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Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells

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Abbreviations: CRT, calreticulin; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; ECT, electrochemotherapy; EPs, electric pulses; ER, endoplasmic reticulum; HMGB1, high mobility group box 1; ICD, immunogenic cell death; IL, interleukin; MTX, mitoxantrone; ROS, reactive oxygen species; TLRs, toll-like receptors

Electrochemotherapy (ECT) is a local cancer treatment that has been used over the course of more than 2 decades for the removal of cutaneous and subcutaneous tumors. Several lines of evidence support the premise that the immune system is an important factor underlying anticancer treatment efficacy, potentially including patient responses to ECT. The concept of immunogenic cell death (ICD) arose a few years ago, stating that some cancer treatments generate danger-associated molecular patterns (DAMPs) that trigger an adaptive immune response against tumors. Hence, dying cancer cells behave as a therapeutic vaccine, eliciting a cytotoxic immune response against surviving malignant cells. In our study, we sought to evaluate the ability of ECT to generate cancer cell death encompassing the immunostimulatory characteristics of ICD. To this end, we assayed CT26 murine colon cancer cells in vitro in response to either electric pulses (EPs) application only or in combination with the anticancer drug bleomycin (that is ECT) by quantification of calreticulin (CRT) membrane externalization, as well as the liberation of adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1) protein. We show here that cell permeabilizing yet non-lethal electric pulses induce CRT exposure on the cell surface of EP-only treated cancer cells, as well as ATP release. However, the association of electric pulses along with the chemotherapeutic agent bleomycin was mandatory for HMGB1 release coincident with regimen-induced cell death. These data obtained in vitro were then substantiated by vaccination protocols performed in immunocompetent mice, showing that the injection of dying ECT-treated cells elicits an antitumor immune response that prevents the growth of a subsequent administration of viable cancer cells. We also confirmed previous results showing ECT treatment is much more efficient in immunocompetent animals than in immunodeficient ones, causing complete regressions in the former but not in the latter. This supports a central role for immunity in this beneficial outcome. In conclusion, we show that ECT not only possesses an intrinsic cytotoxic property toward cancer cells but also generates a systemic anticancer immune response via the activation of ICD. Hence, ECT may represent an interesting approach to treat solid tumors while preventing recurrence and metastasis, possibly in combination with immunostimulating agents.

Introduction

Immunogenic cell death (ICD) emerged few years ago as an ability of some anticancer treatments, such as anthracyclines or oxaliplatin, to kill cancer cells in an immunogenic fashion, thereby secondarily stimulating the immune system.[1,2] Thus, these treatments are not only capable of directly killing cancer cells via intrinsic cytotoxicity, but also by the conversion of dying cancer cells into an anticancer vaccine. The culmination of this process is the generation of a specific immune response against any residual cancer cells, should they be therapy-resistant or metastatic cells.

ICD depends on molecular signals called danger-associated molecular patterns (DAMPs) that activate innate immune cells driving the generation of specific antitumor immunity. Three major DAMPs have been identified and the combination of all 3 has been found to be required for bona fide ICD induction.[1]

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Adenosine triphosphate (ATP) release is one of the hallmark features of ICD.[3] This intracellular metabolite is released from dying cells in an autophagy-dependent fashion.[4] Extracellular ATP is a chemoattractant for the homing of dendritic cells (DCs) and their precursors, immature myeloid cells.[5] Furthermore, ATP binding to P2Y2 purinergic receptors favors the differentiation of precursor myeloid cells into mature DCs with antigen-presenting capacity.[6] Additionally, extracellular ATP can activate the NALP3 inflammasome complex in DCs through P2X7 receptors activation, thus leading to interleukin-1β (IL-1β) secretion, a cytokine required to solicit both IL-17-producing γδ T cells[7] and interferon-γ-producing CD8+ T-cell activation.[3,8] Ultimately, these 2 T cells subsets are responsible for the eradication of the remaining live tumor cells.

The exposure of calreticulin (CRT) on the cell surface is also essential for the elicitation of an immune response after anticancer treatment. Under physiological conditions, this protein is sequestered in the endoplasmic reticulum (ER) lumen and is involved in chaperone-related functions as well as calcium homeostasis and signaling. Upon ER stress induced by some anticancer treatments,[9,10] CRT is externalized to the cell surface and acts as an "eat me" signal for phagocytes, mostly DCs and macrophages, through their CD91 receptor.[11,12] Eventually, this leads to tumor antigen presentation by these professional antigen-presenting cells and to T-cell priming.

Finally, the high mobility group box 1 (HMGB1) protein, that physiologically acts as a DNA chaperone, is passively released from secondary necrotic cells.[13] In the context of ICD, it activates the release of pro-inflammatory cytokines (e.g., tumor necrosis factor, IL-1, IL-6, and IL-8) from innate immune cells such as neutrophils, monocytes, and macrophages.[14,15] Moreover, when bound to toll-like receptor 4 (TLR4) on DCs, it augments the expression of the immature form of IL-1β and favors antigen-processing and presentation.[16]

Electrochemotherapy (ECT) is a local anticancer treatment used for more than 2 decades to treat skin metastases.[17] This treatment modality is a combination of non-permeant cytotoxic molecules, such as bleomycin, with permeabilizing (yet non-fatal) electric pulses[18,19] applied at the tumor site to permit the chemotherapeutic agent to cross the cell membrane and to generate irreversible DNA damages. The multicentric study establishing the protocol for ECT use in clinics known as the European Standard Operating Procedures on Electrochemotherapy (ESOPE) reported that complete tumor regression was observed in 73.7% of the treated nodules and that the overall objective response was around 85%.[20,21] Broadening ranges of cancer types are being treated by ECT and an increasing number of European oncology centers are adopting this treatment regimen, as reported by the 2010 and 2013 International User's Meetings (www.igeamedical.com).

There are accumulating lines of evidence that the immune system contributes to ECT efficiency. In support, relative to outcome of ECT treatments in tumor-bearing immunocompetent mice, ECT-mediated tumor regression was dramatically decreased in animals deficient in functional T lymphocytes, irrespective of whether this immunoinsufficiency was due to prior injection of anti-CD3 (OKT3) monoclonal antibody[22] or genetic factors, such as in immunocompromised nude mice.[23,24] Moreover, DC[25,26] and T lymphocyte[27] recruitment to the site of ECT-treated tumors has been previously reported, along with an elicited antitumor activity of monocytes and T lymphocytes.[28] These observations highlight a functional role of the activation of the immune system in physiological responses to this treatment.

ECT has also been applied in conjunction with histocompatible cells secreting IL-2, a cytokine that possesses tumor-growth inhibitory properties,[29] a treatment course that increased efficiency of ECT on treated tumors but that also generated a systemic response.[30-33] Indeed, following an ECT treatment combined with the inoculation of IL-2-secreting cells, contralateral non-ECT-treated tumors were infiltrated by CD4+ and CD8+ lymphocytes, an effect likely responsible for the observed 50% tumor rejection rate of these contralateral tumors in the mice receiving the combined treatment.[31] Similar protocols resulted in antimetastatic effects following the treatment of subcutaneous[32] or liver-transplanted tumors.[33] Likewise, the injection of ECT-treated tumors with toll-like receptor 9 (TLR9) ligands such as CpG oligodeoxynucleotide, agents known to induce Th1 immune responses,[34] dramatically increased the treatment efficiency in immunocompetent mice.[26] The ECT/CpG combination also revealed a systemic effect since an increase rate of tumor rejection was observed in contralateral non-treated tumors. This systemic effect relied, at least partially, on a T cell-mediated immune response since no sustained tumor-inhibitory effect was observed in nude mice. Altogether, these data point out that immune cells are de facto involved in ECT efficiency and may also play an important role in eliminating cancer cells that have escaped the treatment.

In this report, we sought to determine if ECT-mediated cancer cell death features the hallmark characteristics of ICD.

Results

Toxicity of ECT in CT26 murine colon carcinoma cell line

In order to establish a model system for our ECT study, we assayed a range of bleomycin concentrations from 1 to 100 nM in the context of in vitro ECT in CT26 murine colon cancer cells (**Fig. 1**). A highly significant drop in cell viability (as measured by decreased cloning efficiency of replated cells) was apparent starting from 5 nM of bleomycin only when electric pulses were co-applied ($P < 0.01$ to $P < 0.001$), as had been previously described in similar studies of other cell lines.[35,36] At 100 nM, a slight toxicity of the drug alone was observed, such that we selected 50 nM bleomycin in further experiments. No significant impact on cell viability of electric pulses alone was observed. Mitoxantrone (MTX), a well-known ICD-inducer[11] exhibited a high cytotoxic activity at 1 μM as compared with non-treated cells. Hence, 1 μM MTX was selected for use as an ICD positive control in further experiments.

A kinetic evaluation revealed that when CT26 cells were treated by the application of electric pulses in the presence of 50 nM bleomycin an ECT-mediated decrease in cell viability (as reported by the incorporation of the fluorescent DNA stain YOYO-1 iodide) was initially detected approximately 45 h after the treatment (**Fig. 2A**). Non-treated cells began to die about 20 h later due to confluency (**Fig. 2B**). Control cells (cells treated by either electric pulses alone or bleomycin alone) behaved as the non-treated cells (data not shown).

Electric pulses stimulate CRT externalization

CRT exposure was measured by antibody staining and cytofluorometric analysis of viable (propidium iodide-negative) CT26 cells 30 h after the treatment (**Fig. 3**). No significant effect of bleomycin alone (relative to non-treated cells) was observed. However, cells treated by MTX, electric pulses alone or ECT externalized a similar amount of CRT on the cell membrane, that is approximately twice that of the non-treated cells ($P < 0.05$).

Electric pulses and ECT liberate ATP

Quantification of ATP release was performed 30 h after the treatment (**Fig. 4A**). Similar to MTX-treated control cells, ECT-treated colon cancer cells released a significant amount of ATP with respect to non-treated cells. Exposure to 50 nM bleomycin alone during 30 h also triggered ATP release, although to a lesser extent than ECT. Electric pulses alone did not elicit significant ATP release. A 20% cell viability drop (relative to non-treated cells) was observed when cells were exposed to bleomycin alone for 30 h (data not shown) accounting for the increased amount of released ATP detected.

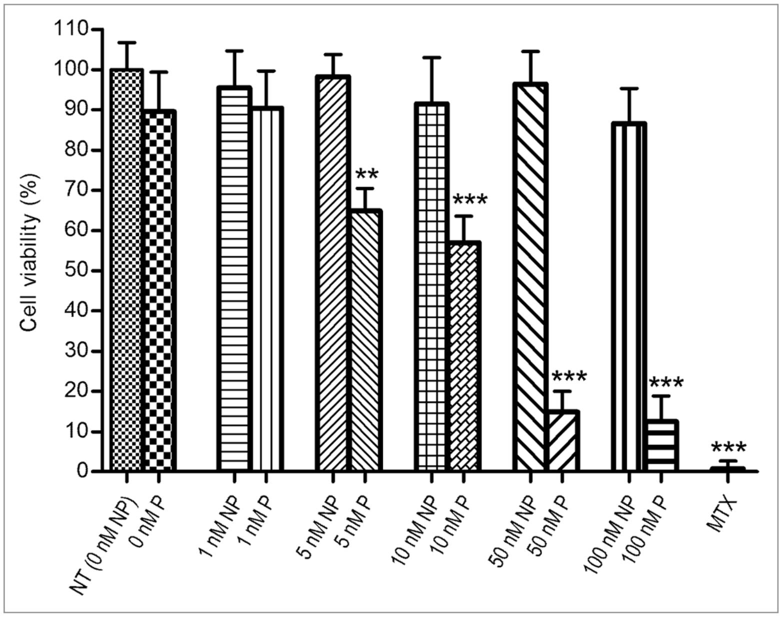

Figure 1. Cytotoxicity of electrochemotherapy and mitoxantrone treatments on CT26 cancer cells. Cultured CT26 cells were treated by electrochemotherapy (ECT) using various doses of bleomycin or by 1 µM mitoxantrone (MTX) over the course of 30 h. Cytotoxicity was assessed by cloning efficiency assay in which 200 cells/well per treatment group were replated in a 6-well plate and calculated as the number of colonies formed 1-wk later relative to the number of colonies obtained in the non-treated condition. The concentrations mentioned in the figure are those of bleomycin. NT = non-treated cells, NP = without electric pulses, *P* = with electric pulses. Statistical analyses were performed by Kruskal-Wallis test with Dunn's multiple comparison test: $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to the non-treated cells. Means ± SD are shown from n = 9 from 3 independent experiments.

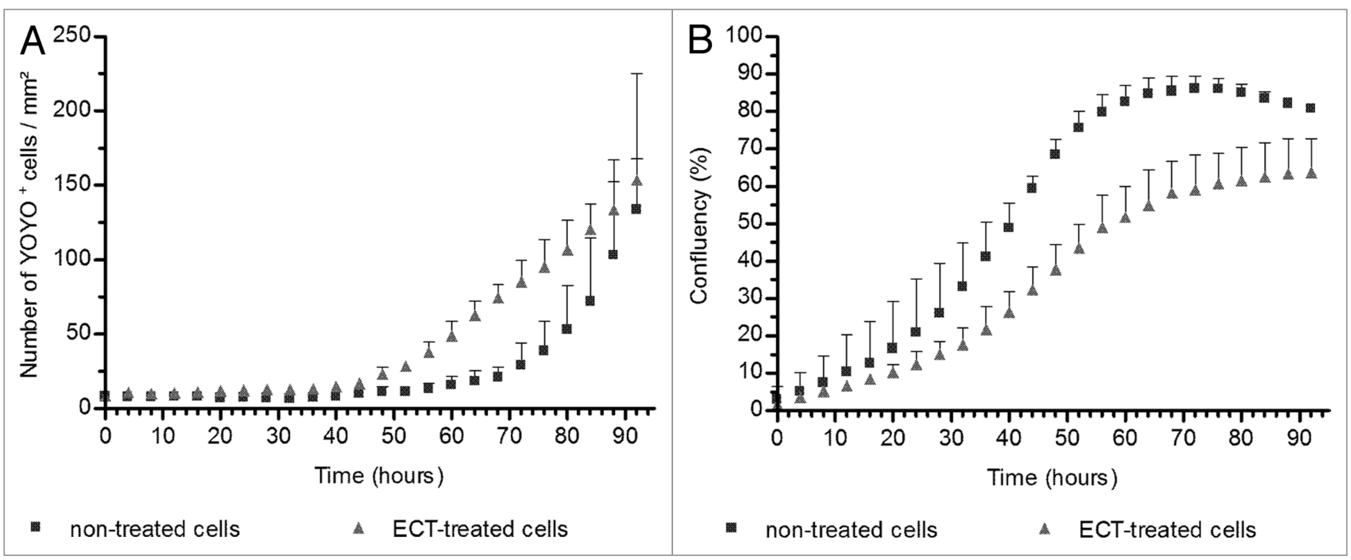

Figure 2. Kinetics of ECT-mediated cell death and confluency. (**A and B**) Cultured CT26 cells (5000 cells/group) were treated by electrochemotherapy (ECT) comprising electric pulses + 50 nM bleomycin over the indicated time frames. After the treatments, cells were seeded back into complete medium containing the fluorescent cell viability reporter YOYO-1 iodide. Cell viability (**A**) and confluence (**B**) were monitored every 4 h using the IncuCyteTM FLR live-cell imaging system. ECT-treated (triangles) vs. non-treated (squares) results are shown. Data are representative of 3 independent experiments each performed in triplicate. Means ± SD are pictured.

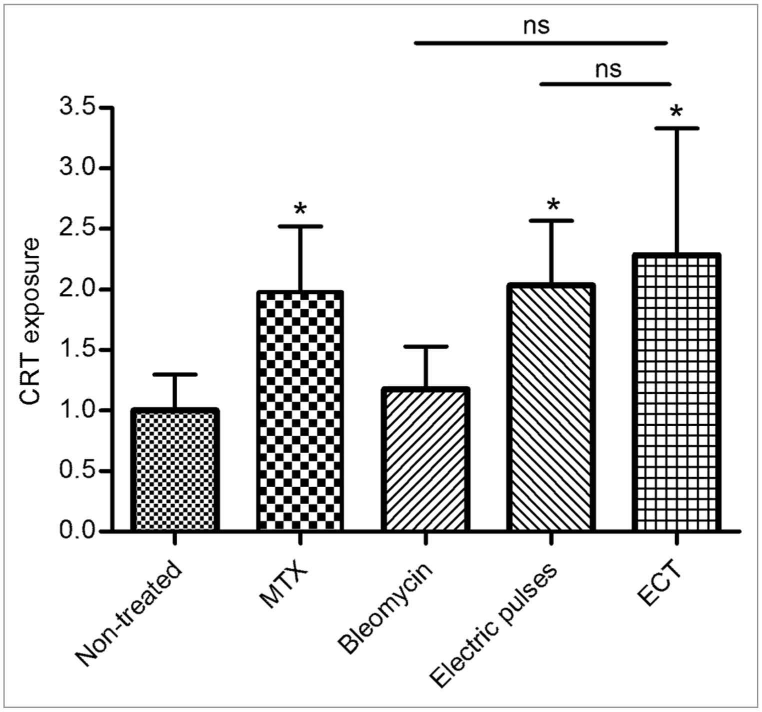

Figure 3. Electric pulses stimulate calreticulin exposure. The levels of calreticulin (CRT) on the surface of CT26 cells were measured in response to electrochemotherapy (ECT). Cells were treated with ECT (electric pulses + 50 nM bleomycin), 50 nM bleomycin only, electric pulses only or 1 μM mitoxantrone (MTX) for 30 h. Treated cells were stained using anti-CRT antibody followed by cytofluorometric analysis. Statistical analysis was performed by Mann-Whitney-Wilcoxon test; ns = not statistically significant,*$P < 0.05$ with respect to the non-treated cells (unless otherwise specified). Means ± SD are shown from n = 5 from 5 independent experiments.

It should be noted that the electropermeabilized cells were kept 30 min on the bench after the electropermeabilization procedure and then washed before being put back in culture. Hence, the ATP detection mentioned above is only correlated with the ATP released during the cell death process, far after the cells had their membrane resealed (cell membrane reseals in minutes after the electric pulses application[37]). We also measured the ATP released in the pulsing buffer, during the 30 min following the electropermeabilization (**Fig. 4B**). We detected about 400 times more ATP than in the cell culture medium of non-treated cells.

Cell death triggered by ECT induces HMGB1 release

HMGB1 release was measured in the CT26 cell culture supernatants 72 h after treatment. As shown in **Figure 5**, both ECT and MTX treatment stimulated a 2.5-fold increase in the amount of free HMGB1 in comparison to non-treated cells. Consistently with the fact that the electric pulses used in ECT are not meant to kill cells when they are used without bleomycin, we did not detect a significant difference in the amount of HMGB1 released between non-treated cells and cells treated by electric pulses alone. However, exposure to 50 nM bleomycin over 72 h decreased cell viability by 30% (data not shown) probably contributing to the significant release of HMGB1 (relative to non-treated cells) that we observed under this condition.

ECT-treated cancer cells elicit a protective immune response against tumor challenge in syngenic mice

In order to assay the immunogenicity of ECT-treated CT26 cells, we next applied a vaccination protocol in

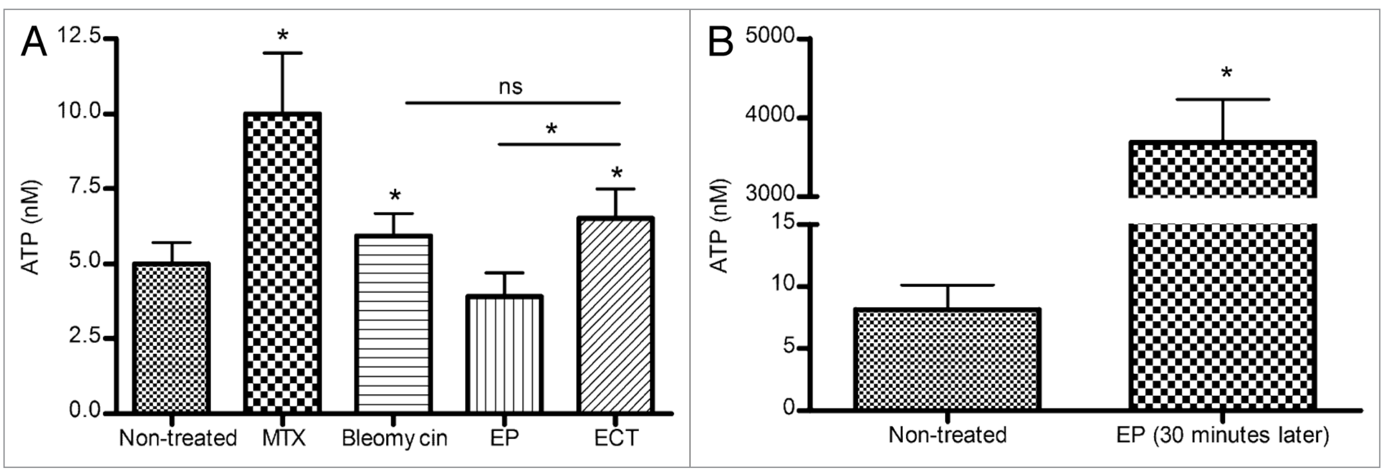

Figure 4. Electrochemotherapy and electric pulses liberate ATP. (**A and B**) The concentration of extracellular ATP in CT26 culture media of cells treated by electrochemotherapy (ECT) was measured by bioluminescence ATP reporter assay. Cells were treated with ECT (electric pulses + 50 nM bleomycin), 50 nM bleomycin only, electric pulses only or 1 μM mitoxantrone (MTX) and 250 000 cells/group were cultured for 30 h. ATP quantification was performed in (**A**) cell culture supernatants 30 h after the treatment or (**B**) in the pulsing buffer directly after the delivery of electric pulses (EP). Statistical analysis was performed by Wilcoxon signed rank test; ns = not statistically significant; *$P < 0.05$ with respect to the non-treated cells (unless otherwise specified). Means ± SD are shown from n = 6–8 from 3-4 independent experiments.

which dying ECT-treated CT26 cells (or MTX-treated control cells) were injected into immunocompetent syngenic BALB/c mice (**Fig. 6**). One week later, non-treated viable CT26 cells were injected in the contralateral flank. We found that BALB/c mice vaccinated with ECT-treated cells were protected against a subsequent challenge with viable colon cancer cells. The protective effect of vaccination with ECT-treated cells was nearly equivalent to that of MTX-treated cells, such that tumor take only reached 8% and 0%, respectively. Conversely, a 92% tumor take was obtained when control phosphate buffered saline (PBS) injection was performed as the immunization procedure, that is a highly significant ($P < 0.001$) difference relative to that of ECT- or MTX-treated cell vaccination. Vaccination with untreated viable cells could not be evaluated as mice had to be sacrificed before the challenge could potentially generate a contralateral tumor due to the growth of the vaccinal inoculum.

It should be noted that as a result of cell washing procedures of the vaccinal inoculum in vitro, the majority of ATP released as a direct consequence of the application of electric pulses was discarded.

ECT is much more efficacious in immunocompetent mice than in immunodeficient animals

Given that we almost reached an optimal protection in mice vaccinated with ECT-treated cells, we could surmise that the application of ECT on tumors in vivo might lead to complete antitumor protection via the massive amount of ATP released directly into the tumor cell microenvironment.

In order to mimic the treatment of human disease, and to determine the necessity for an intact immune system in eliciting ECT-mediated anticancer effects, ECT was performed on established CT26 tumors in both immunocompetent wild-type and immunodeficient nude BALB/c mice. As shown in **Figure 7**, we observed that 7 out of 8 immunocompetent mice were disease-free 24 d after the ECT treatment whereas all the treated nude mice presented progressive disease.

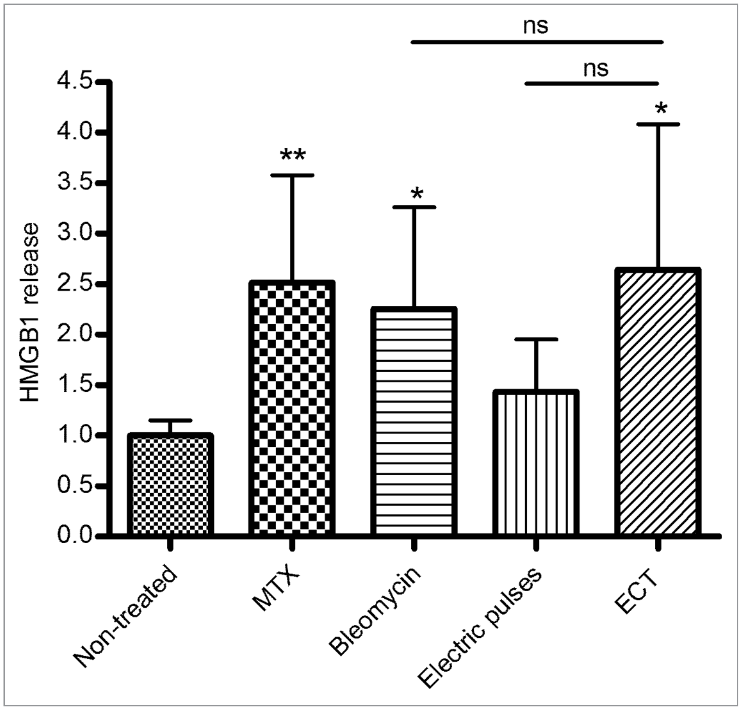

Figure 5. Electrochemotherapy induces HMGB1 release. To assay electrochemotherapy (ECT)-stimulated release of high mobility group box 1 (HMGB1) protein, CT26 cells were treated with ECT (electric pulses + 50 nM bleomycin), 50 nM bleomycin only, electric pulses only, or 1 μM mitoxantrone (MTX) and seeded back in culture for 72 h. HMGB1 release was quantified in cell culture supernatants from 20 000 cells using an ELISA-based technique (HMGB1 detection kit; Gentaur). Statistical analysis was performed by Mann-Whitney-Wilcoxon test; $*P < 0.05$ and $**P < 0.01$ with respect to the non-treated cells (unless otherwise specified); ns = not statistically significant. Means ± SD are shown from n = 5–6 from 3 independent experiments.

Discussion

ECT is an effective antitumor treatment used for the management of superficial tumors and is under preclinical and clinical evaluation for deep-seated tumors.[18,38-44] In this report, we evaluated the ability of ECT to induce ICD so that dying cancer cells act as a vaccine by eliciting an antitumor immune response.

ICD relies on the generation of DAMPs that activate specific functions of immune cells.[1,2] ATP, released by dying cells undergoing ICD,[3] acts as a "find me" signal for DCs and their precursors, favoring their maturation and triggering the secretion of IL-1β that ultimately leads to cytotoxic cell activation.[5-8] CRT, the major ER protein, is exposed on dying cells undergoing ICD and constitutes an "eat me" signal for DCs.[9-12] This leads to antigen presentation of tumor-associated antigens, which is favored by the passively released HMGB1 protein,[16] ultimately leading to the priming of specific anticancer effector cells.

Here, we first determined that 50 nM bleomycin combined with EPs resulted in massive cell death starting 45 h after the treatment (refer to **Figs. 1 and 2A**). We next measured the capacity of ECT to solicit hallmark features of ICD, including evaluation of both CRT translocation to the cell membrane and ATP release in the pre-mortem state and HMGB1 release post-mortem.[1]

A recent study revealed that bleomycin alone has intrinsic immunogenic properties since, at a 200-fold higher dose than the one used in our study or in classical ECT, bleomycin was able to induce CRT translocation to the surface of dying cells and both ATP and HMGB1 release, eventually leading to ICD.[45] Indeed, bleomycin can be internalized into cells through an endocytotic mechanism that involves binding to its membrane receptor.[46-48] A high drug concentration or a prolonged exposition to the drug increases the probability of internalization and thus may allow bleomycin to generate DAMPs. In our study, CT26 cells treated with 50 nM bleomycin (without electric pulses) did not expose CRT on their cell membrane but did release a significant amount of ATP and HMGB1 (refer to **Figs. 3–5**). This probably results from the increased uptake of

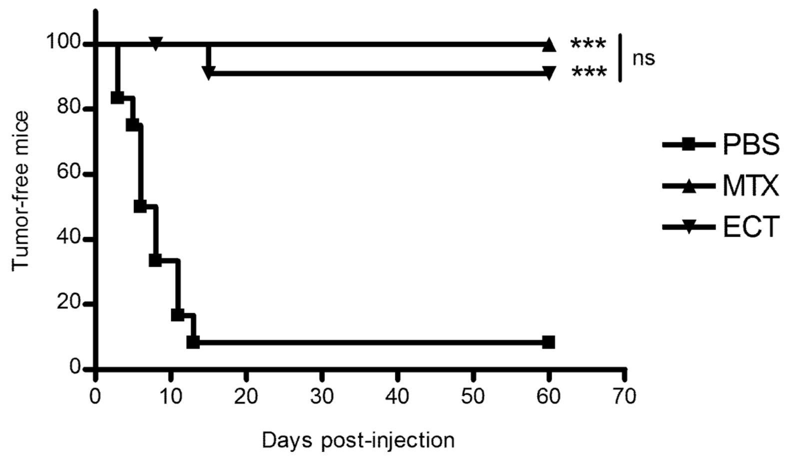

Figure 6. Vaccination with electrochemotherapy-treated cancer cells elicits a protective effect against tumor challenge in syngenic mice. CT26 cells were treated with electrochemotherapy (ECT) comprising electric pulses + 50 nM bleomycin. Thirty min later, 3×10^6 cells were subcutaneously injected in the left flank of immunocompetent BALB/c mice. A negative control of PBS injection and a positive control of immunization with cancer cells treated with 1 μM MTX over 30 h were also performed. One week later, 5×10^5 CT26 viable cells were injected in the contralateral flank and tumor take was monitored over a 2-mo period. Statistical analysis was performed by Chi-2 test; ns = not statistically significant; ***$P < 0.001$ with respect to the PBS control immunization (unless otherwise specified); n = 12 animals from 3 independent experiments.

bleomycin and consecutive toxicity (from 20% to 30%, data not shown) due to the 30 to 72 h drug treatment times.

In our study, we also demonstrated that CRT externalization was similarly enhanced in both ECT-treated cells and cells treated with electric pulses alone. This reinforces the fact that, at a very low drug concentration such as the one used for ECT, bleomycin alone does not induce CRT exposure on the cell surface, a response that does occur at a 200-fold higher bleomycin concentration.[45] Therefore, one may surmise that CRT exposure on ECT-treated cells is due to the application of the electric pulses.

There are several hypotheses that could support our findings regarding CRT exposure after the application of electric pulses. Microsecond electric pulses were initially thought to affect only plasma membrane, leaving intact the membranes of organelles, such as the one surrounding the ER. However, recent mathematical calculations have shown that such pulses can actually have effects on internal membranes.[49] This theoretical model has been confirmed in vitro by our laboratory (unpublished data) and by others, although a 2-fold higher field amplitude was used in this latter study.[50] Hence, the microsecond electric pulses used in our study and in ECT might directly provoke CRT translocation to the cell membrane. Another plausible scenario that may underlie our findings is that the application of intense electric pulses to cells has been correlated with the generation of reactive oxygen species (ROS).[51,52] Such ROS could be responsible for ER stress[53,54] that could indirectly lead to CRT exposure on the cell surface as occurs in response to anthracycline-based treatments.[9] Finally, electric fields have been shown to increase endocytotic and exocytotic processes[55,56] and could thus favor the endocytosis-dependent pathway and CRT translocation to the cell membrane.[9]

Regarding the ATP release stimulated by ECT, a massive and potentially relevant amount of ATP was detected in the pulsing buffer 30 min after the electropermeabilization procedure (refer to **Fig. 4B**). This result supports previous data reporting that electric pulses liberate both ATP[57,58] and inflammatory factors, ultimately leading to APC recruitment to the site of electric pulse application.[59-61] We hypothesize that this immune cells recruitment is due to ATP released directly following electric pulse-mediated cell permeabilization. Indeed, no ATP was detected 30 h after the application of electric pulses alone when cells were washed and put back in culture following membrane resealed. However, although to a lesser extent than directly after electric pulses-mediated permeabilization, a significant amount of ATP was detected 30 h after ECT treatment, despite the fact that the cells were washed thereby removing the ATP released by the electric pulses. This suggests a continued leakage of ATP from the ECT-treated cells, even after cell membranes were completely resealed. Since no significant release of ATP was observed 30 h after the application of electric pulses alone when the cell membrane was resealed, this demonstrates that bleomycin is mandatory for ATP leakage in cells with resealed membrane.

In regards to HMGB1, we demonstrated that its release occurred only when electric pulses application was combined with the bleomycin administration, consistently with the induction of cell death associated with the disruption of cellular compartments.[13] Likewise, a 72-h exposition to 50 nM bleomycin alone induced a 30% drop in cell viability (data not shown) along with HMGB1 release. However, electric pulses alone did not kill cells and thus, naturally did not trigger HMGB1 release.

Our in vitro data correlated well with vaccination experiments performed in syngenic BALB/c mice with intact immune systems, revealing that ECT-treated dying CT26 cells could elicit immunity against subsequent tumor challenge. Less than 10% of tumor take was observed using a vaccination protocol that involved ECT-treated cells (refer to **Fig. 6**). By contrast, tumor take surpassed 90% when control immunization was performed with PBS only. Hence, an antitumor immune response was elicited by ECT-treated cells, presumably by the induction of ICD, as revealed by the protection afforded to mice subsequently injected with live tumor cells.

Likewise, we demonstrated that ECT treatment performed in immunocompetent mice was much more efficient than those that were immunodeficient (refer to **Fig.** 7). Indeed, a large number (7 out of 8) of complete responses was observed in wild-type BALB/c mice whereas all the tumors progressed in nude mice, despite a temporary decrease in volume and an initial arrest in tumor development caused by the debulking effect

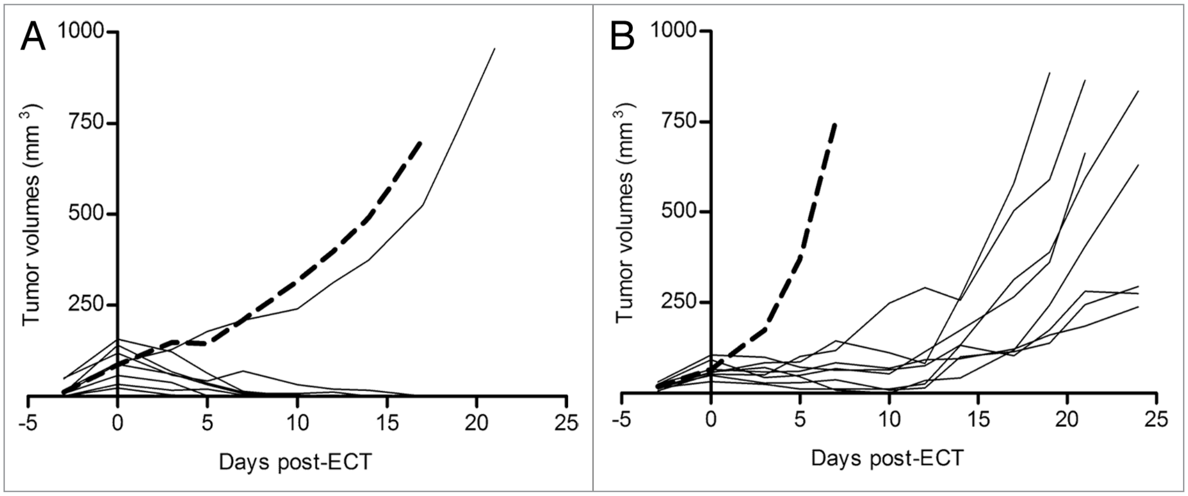

Figure 7. ECT is much more efficacious in immunocompetent mice than in immunodeficient animals. (**A and B**) Comparison of electrochemotherapy (ECT) efficiency in wild-type and in nude BALB/c mice bearing established CT26 tumors. Once the mean volume of tumors reached approximately 60 mm^3, electrochemotherapy was applied by i.v. injection of 15 µg of bleomycin in 100 µL PBS into each anesthetized mouse and 4 min later, 8 electric pulses of 100 µs at 1300 V/cm were delivered to the tumors by Cliniporator. (**A**) ECT treatment in wild-type mice (n = 7 untreated mice and n = 8 treated mice). (**B**) ECT treatment in immunocompromised nude mice (n = 8 mice per group). Dotted bold lines represent the mean volume of the untreated tumors. The volume of each ECT-treated tumor is depicted with a thin full line.

of ECT. These results support previous data from our group[22-24] and show that the immune system can actually destroy the cancer cells that escaped the direct antitumoral effect of ECT, either because they were not sufficiently permeabilized by the electric pulses or not surrounded by enough bleomycin molecules for toxicity.

To summarize, ECT generated ICD, partially due to the application of electric pulses, that triggered on their own both CRT translocation to the cell membrane and an early and physiologically relevant ATP release. Thus, electric pulses might be combined with other non-immunogenic cancer treatments such as *cis*-platin, not only to enhance cancer cell drug uptake but also in an attempt to generate DAMPs, i.e., CRT exposure on the cell membrane and ATP leakage, thereby inducing bona fide ICD.

Implications of ECT elicitation of cancer cell ICD encompasses long-term antitumor benefits. Indeed, there is evidence that cancer stem cells, purportedly a major factor underlying cancer recurrence,[62] are depleted in response to extracellular ATP.[63] Furthermore, it has been shown that cancer stem cells are efficiently recognized by cytotoxic T lymphocytes,[64] the main antitumor effector activated as a consequence of ICD induction.[1] Our study also supports clinical observations describing the absence of tumor recurrence in ECT-treated areas, suggesting all tumor cells had been ablated in these sites due to sustained ECT effects. We hypothesize that the residual tumor cells not directly affected by ECT treatment (due to insufficent permeabilization or paucity of bleomycin molecules) were secondarily killed by the cytotoxic immune cells activated by ICD processes. Finally, preclinical evidence[22,26,31-33] suggests that the generation of a systemic antitumor immune response initiated by ECT-mediated immunogenic cancer cell death may be potentiated by combination with immunostimulatory agents, thus offering an elegant and efficient means to cure both the ECT-treated nodules as well as undetected metastases elsewhere.

Materials and Methods

Cultured cancer cells

CT26 murine colon carcinoma cells (kindly provided by Pr. Guido Kroemer) were cultivated in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were propagated in this complete medium at 37 °C in a 95% humidity atmosphere containing 5% CO_2 and passaged upon confluency at a 1:10 dilution using a TrypLE express solution. Unless otherwise specified, all components mentioned above were purchased from Life Technologies.

Electrochemotherapy treatment

A solution of 5 × 10^6 CT26 cells/mL containing bleomycin (0–100 nM) was prepared in a pulsing buffer consisting of modified Eagle's medium (MEM) specifically modified for suspension cultures without calcium and without glutamine (S-MEM; Life Technologies). A 100 µL or 200 µL volume of this solution was placed into a 1 mm or 2 mm gap electroporation cuvette (Cellprojects), respectively and submitted to 8 electric pulses 100 µs-long at 1200 V/cm by a Cliniporator generator (IGEA). Cells were subsequently kept at room temperature for 30 min before being washed and replaced back in culture.

Cytotoxicity clonal growth assay

Following ECT treatment as described above, 200 total CT26 cells were seeded into a well of a 6-well plate containing complete medium. After 1 wk, medium was discarded, cells were washed with PBS, and fixed and stained using an aqueous solution containing 20% ethanol, 3.7% formaldehyde, and 0.2% crystal violet. Cytotoxicity was calculated as the number of colonies formed in the treated condition relative to the number of colonies formed in the non-treated condition. Controls with electric pulses alone or exposed to bleomycin alone for 30 min or to 1 µM MTX (Sigma–Aldrich) alone for 30 h were also performed.

ECT-mediated cell death kinetics

To assay the kinetics of ECT-mediated cell death in our system, 5000 CT26 cells treated by electric pulses in the presence of 50 nM bleomycin (see above) were transferred into a well of a 96-well plate containing complete medium with 0.1 µM YOYO-1 (Life Technologies) and then placed into an IncuCyte™ FLR imaging system (Essen Biosciences) inside a regular cell culture incubator. The IncuCyte™ FLR device monitored the degree of confluency and the percentage of fluorescently stained dead cells over the course of 90 h. Control cells exposed to bleomycin alone for 30 min or electric pulses alone were also performed.

Detection of CRT exposure

In order to evaluate CRT externalization in response to ECT, treated CT26 cells were evaluated 30 h after being treated by ECT with 50 nM bleomycin, that is in the pre-mortem time scale.[1] Cells were harvested, washed twice with cold PBS, and stained for 30 min with rabbit polyclonal anti-mouse CRT antibodies (Abcam) at a 1:100 dilution in a 5% bovine serum albumin (BSA) solution in PBS. Cells were then washed twice with cold PBS and stained for 30 min with goat anti-rabbit Alexafluor488-conjugated monoclonal secondary antibodies (Life Technologies) at a 1:500 dilution in a 5% BSA solution in PBS. Cells were then washed 3 times with cold PBS and analyzed using an Accuri C6 flow cytometer (BD Biosciences). Control cells were treated with electric pulses alone, exposed to bleomycin alone or to 1 µM MTX alone over 30 h.

Quantification of extracellular ATP

In order to evaluate release of cellular ATP in response to ECT, treated CT26 cells were evaluated 30 h after being treated by ECT with 50 nM bleomycin, that is in the pre-mortem time scale.[1] Cell culture supernatants of 250 000 ECT (vs. control) treated cells (see above) were analyzed using an ATP Bioluminescent Assay kit (Sigma-Aldrich), according to the manufacturer's instructions. Control cells were treated with electric pulses alone, exposed to bleomycin alone or to 1 µM MTX alone over 30 h. ATP release was also measured in the pulsing buffer 30 min after the application of electric pulses in the absence of drug.

Quantification of HMGB1 release

In order to evaluate release of HMGB1 in response to ECT, treated CT26 cells were evaluated 72 h after being treated by ECT with 50 nM bleomycin, that is in the post-mortem time scale.[1] Cell culture supernatants of 20 000 cells were analyzed using an ELISA-based HMGB1 detection kit (Gentaur), according to the manufacturer's instructions. Control cells were treated with electric pulses alone, exposed to bleomycin alone or to 1 µM MTX alone during 72 h.

Antitumor vaccination activity of ECT-treated CT26 cells

To determine the potential of ECT to generate systemic anticancer immunity, 30 min after being treated by ECT as above with 50 nM bleomycin, 3×10^6 total CT26 cells were injected subcutaneously in 200 µL of PBS in the left flank of 7-wk-old wild-type BALB/c mice (Janvier). A negative control of PBS injection and a positive control of immunization with cancer cells treated with 1 µM MTX over 30 h were also performed. One week later, 5×10^5 non-treated cells were injected subcutaneously in the contralateral flank. Tumor take was monitored 2 to 3 per week for 2 mo.

ECT treatment of established CT26 tumors

In vivo ECT experiments were performed by injecting 500 000 viable CT26 cells in 100 µL PBS subcutaneously into the flanks of 7-wk-old BALB/c mice, either wild-type or nude (Janvier). Once the mean volume of tumors reached approximately 60 mm^3, electrochemotherapy was applied by i.v. injection of 15 µg of bleomycin in 100 µL PBS into each anesthetized mouse and 4 min later, 8 electric pulses of 100 µs at 1300 V/cm were delivered to the tumors using the Cliniporator. Non-invasive electrodes (IGEA) with 0.5 cm-spaced metallic plates were used and conductive gel (NM Médical) was applied on the treated-to-be area in order to improve the contact between the metallic plates and the tumor. Tumor progress was monitored 3 times a week and the tumor response was determined according to the RECIST 1.1 guidelines.[65]

Statistical analyses

Data are presented as mean ± standard deviation (unless otherwise specified). Data were analyzed with GraphPad Prism 4 software. Statistical analyses were performed using Kruskall–Wallis test with Dunn's multiple comparison test, Mann–Whitney–Wilcoxon test, Wilcoxon signed rank test or Chi-2 test and $P < 0.05$ was considered statistically significant.

Ethics statement

All animal experiments were performed in strict compliance with the ethical guidelines issued by the European Committee (Directive 86/609/CCE). The Université Paris-Sud Animal Ethics Committee #26, registered by the French Department of Research, specifically approved this protocol (protocol registration number #2012_027).

Disclosure of Potential Conflicts of Interest

LMM has a consulting activity for IGEA and holds several patents on electroporation technologies.

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