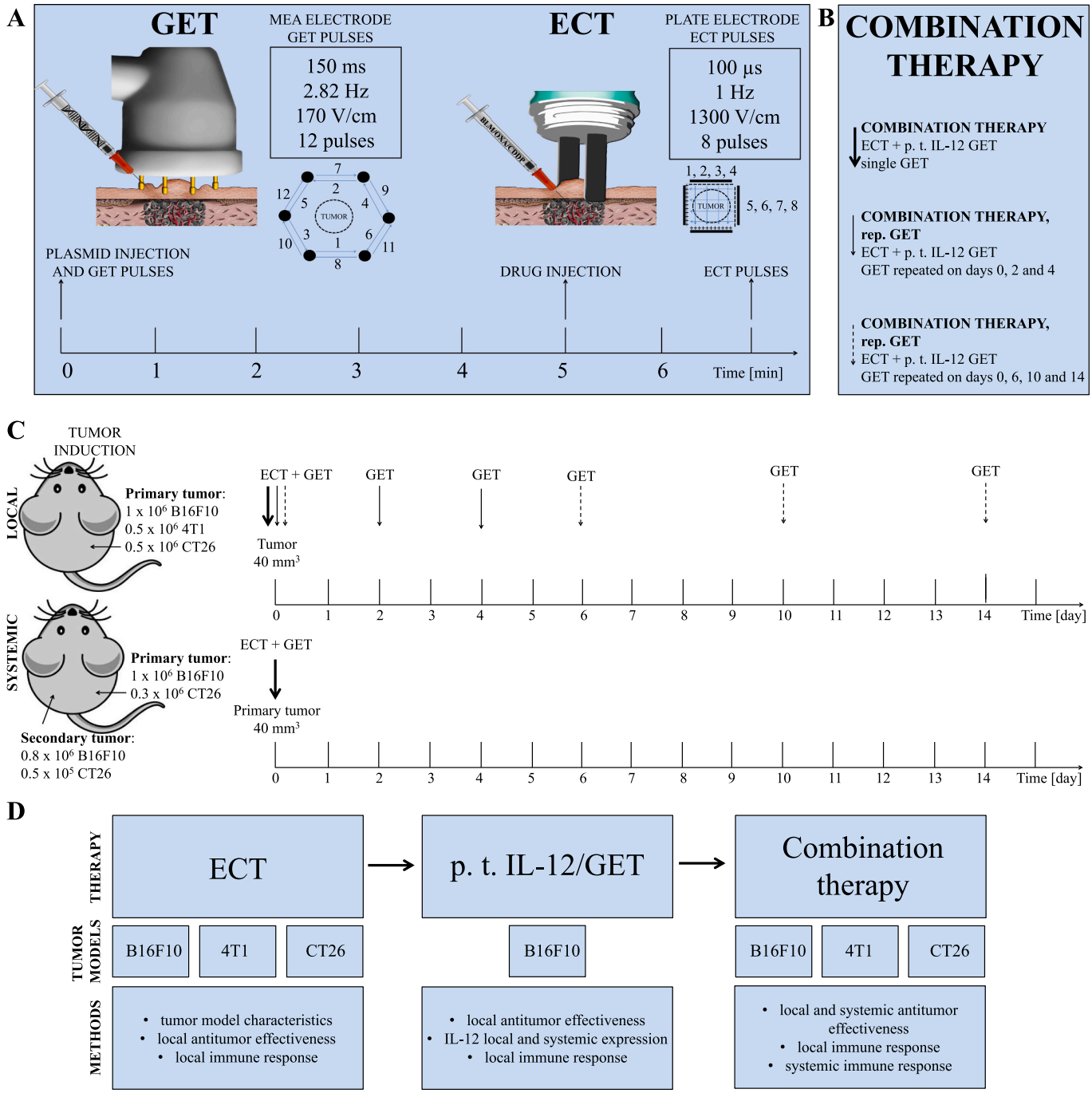


Fig. 1. Treatment protocol and study design. A, Treatment protocol consisting of p. t. IL-12 GET and ECT is presented. B, Three different combination therapies were tested: 1) combination therapy, including ECT and p. t. IL-12 GET, both on day 0 (bold arrow); 2) combination therapy, including ECT and p. t. IL-12 GET, both on day 0 with additional p. t. IL-12 GET on day 2 and day 4 (nonbold arrow); and 3) combination therapy, including ECT and p. t. IL-12 GET, both on day 0 with additional p. t. IL-12 GET on day 6, day 10 and day 14 (dashed arrow). C, Local and systemic antitumor effects of the therapy were evaluated. The therapy was administered when primary tumors reached 40 mm^3. D, Study design.

as resistant to GAR.

2.5. Histological analysis

Mice were sacrificed, and primary and contralateral tumors were excised 6 and 4 days, respectively, after therapy. Tumors were formalin-fixed and paraffin embedded [19], and consecutive sections were cut. Hematoxylin and eosin staining (H & E; cell density), Masson's trichrome staining (collagen content), Periodic acid-Schiff staining (PAS; proteoglycan content) and immunohistochemical staining (IHC; granzyme B (GrB), Foxp3, IL-12 and CD31; Abcam; **Supplementary Table S1**) were performed. IHC was performed using EXPOSE Rabbit-specific HRP/AEC or HRP/DAB detection IHC kit (Abcam) as previously described [19]. A brightfield microscope (BX-51 microscope, Olympus) connected to a DP72 CCD camera (Olympus) was used to capture images (40 ×, 100 × and 400 × magnification). Given a low number of IHC^+ cells per field of view, a semi-quantitative scoring system was used as follows: (−) negative staining, (+) low positivity (≤5 IHC^+ cells), (++) moderate positivity (6–10 IHC^+ cells), and (+++) high positivity (>10 IHC^+ cells).

In vivo cell size was measured using ImageJ. From each of the three H & E stained tumor sections, 30 cells (10 per section) were randomly selected, and an average diameter was calculated from two perpendicular cell diameters. To compare the composition of the extracellular matrix (ECM) in the tumors, the collagen and proteoglycan content (percentage of positive area per field of view) and cell density (number of nuclei per field of view) were analyzed using ImageJ (400 × magnification). The vascular parameters (400 × magnification) were determined as described previously [25].

2.6. ELISA

Quantification of m-IL-12 serum concentrations was performed using an ELISA assay (ELISA Quantikine Mouse IL-12 p70 Immunoassay, R&D Systems) according to manufacturer's instructions. Serum was collected on days 0, 2, 4, 6 and 10 post single or repetitive p. t. IL-12 GET as described previously [15].

2.7. Flow cytometry

The antibodies used are listed in **Supplementary Table S1**. For *in vitro* analysis, one day after ECT *in vitro* [19], cells were trypsinized, washed twice with PBS, counted and 1.5×10^6 cells were stained on ice for 15 min. In case of Annexin V staining (FITC Annexin V Apoptosis Detection Kit with 7AAD (Biolegend)) cells were stained according to manufacturer's instructions.

For *in vivo* analysis (tumor immune status and systemic immune response) the 40 mm^3 tumors, spleens or draining lymph nodes were finely chopped with scalpels and the obtained fragments digested in Hanks' Balanced Salt solution (with Calcium and Magnesium; GIBCO) containing Collagenase Type 2 (2 mg/ml, Worthington Biochem) and DNase I (2 U ml, Thermo Fisher Scientific) for 45 min with gentle shaking at 37 °C and strained through 50 μm strainers (Sysmex). Cells were then incubated on ice for 5 min in Red Blood Cell Lysis Buffer (BioLegend) and counted. Then, 2×10^6 cells were stained on ice for 30 min for surface antigens followed by staining of intracellular antigens (when stained) using the Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific). When intracellular antigens were not stained, the cells were fixed in IC Fixation Buffer (Thermo Fisher Scientific) after staining of surface antigens.

Cells were analyzed with FACSCanto II flow cytometer (BD Biosciences). FMOs or isotype controls were used to determine the gating strategy (**Supplementary Fig. S1**). Data were analyzed using FlowJo software (Tree Star Inc.).

2.8. Statistical analysis

For statistical analysis and graphical representations, SigmaPlot Software (version 13.0, Systat Software Inc.) was used. All data were tested for distribution normality using the Shapiro-Wilk test. Data are presented as the arithmetic mean (AM) ± the standard error of the mean (SE). One-way ANOVA followed by the Holm-Sidak test for multiple comparisons was used for the determination of significant differences ($p < 0.05$) between groups.

In Kaplan-Meier analysis (Survival Log-Rank Test), tumor volumes of 300 mm^3 were counted as events for the construction of the curves. Additionally, average survival, *i.e.*, time after treatment when tumors reached 300 mm^3, was calculated. Fold change in programmed death-ligand 1 (PD-L1) and major histocompatibility complex class 1 (MHC-1) expression was calculated by dividing the average expression of the control group (percentage or MFI) with the average expression of treatment groups.

3. Results

Firstly we wanted to elaborate the ECT in B16F10, 4 T1 and CT26 tumor models. Thus, we examined whether tumor cell characteristics, tumor immune status, ECM and vasculature affect ECT's antitumor effectiveness. Next, to boost the local effectiveness of ECT and especially to induce a systemic antitumor effect, we combined it with p. t. IL-12 GET in B16F10 tumor model. Importantly, the p. t. IL-12 GET was also investigated as a monotherapy. Two additional treatment protocols were tested to improve the combination therapy, exploiting higher doses of either chemotherapeutic drugs or IL-12. We also investigated some of the immune-related background mechanisms and systemic effect of the therapies. Lastly, the antitumor effect of the combination therapy was investigated also in 4 T1 and CT26. The study design is presented in Fig. 1D.

3.1. Effectiveness of ECT depends on tumor immune status

Biologically equivalent doses of BLM (5 μg), OXA (85 μg) or CDDP (40 μg) in ECT, which were determined in B16F10 melanoma, were tested in 4 T1 and CT26 tumor models. CT26 tumors were the most sensitive to ECT, and up to 100% curability was observed. Mice bearing 4 T1 tumors exhibited up to 50% CR; however, no cures were observed in B16F10 tumors (Fig. 2A**, Supplementary Table S2, Fig. S2**). As s measure of systemic toxicity, the body weight loss of the treated animals was evaluated and it did not change more than 10% (data not shown). We compared the intrinsic sensitivity of tumor cells *in vitro* (Fig. 3A**, Supplementary Table S3, Fig. S4A**) with the antitumor response *in vivo* and found that intrinsic sensitivity of tumor cells could be an indicator of tumor response to ECT with OXA and CDDP, but not with BLM.

We next examined whether cell size [26], ECM [27] and vasculature [25] also affect ECT effectiveness. No significant differences in tumor cell size were noted between the three tumor models. Regarding ECM, significantly fewer cells per field of view and significantly less collagen and proteoglycan were observed in B16F10 compared with 4 T1 and CT26 tumors. Vasculature in B16F10 melanoma presented with large, lacuna-like vessels with microvascular density compared with 4 T1 and CT26 tumors. In the latter tumors, vessels were thinner and denser. None of the three stromal characteristics could explain the observed differences in the response of tumors to ECT (Fig. 3**, Supplementary Table S3, Fig. S4B**).

Tumor immune status was determined in untreated B16F10, 4 T1 and CT26 tumors. These tumors exhibited differences in the infiltration of $CD4^+$, $CD8^+$ and GrB^+ immune cells and expression of MHC-1 and PD-L1. Specifically, B16F10 tumors exhibited significantly less infiltration compared with 4 T1 and CT26 tumors. Few B16F10 cells expressed MHC-1, whereas most 4 T1 and CT26 cells were MHC-1^+. PD-L1 was expressed in >70% cells, and the difference was not significant *in vivo*. According to tumor mutational burden [28,29], 4 T1 tumors are the least mutated followed by B16F10 and CT26 tumors. Based on these data, B16F10 tumors were categorized as poorly immunogenic, 4 T1 tumors as moderately immunogenic and CT26 tumors as highly immunogenic (Fig. 3**, Supplementary Table S3, Fig. S4B**). The highest response rates to ECT were observed in highly immunogenic tumors, whereas the least immunogenic tumors exhibited the lowest response rates.

ECT also modulated the immunological features of the tumor cells. Specifically, ECT of cells with BLM, OXA or CDDP (IC_{50}) significantly increased the percentage of MHC-1^+ B16F10 cells (up to 3-fold). MHC-1 expression (MFI) significantly increased after OXA/EP and CDDP/EP but not with BLM/EP in 4 T1 cells; however, the changes were not significant in CT26. Electroporation alone or in combination with BLM, OXA or CDDP significantly increased the expression of PD-L1 in B16F10 cells. Contrary, chemotherapeutic drugs administered alone or with ECT did not affect PD-L1 expression in 4 T1 and CT26 cells (Fig. 2B**)**.

3.2. Peritumoral IL-12 GET delays B16F10 melanoma growth

The antitumor and immunostimulating effectiveness of single or

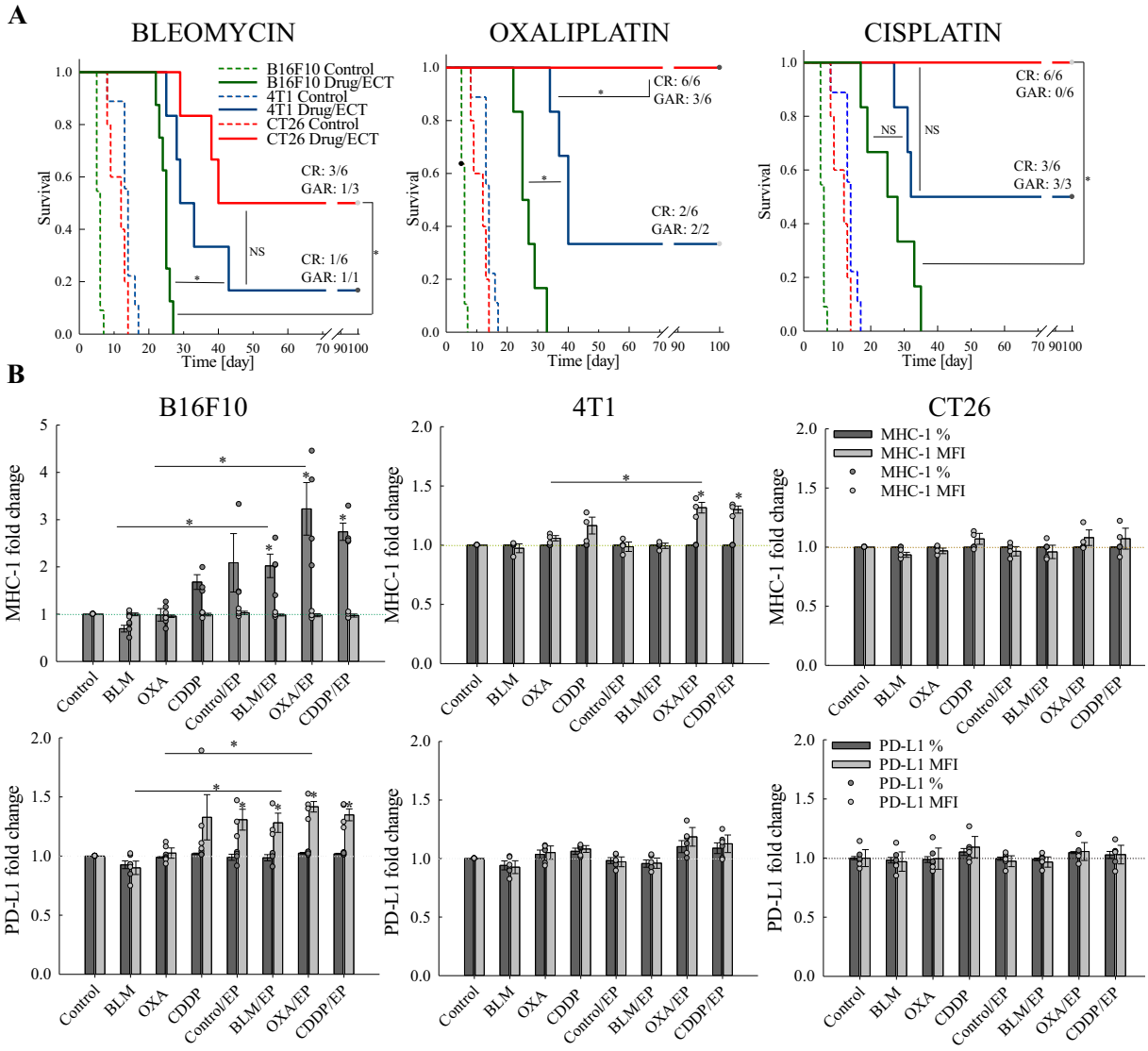


Fig. 2. The effectiveness of ECT varies in B16F10, 4 T1 and CT26 tumors. A, The response to ECT is presented with Kaplan-Meier graphs ($n = 6$; Survival Log-Rank Test; *$p < 0.05$; NS: not statistically significant). B, ECT impacts PD-L1 and MHC-1 expression *in vitro*. Fold change (percentage (%) and MFI) in the expression of MHC-1 and PD-L1 after incubation with BLM, OXA or CDDP alone or in combination with electroporation is presented ($n = 3$–4; Data represents AM ± SE and individual values; one-way ANOVA; *$p < 0.05$).

triple p. t. IL-12 GET were assessed in B16F10 melanoma given its potential in combined treatment with ECT. Only triple p. t. IL-12 GET resulted in significantly prolonged survival compared with Control and Control/EP (Fig. 4A, **Supplementary Table S2, Fig. S2A**). The therapies were well tolerated, and no adverse effects were observed. The weight of treated animals as a measure of systemic toxicity did not change more than 10% (data not shown).

Serum IL-12 concentrations did not increase significantly after a single or triple p. t. IL-12 GET as measured by ELISA (data not shown). However, IL-12 expression was directly confirmed in muscles of *panniculus carnosus* and other cells after single or triple p. t. IL-12 GET (Fig. 4B). Intratumoral IL-12$^+$ cells were present after p. t. IL-12 GET and also in the control group. Thus, we assume that some of the cells express endogenous IL-12 (Fig. 4B). IL-12 expression was also indirectly confirmed by immune cell infiltration to the peritumoral region after p. t. IL-12 GET, which was absent in the control group (Fig. 4C). Furthermore, the presence of GrB$^+$ cells was observed at the site of the GET, in the peritumoral region and in tumors after p. t. IL-12 GET using both treatment protocols. Contrary to single p. t. IL-12 GET, triple p. t. IL-12 GET attracted Foxp3$^+$ cells peritumorally (Fig. 4C).

3.3. Adjuvant p. t. IL-12 GET potentiates ECT effectiveness in B16F10 melanoma

The contribution of p. t. IL-12 GET to local tumor control of biologically equivalent ECT with suboptimal doses of OXA (85 μg), BLM (5 μg) or CDDP (40 μg) was determined in B16F10 melanoma. Immunostimulation with single p. t. IL-12 GET significantly prolonged survival of mice treated with ECT using either OXA, BLM or CDDP. Among the three chemotherapeutic drugs, only combination therapy with CDDP/ECT resulted in 38% of CR (Fig. 5A, **Supplementary Table S2, Fig. S2A**).

To evaluate the induction of immune response by combination therapy, 1) tumor IHC staining, 2) flow cytometry analysis of spleen and draining lymph nodes and 3) a secondary challenge were performed. In peritumoral region, GrB$^+$ cells were observed after OXA/ECT, CDDP/ECT and all combined therapies. ECT treatment increased the intratumoral infiltration of GrB$^+$ cells compared to untreated tumor. However, p. t. IL-12 GET immunostimulation did not further increase GrB$^+$ cell infiltration (**Supplementary Fig. S3**).

Induction of a systemic immune response was investigated after administration of the combination therapy with the highest antitumor

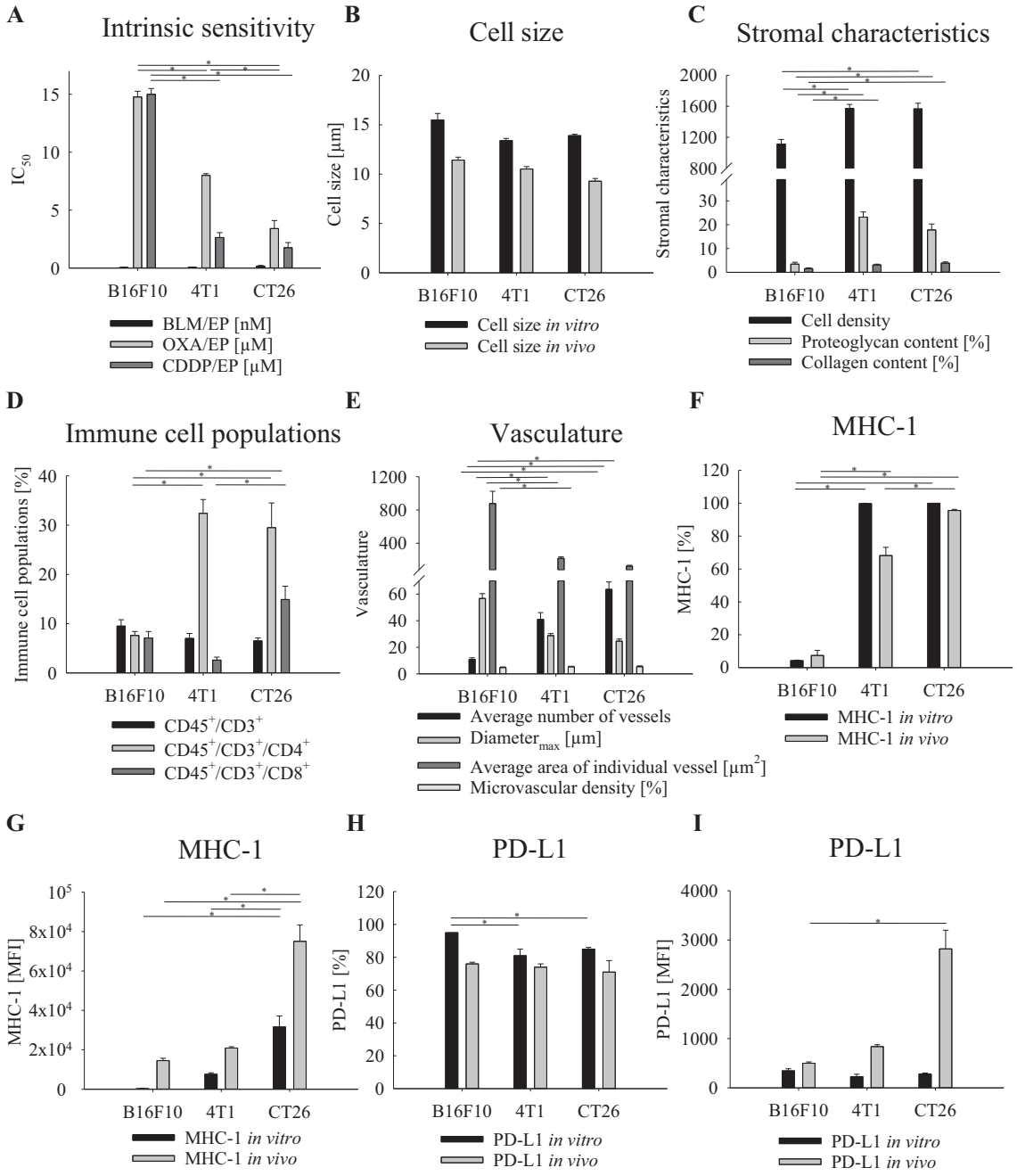


Fig. 3. Classification of tumors by tumor immune status. A, Intrinsic sensitivity of B16F10, 4 T1 and CT26 cells to BLM/EP, OXA/EP and CDDP/EP. IC_{50} values of BLM, OXA, and CDDP in combination with electric pulses in B16F10, 4 T1 and CT26 cells *in vitro* (n = 3; AM ± SE; one-way ANOVA; $*p < 0.05$). B, Tumor cell size *in vitro* ($n = 3$; Cytosmart; AM ± SE; one-way ANOVA; $*p < 0.05$) and *in vivo* ($n = 30$; AM ± SE; one-way ANOVA; $*p < 0.05$). C, Tumor cell density (the number of cells per field of view of tumor section; $n = 9$ fields of view (3 tumors); AM ± SE; one-way ANOVA; $*p < 0.05$) as well as tumor stroma characteristics as collagen or proteoglycan content (% area; n = 9 fields of view (3 tumors); AM ± SE; one-way ANOVA; $*p < 0.05$). D, Tumors were classified according to intratumoral immune cell infiltrate: $CD45^+/CD3^+$, $CD3^+/CD4^+$ and $CD3^+/CD8^+$ (flow cytometry; $n = 6$; AM ± SE; one-way ANOVA, $*p < 0.05$). E, Vasculature (presented as average number of vessels per field of view, $diameter_{max}$, average area of individual vessel per field of view and microvascular density; the images were taken under the 400 × magnification; $n = 12$ fields of view (3 tumors); AM ± SE; one-way ANOVA; $*p < 0.05$). F, G, Tumor sensitivity to immune effectors, (MHC-1 expression; flow cytometry; $n = 3$ *in vitro* and n = 6 *in vivo*; AM ± SE; one-way ANOVA; $*p < 0.05$), and H, I, presence of one of the immune checkpoints (PD-L1 expression; flow cytometry; n = 3 *in vitro* and n = 6 *in vivo*; AM ± SE; one-way ANOVA; $*p < 0.05$). Where not indicated with *, differences are not significant.

effect (CDDP/ECT + IL-12 p. t./GET). The therapy did not induce systemic immune response. Namely, after combination therapy, immune cell populations from spleen or draining lymph node on days 3, 5 and 8 after the therapy were comparable to those of untreated mice (**Supplementary Fig. S5**). Moreover, none of the cured mice were resistant to secondary challenge (Fig. 5A**, Supplementary Fig. S2A**).

3.4. An increased chemotherapeutic dose in ECT but not repetition of p. t. IL-12 GET improves the therapeutic outcomes of the combination therapy in B16F10 melanoma

Two treatment protocols were tested to improve the combination therapy: 1) additional p. t. IL-12 GET after the combination therapy or 2) increased drug concentration in ECT.

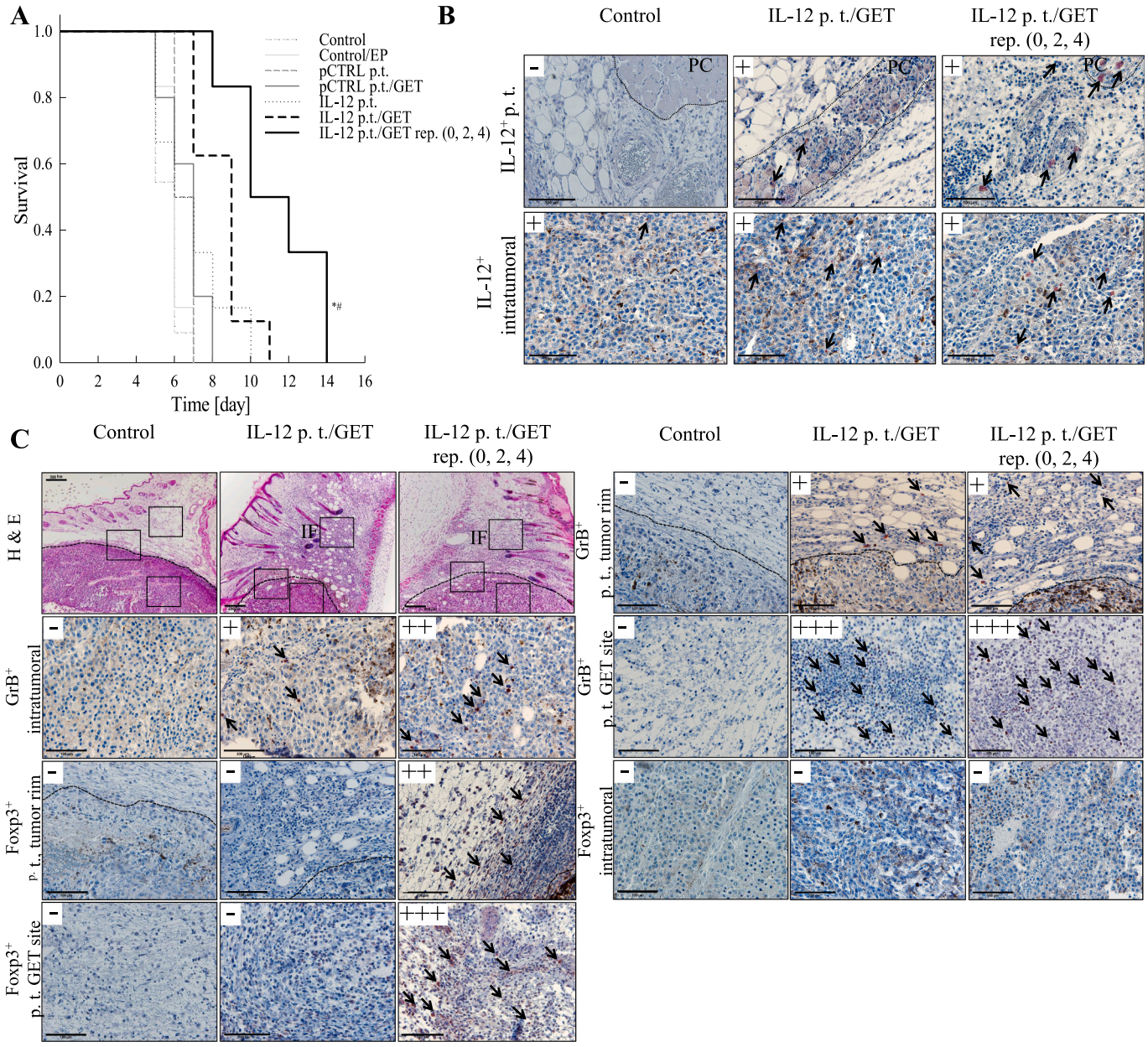


Fig. 4. Triple p. t. IL-12 GET is effective in treating B16F10 melanoma with no systemic toxicity. A, Prolonged survival of the treated animals was observed after triple p. t. IL-12 GET performed on days 0, 2, 4 as presented with Kaplan-Meier graph ($n = 6$; Survival Log-Rank Test; $^*p < 0.05$ compared to Control or $\#p < 0.05$ compared to Control/EP). B, Direct histological confirmation of the IL-12 expression after single or triple p. t. IL-12 GET. Representative micrographies after IHC staining of IL-12 are presented. *Panniculus carnosus* is surrounded by dashed line and marked with PC and IL-12$^+$ cells are marked with black arrows (n = 6 fields of view (3 tumors); a semi-quantitative scoring system for IHC$^+$ cells was used: (−) negative staining, (+) low, (++) moderate, and (+++) high positivity. The images were obtained under the 400 × magnification, scale bars are 100 μm). C, Histological analyses of tumors, treated with single or triple p. t. IL-12 GET. Representative micrographies after H & E and IHC staining of GrB$^+$ and Foxp3$^+$ cells are presented. Tumors are surrounded with dashed line, peritumoral immune infiltrate is marked with IF, GrB$^+$ and Foxp3$^+$ cells are marked with black arrows (n = 6 fields of view (3 tumors); a semi-quantitative scoring system for IHC$^+$ cells was used: (−) negative staining, (+) low, (++) moderate, and (+++) high positivity. The images were obtained under the 100 × and 400 × magnification, scale bars are 200 μm (H & E) and 100 μm (IHC)).

Additional treatments of p. t. IL-12 GET with 1) additional GET every second day (days 2 and 4), or 2) additional GET with longer intervals (days 6, 10 and 14) did not significantly improve the local effectiveness of the combination therapy with CDDP/ECT (Fig. 5B, **Supplementary Table S2, Fig. S2A)**. Tumors were equally infiltrated by GrB$^+$ cells after combination therapy with single p. t. IL-12 GET or repetitive p. t. IL-12 GET. However, the latter exhibited an increased level of peritumoral infiltration (**Supplementary Fig. S3**). Conversely, Foxp3$^+$ cells were detected intatumorally and peritumorally after combination therapy with repetitive p. t. IL-12 GET only (**Supplementary Fig. S3**).

To explore the contribution of p. t. IL-12 GET with more effective ECT, the concentrations of BLM, OXA and CDDP were doubled. The CR rates of the tumors to OXA/ECT and CDDP/ECT but not BLM/ECT were significantly increased compared with ECT with low doses; however, potentiation with p. t. IL-12 GET was lost. The best outcome was observed when p. t. IL-12 GET was combined with CDDP/ECT; the CR rate was 57%. Again, tumor-free mice were not resistant to secondary challenge (Fig. 5C, D, D**, Supplementary Table S2, Fig. S2A**).

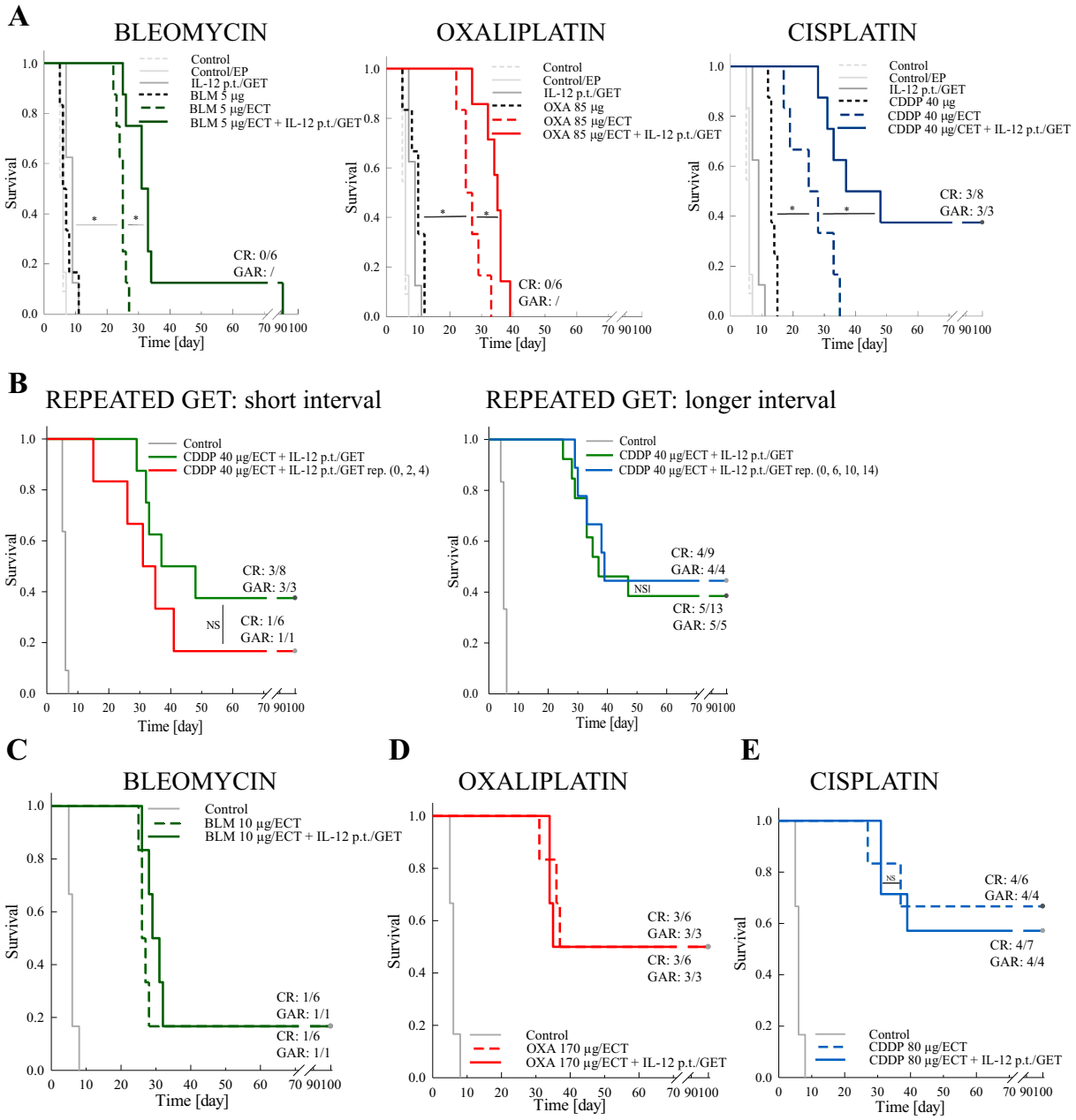


Fig. 5. Immunostimulation with p. t. IL-12 GET potentiates local effectiveness of ECT in B16F10 melanoma. A, Survival of the animals is prolonged after combination therapy with BLM, OXA or CDDP as presented with Kaplan-Meier graph ($n = 6–8$; Survival Log-Rank Test; $*p < 0.05$; NS: not significant). B, Additional repetitions of GET (short or longer interval) did not significantly improve the effectiveness of the combination therapy (n = 6–13; Survival Log-Rank Test; NS: not statistically significant). Adjuvant p. t. IL-12 GET did not significantly contribute to the antitumor effect of high dose C, BLM/ECT, D, OXA/ECT or E, CDDP/ECT (n = 6–8; Survival Log-Rank Test; NS: not statistically significant).

3.5. Adjuvant effects of p. t. IL-12 GET to ECT are more pronounced in poorly immunogenic tumors

Since the combination therapy with additional repetitions of GET did not outperform combination therapy with single GET in B16F10 tumor model, only single GET with ECT was tested in 4 T1 and CT26 tumor models. The aim was to associate its effectiveness with tumor immune status and with a specific drug in ECT. First, the combination therapies with biologically equivalent doses of BLM (5 µg), OXA (85 µg) or CDDP (40 µg) in ECT, which were determined in the B16F10 model, were tested in 4 T1 and CT26 tumors. Next, the effectiveness of combination treatment with reduced doses of BLM and CDDP in ECT was tested.

In 4 T1, adjuvant single p. t. IL-12 GET significantly improved OXA/ECT only. The contribution was not significant when p. t. IL-12 GET was combined with BLM/ECT or CDDP/ECT nor with the BLM/ECT or CDDP/ECT with reduced drug doses in the combination therapy (Fig. 6A, B, **Supplementary Table S2, Fig. S2B**). In CT26, single p. t. IL-12 GET did not prolong the survival of mice when combined with ECT (Fig. 6C). Biologically equivalent low doses were assessed for BLM and CDDP; however, ECT resulted in CR even with up to a 3-fold reduced dose of BLM and 16-fold reduced dose of CDDP **(Supplementary Table S2, Fig. S2C**). Therefore, combination therapies were not tested.

These results implied that the adjuvant effect of single p. t. IL-12 GET depends on tumor immune status (Fig. 6D). In poorly immunogenic B16F10 tumors, single p. t. IL-12 GET significantly contributed to biologically equivalent suboptimal ECT with either BLM, OXA or CDDP;

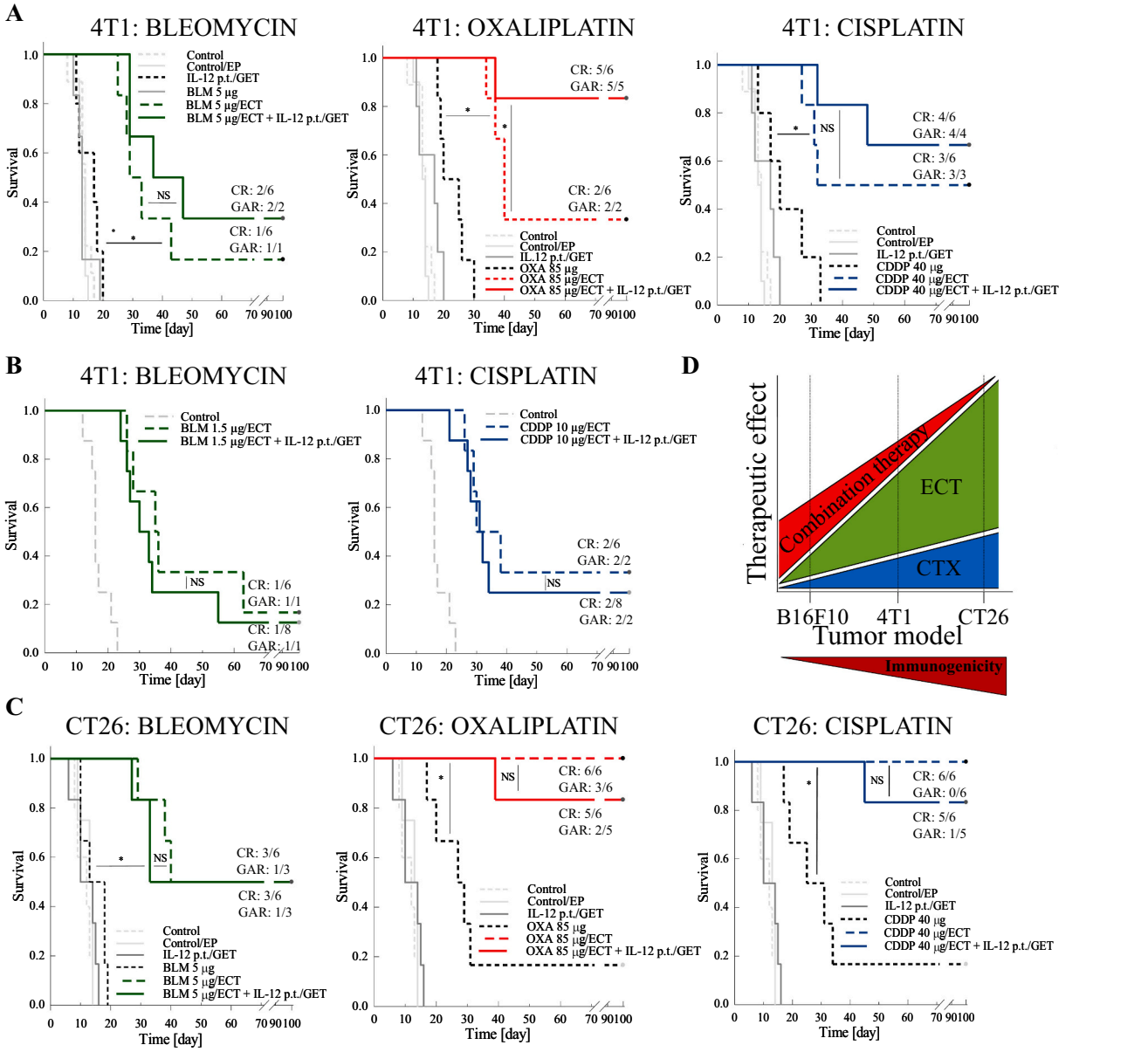


Fig. 6. Adjuvant effect of single p. t. IL-12 GET to ECT is more pronounced in poorly immunogenic tumors. Survival of animals, treated with ECT or combination therapy, as presented with Kaplan-Meier graphs. A, 4 T1 with 5 µg BLM, 85 µg OXA and 40 µg CDDP and B, 4 T1 with decreased chemotherapeutic drug dose in ECT *i.e.*, 1.5 µg BLM and 10 µg CDDP ($n = 6–8$; Survival Log-Rank Test; NS: not significant). C, Survival of CT26 tumor bearing mice treated with combination therapy with 5 µg BLM, 85 µg OXA and 40 µg CDDP (n = 6; Survival Log-Rank Test; NS: not significant). D, Proposed model represents therapeutic effect of chemotherapy (CTX), ECT and combination therapy in tumors with different tumor immune status.

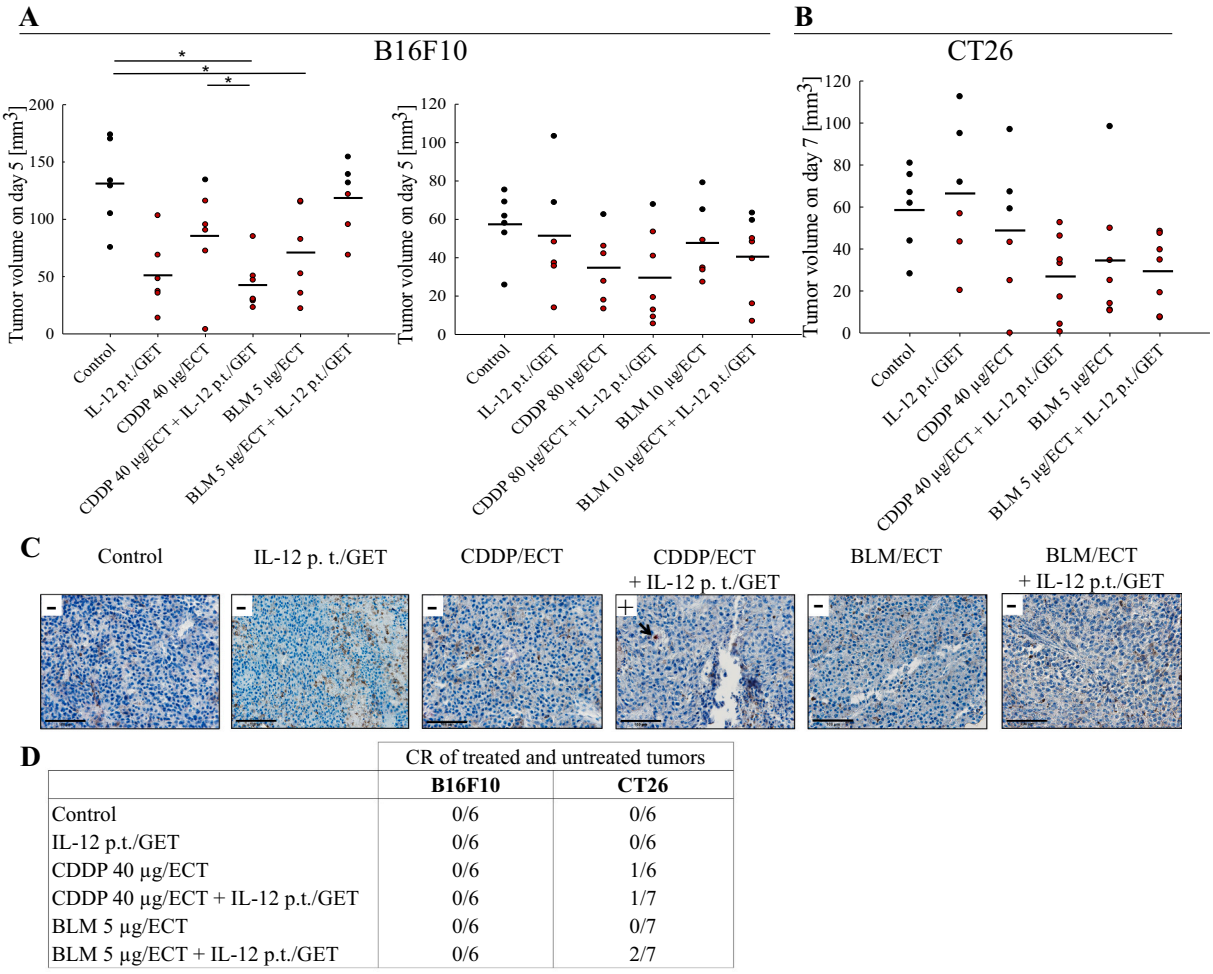
however, in 4 T1, the contribution was significant with OXA/ECT only (Fig. 6, **Supplementary Table S2, Fig. S2**).

3.6. Combination therapy or ECT alone impacts growth of the distant untreated tumors

To determine whether single p. t. IL-12 GET adds a systemic component to the local effectiveness of ECT using CDDP or BLM, dual-flank B16F10 and CT26 models, which mimic systemic disease, were introduced.

In B16F10, combination therapy, including CDDP/ECT, but not CDDP/ECT alone, significantly delayed growth of distant untreated tumors (Fig. 7A, **Supplementary Fig. S2D**). This finding could be ascribed to the induction of the systemic immune response. Namely, infiltration of GrB^{+} cells was observed in distant untreated tumors (Fig. 7C). However, this trend was not observed with BLM. The abscopal effect was detected after BLM/ECT but not after combination therapy with BLM/ECT (Fig. 7A, **Supplementary Fig. S2D**). With both chemotherapeutic drugs, the abscopal effect was lost when the dose of CDDP or BLM was doubled (Fig. 7A, **Supplementary Fig. S2D**).

Contrary, in the CT26 dual-flank model, an abscopal effect was observed (NS) after CDDP/ECT alone. Additional p. t. IL-12 GET did not significantly improve the effect (Fig. 7B, **Supplementary Fig. S2D**). Specifically, after CDDP/ECT alone and combination therapy, CR of treated and untreated distant tumors were observed in 16.7% and 14.3% of mice, respectively. Moreover, in the CT26 model, both BLM/ECT and combination therapy indicated a systemic effect, and the latter exhibits a 28.6% CR of treated and untreated distant tumors (Fig. 7D, **Supplementary Fig. S2D**).



D

	CR of treated and untreated tumors	
	B16F10	**CT26**
Control	0/6	0/6
IL-12 p.t./GET	0/6	0/6
CDDP 40 μg/ECT	0/6	1/6
CDDP 40 μg/ECT + IL-12 p.t./GET	0/6	1/7
BLM 5 μg/ECT	0/6	0/7
BLM 5 μg/ECT + IL-12 p.t./GET	0/6	2/7

Fig. 7. Combination therapy or ECT alone impacts growth of distal untreated tumors. Volume of distal untreated A, B16F10 tumors after the combination therapy or ECT with 5 μg or 10 μg of BLM and 40 μg or 80 μg of CDDP and B, CT26 tumors after the combination therapy or ECT with 5 μg of BLM and 40 μg of CDDP ($n = 6$–7; line represents AM; dot represents tumor volume; tumors, larger (black dot) or smaller (red dot) compared to Control AM; $^*p < 0.05$; one-way ANOVA). C, After the combination therapy with CDDP/ECT (CDDP/ECT + IL-12 p. t./GET) the distal untreated B16F10 tumors were infiltrated by GrB^+ cells. Representative micrographies after IHC staining of GrB^+ cells are presented ($n = 9$ fields of view per 3 tumors; a semi-quantitative scoring system for IHC^+ cells was used: (−) negative staining, (+) low, (++) moderate, and (+++) high positivity. GrB^+ cells are marked with black arrows. The images were obtained under the 400 × magnification, scale bars are 100 μm). D, Fraction of mice bearing B16F10 and CT26 tumors that responded with CR of the treated and untreated distant tumors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, a treatment combination utilizing adjuvant effects of p. t. IL-12 GET to enhance ECT-mediated tumor ablation was systematically assessed. The aim was to increase the already high local effectiveness of ECT [17] and, most importantly, to induce a systemic antitumor response. We demonstrated that the effectiveness of the combination therapy was dependent on intrinsic sensitivity and tumor immune status. ECT was more effective in more immunogenic tumors, whereas GET exhibited a greater contribution in less immunogenic tumors.

We observed different responses to ECT according to the tumor model and the drug used in ECT. Specifically, the CT26 tumor model exhibited the greatest response, whereas the B16F10 melanoma model was the least responsive. First, we explored whether this finding is due to intrinsic tumor cell sensitivity to ECT. Various previous studies have demonstrated this correlation [30], whereas others have not [25,31]. Our results do not demonstrate a clear correlation; *in vitro* sensitivity indicated the *in vivo* tumor response when OXA and CDDP were used in ECT but not BLM. Furthermore, tumor and stromal characteristics, such as tumor cell size [26], vascularization [25] and ECM [27], may also impact the effectiveness of ECT. Among these stromal characteristics, our study indicates that drug pharmacokinetics may represent an important characteristic that contributes to ECT effectiveness due to the vascularization of the tumors. Better drug distribution would be expected in 4 T1 and CT26 tumors as they are more vascularized, but this increase in vascularization could also increase wash out. This would be the case in intravenous drug administration but is questionable in intratumoral administration [32] as is the case in our study. Therefore, these three characteristics may not be the main reason for the differences in intratumoral ECT effectiveness among the three tested tumor models.

Another important factor may be tumor immune status. Since the immune system intervention is indispensable in tumor curability after ECT [31,33], it was proposed that more immunogenic tumors would respond better to ECT [31]. Here, tumor immune status was evaluated following the cancer immunogram [34] and immunoscore [35]. According to published literature, tumors differ in tumor mutational burden [28,29]. Here, differences in tumor immune infiltrate and MHC-1 expression were detected, which is consistent with published data [36]. The highest response rate to ECT was demonstrated for the highly immunogenic CT26 tumor, whereas the lowest response rate was noted

for the poorly immunogenic B16F10 tumor. Specifically, in B16F10 tumors, low-dose ECT may not be completely effective in overcoming the immunosuppressive networks. Presumably, effector immune cells are not present to intervene in the response. To further assess this notion, additional experiments with immunocompromised mice are needed.

If the tumor response to ECT is dependent on tumor immune status, this effect should be mediated though *in situ* vaccination [37] together with immunogenic cell death [19,21]. Immunomodulatory properties of BLM [21], OXA [19,38] and CDDP [19,22] improve the therapeutic effect. BLM/ECT induces immunogenic cell death in CT26 tumors. Furthermore, mice vaccinated with ECT-treated cells are protected against rechallenge [21]. We demonstrated that electroporation in combination with BLM, OXA or CDDP increased the expression and percentage of MHC-1$^+$ cells *in vitro* up to 3-fold. An increase in MHC-1 expression was previously described after chemotherapy or irradiation [39]. Electroporation with BLM, OXA or CDDP also stimulated PD-L1 expression. Based on these data, it is rational to combine ECT with checkpoint inhibitors, which are currently being used in clinics [40].

To boost the antitumor response locally and specially to induce an abscopal effect, we combined ECT with immune-stimulatory p. t. IL-12 GET. The combination therapy aimed at relieving the immunosuppressive tumor microenvironment [6] by enhancing the antigenicity and immunogenicity with ECT (*in situ* vaccination) and potentiating the response with IL-12. The combination strategy was already proposed in [37]. Furthermore, the combination of ECT using either BLM or CDDP with p. t. or intramuscular IL-12 GET was already demonstrated to be effective in a variety of tumors in a preclinical setting [31,41] and in veterinary clinics [42]. Despite published data, a systematic experimental approach addressing the effectiveness of different combinatorial strategies in different tumor types is needed to improve the treatment outcomes in clinics. Here, intratumoral ECT and p. t. GET were chosen for two reasons: 1) to confine the chemotherapeutic drug to tumors where they cannot degrade the plasmid and 2) to segregate the ablative effect of ECT from the immune-stimulatory effect of the peritumoral IL-12 expression. This is the first study that systematically compares the combination therapy effectiveness based on 1) different chemotherapeutic drugs, of which OXA is for the first time used in combination with GET, 2) different chemotherapeutic drug doses, 3) variations in repetitions of p. t. IL-12 GET, 4) local and systemic effectiveness as well as some of the background immune-related mechanisms and last, but not least 5) (immunologically) different tumor models.

Skin delivery of IL-12 plasmid was performed according to a previously established protocol [15]. Significant antitumor effectiveness was demonstrated with triple but not single p. t. IL-12 GET in B16F10 tumors. However, in B16F10 melanoma, IL-12 was not detected systemically in the serum. The latter further supports the fact that immune cells but not systemically elevated IL-12 concentrations are the mediators of the antitumor-immune response [9]. On the other hand, it is possible that systemic concentrations of IL-12 were too low to be detected by ELISA. The empty plasmid did not affect B16F10 tumor growth and was thus excluded from the subsequent experiments. Elevated levels of Foxp3$^+$ cells after triple p. t. IL-12 GET presumably did not impact local antitumor effectiveness. However, it is possible that these levels reflect negative feedback activation due to high local levels of IL-12 [9,43]. Regardless of the increased GrB$^+$ cell infiltration intra- and peritumorally, demonstrated already after single GET, a systemic antitumor effect was not observed.

We showed that single p. t. IL-12 GET potentiates the local antitumor effect of suboptimal ECT with all three chemotherapeutic drugs in B16F10 melanoma. This finding suggests that IL-12 effectively boosts ECT *in situ* vaccination. The most pronounced potentiation was observed with CDDP/ECT. Additional repetition of p. t. IL-12 GET did not improve the response. Using increased drug doses, we obtained significantly better antitumor effect of OXA/ECT and CDDP/ECT, but the significant contribution of p. t. IL-12 GET was lost. The antitumor effect of ECT is dose dependent [19]; however, as reported with combined radiotherapy and IL-12 [44], the "quantity" and the "quality" of induced tumor cell death should be addressed. Presumably, the same is true with ECT, where the *in situ* vaccination effect does not necessarily increase with the increased drug dose. Further research is needed to resolve the hypothesis. Low MHC-1 expression on B16F10 tumor cells could represent one of the obstacles that could impede immune intervention and resistance to rechallenge [45]. Namely, in cured mice, the addition of p. t. IL-12 GET to ECT did not increase resistance to rechallenge.

To associate tumor status with the antitumor effect of the combination therapy, 4 T1 and CT26 tumors were used. Using the biologically equivalent ECT established in B16F10, p. t. IL-12 GET potentiated the antitumor effect of OXA/ECT exclusively in 4 T1. In CT26, the local antitumor effect of ECT was too high to enable potentiation of p. t. IL-12 GET. To compare the contribution of p. t. IL-12 GET in all three tumor models, p. t. IL-12 GET should be combined with a suboptimal biologically equivalent ECT. In 4 T1 tumors, p. t. IL-12 GET did not improve the antitumor effect of suboptimal BLM/ECT or CDDP/ECT. Suboptimal doses of ECT were also evaluated in the CT26 tumor model, and an up to 16-fold drug dose reduction in ECT still resulted in CR and long-term immunity. Due to the high efficacy of ECT, additional immunotherapy was not tested. Altogether, more immunogenic tumors respond better to ECT, where adjuvant p. t. IL-12 GET leads to the lowest potentiation (Fig. 6D).

Immunostimulation with p. t. IL-12 GET in the current study and in the veterinary study on mast cell tumors [42], potentiated the effect of ECT on treated tumors. Moreover, in the veterinary study it also prevented recurrences or metastases [42]. However, an abscopal effect has not been directly confirmed. In our study, although not detected in spleen and draining lymph nodes, an immune response was indicated by delayed growth of distant untreated tumors. The unexpected results could be explained by the inappropriate time points when systemic immune response was evaluated or by the intervention of other unmeasured immune cell populations. Therefore, to resolve this, our future studies would more precisely evaluate the background immune events in the draining lymph nodes and spleen. Abscopal effect was detected in B16F10 tumors after combination therapy with CDDP/ECT and after BLM/ECT alone. Accordingly, an abscopal effect of BLM/ECT was also described in human patients with skin melanoma metastases [23]. Although the mechanism of the abscopal effect is not completely understood, the intervention of cytotoxic T cells is essential [46]. In our study, GrB$^+$ cells were observed in distal untreated tumors after combination therapy with CDDP/ECT only, which may contribute to the abscopal effect. It is possible that after BLM/ECT other immune cells, *e.g.*, regulatory T cells, mediate the abscopal effect [47]. Interestingly, the abscopal effect was abolished when the drug dose in the ECT of combination therapy was doubled. This finding further supports the hypothesis that the "quality" of cell death affects *in situ* vaccination of ECT [44], which we are resolving in our ongoing studies. The abscopal effect was associated with tumor immune status. Animals bearing highly immunogenic CT26 tumors, though few, responded to ECT and combination therapy with CR of treated and even untreated tumors. However, the limitation of the current study is that the immune-related background mechanisms are not fully explored. The study indicates that exploration of immune-related aspects as innate and adaptive immunity, immunosuppression, *in situ* vaccination effects as well as immunogenic cell death and other antitumor immune responses are important and thus the aim of our future studies.

5. Conclusion

The study was initiated to systematically evaluate local and systemic effects of ECT alone or in combination with p. t. IL-12 GET in three murine tumor models. We demonstrated that p. t. IL-12 GET significantly potentiates the antitumor effect of BLM/ECT, OXA/ECT or CDDP/ECT in poorly immunogenic B16F10 melanoma. This regimen also has a notable antitumor effect on distant untreated tumors as demonstrated

with CDDP/ECT. In B16F10, an abscopal effect was also detected after BLM/ECT. The effectiveness of the combination therapy or ECT alone depends on intrinsic sensitivity and tumor immune status. ECT is more effective in more immunogenic 4 T1 and CT26 tumors, where the local antitumor contribution of p. t. IL-12 GET was not significant or was completely absent. Moreover, in the most immunogenic CT26 tumors, ECT alone is sufficient to achieve an abscopal effect and long-term immunity. This study indicates that the selection of the therapy should be predominantly based on the tumor immune status.

Ethics approval and consent to participate

All procedures involving animals were performed in compliance with guidelines for animal experiments of the EU Directives, ARRIVE Guidelines and the permission of the Ministry of Agriculture Forestry and Food of the Republic of Slovenia (Permission No. U34401–1/2015/43).

Consent for publication

Not applicable.

Availability of data and material

All data needed to evaluate the conclusions are presented in the paper. All materials, data, and protocols described in the manuscript will be made available upon request, if the request is made within six years of publication.

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Credit author statement

Conceptualization and study design: KU, GS, MC and RH. Supervision: GS, MC, RH and VKP. Investigation, data curation, analysis, statistical analysis, validation, visualization, methodology: KU, SK, UK, SM, SB, BM and BS. Original draft: KU and GS. Review, editing and final approval of manuscript: all authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2021.03.009.

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