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## A Study of the Immunological Response to Tumor Ablation with Irreversible Electroporation

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Immune cell recruitment during the treatment of sarcoma tumors in mice with irreversible electroporation was studied by immunohistochemistry. Irreversible electroporation is a non-thermal tissue ablation technique in which certain short duration electrical fields are used to permanently permeabilize the cell membrane, presumably through the formation of nanoscale defects in the membrane. Employing irreversible electroporation parameters known to completely ablate the tumors without thermal effects we did not find infiltration of immune cells probably because of the destruction of infiltration routes. We confirm here that immune response is not instrumental in irreversible electroporation efficacy, and we propose that irreversible electroporation may be, therefore, a treatment modality of interest to immunodepressed cancer patients.

Key words: Tumor ablation; Irreversible electroporation; Immunological response; Tumor infiltration; and Immunocytochemistry.

### Introduction

Electroporation, also known as electropermeabilization, is a term used to describe the permeabilization of the cell membrane as a consequence of the application of certain short and intense electric fields across the cell membrane, the cells or the tissues. The permeabilization can be temporary (reversible electroporation) or permanent (irreversible electroporation), as a function of the electrical field magnitude and duration and number of the pulses (1). Reversible electroporation is commonly used to facilitate the penetration of various otherwise non-permeable macromolecules across the cell membrane (2-4).

Irreversible electroporation, the ability of certain electrical pulses to permanently permeabilize the cell membrane has been known for several decades. For most of this period irreversible electroporation was primarily studied as an upper limit for the electrical parameters for reversible tissue electroporation applications (5). Irreversible electroporation has also been studied as method to destroy prokaryotic (6) and eukaryotic cells *in vitro* and has gained momentum recently as a method to kill microorganisms (7), mammalian normal cells (8) as well as mammalian cancer cells (9) *in vitro*. These studies have demonstrated the ability of irreversible electroporation (IRE), to completely eradicate an entire population of cells *in vitro* without inducing any thermal damage.

Davalos, Mir, and Rubinsky (10) recently showed that irreversible electroporation (IRE) can serve as an independent and new method to ablate substantial volumes

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**Abbreviations:** IRE, Irreversible electroporation; ECT, Electrochemotherapy.

of tissue. This study opened the way to the large scale use of IRE in surgery. The procedure would involve placing minimally invasive electrodes near the targeted region to deliver the electroporation pulses. Edd *et al.* were the first to study tissue ablation with IRE *in vivo* in the normal liver of rats (11). Complete ablation of the targeted liver tissue was achieved by exposing the tissue to electric pulses with parameters that do not induce thermal damage (11) but irreversibly permeate the cells and induce necrosis. Massive blood vessel congestion was observed in the sinusoids of the treated volume. The study concluded that the ablation zones produced by IRE are precisely delineated, with cell scale resolution between ablated and non-ablated areas. Another recent study evaluated the long term effects of IRE in a large animal model (12). The results demonstrated that irreversible electroporation has the potential to ablate large volumes of tissue. The electrical parameters used do not induce substantial thermal effects even though they are above those conventionally used in reversible electroporation in terms of electrical field magnitude, pulse duration and number of pulses. Histology has reconfirmed the results: showing that mathematical modeling of electrical and thermal fields are a powerful tool in designing IRE ablation, that IRE can be used to ablate tissue with cell scale resolution and that IRE affects only the cell membrane and spares connective tissues (11, 12).

In this study we report the first attempt to systematically study the immune system reactions in cancer tissues treated with IRE. We have recently reported that IRE is also effective in ablating transplanted tumors in mice: cell membrane destruction in the whole of the tumor tissue results in long term complete regressions lasting more than 60 days (cures). Employing electrical parameters which were shown to induce tumor ablation via IRE without a thermal effect, we did not observe an increase in the immune cells. This suggests that the immune system is not needed to ablate tumors with IRE and therefore IRE may be a viable option to consider within the choice of treatments for immunodepressed cancer patients.

## Materials and Methods

### Tumor Cells Culture and Tumor Production

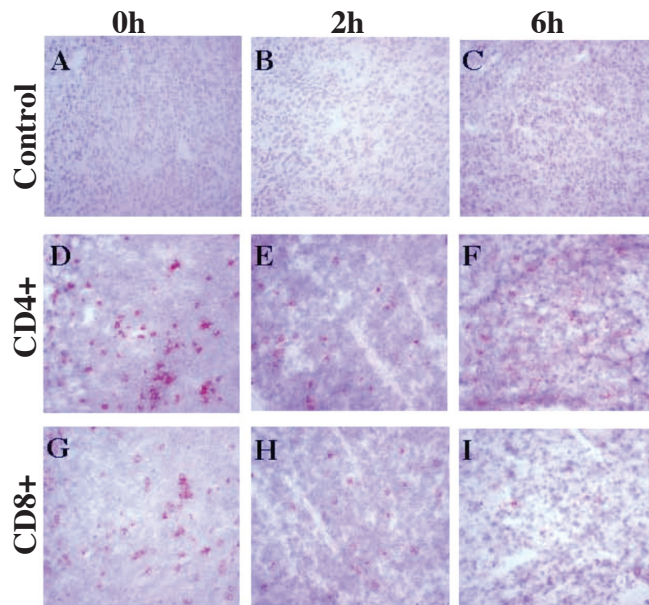
Cells from a LPB cell line, a methylcholanthrene-induced C57Bl/6 mouse sarcoma cell line (13), were cultured using standard procedures in a minimum essential medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 100 U.ml<sup>-1</sup> penicillin, 100 mg.ml<sup>-1</sup> streptomycin (Sarbach, France) and 8% foetal calf serum (Gibco). C57Bl/6 female mice, 6-8 weeks old, were inoculated subcutaneously in the left flank with  $1 \times 10^6$  cells, producing in 9 days tumors of 4 to 5 mm in diameter. Animals were housed and handled according to the recommended guidelines (14).

### Tumor Treatment

At the start of the procedure mice were anaesthetised using a mixture of xylazine 12.5 mg.kg<sup>-1</sup> (Bayer Pharma, Puteaux, France) and ketamine 125 mg.kg<sup>-1</sup> (Parke Davis, Courbevoie, France). A small incision was performed on the skin near the tumor and the blood vessels were located. Then the skin flap containing the tumor was lifted, taking particular care to avoid cutting the main blood vessels nourishing the tumor. Stainless-steel plate electrodes were placed in direct contact with both sides of the cutaneous tumor, with the tumor sandwiched between the parallel plates, to have a good control of the electric field amplitude and distribution in the tumor tissue. Good contact of the electrodes with the tumor tissue was produced using electrocardiography paste (Eko-gel, Camina, Egna, Italy). The distance between the electrodes was 4 mm and the pulse generator was set to 1000 V to deliver an applied field of 2500 V/cm. We delivered 4 trains of 16 pulses of 100  $\mu$ s and 2500 V/cm, at a repetition frequency of 1 Hz. Between each train of pulses the electrodes were rotated 90°, and pulses started again after an interval of 45 seconds. In our previous studies (Al Sakere *et al.*, submitted) these parameters were found to induce tumor tissue ablation (37% of long term complete regressions) without a thermal effect. The square-wave electric pulses (EP) were generated by an electroporation power supply (Cliniporator™, Igea, Carpi, Italy). After the electroporation pulses were delivered, the skin incisions were closed with metallic clips, the mice were returned to their cages, kept for different periods of time (between 1 and 72 hours) and then humanely sacrificed by CO<sub>2</sub> inhalation before the tumors were removed and processed for immunohistochemical analysis.

### Immunohistochemistry for Immunocompetent Cell Subtypes Determinations

Frozen sections of both IRE treated LPB and control tumors were processed. 5  $\mu$ m-thick sections were prepared, air-dried for 1 h and fixed with cold acetone (4 °C) for 10 min. Slides were then washed with PBS for 4 min and incubated with anti-CD11c, anti-CD80 (Caltag Laboratories, Burlingame, CA) or with anti-CD4+ 1:3000, anti-CD8+ 1:1000, anti-CD86 1:50, and anti-Mac1 1:600 antibodies (Pharmingen International, Carpinteria, CA) for 1 h. The sections were incubated with biotinylated goat anti-rat for CD4+, CD8+, CD86, and Mac1 and with biotinylated goat antihamster for CD11c, CD80 in 1:50 dilution for 30 min. The sections were further incubated with an APAAP rat antibody for 30 min (DakoCytomation Ltd, Ely, UK). Finally, signal was visualised with Fast Red substrate (Dako Corporation, Carpinteria, CA). Slides were counterstained with Mayer's haematoxylin and mounted with aqueous medium (Aquaperm Shandon Aqua-Perm™ Thermo Electron IVDD Compliant, CE).



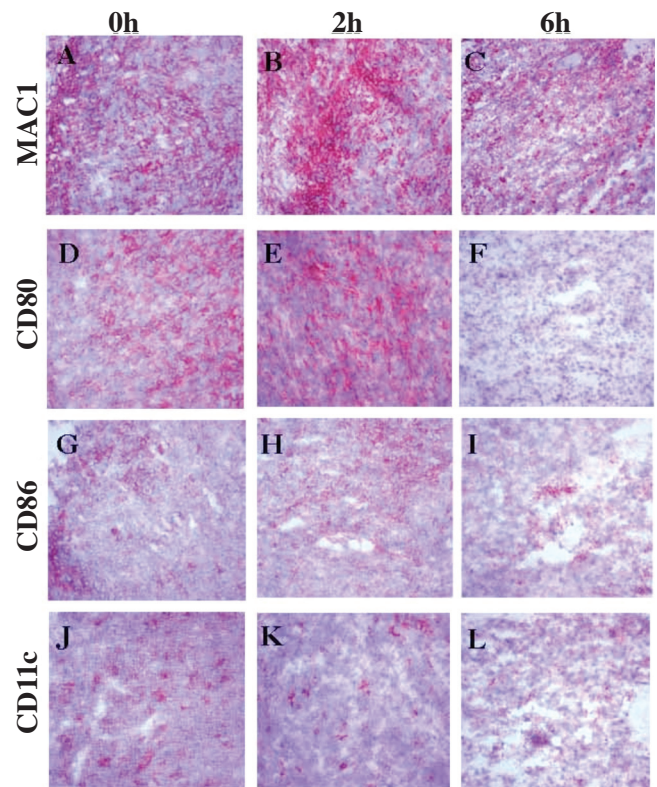
**Figure 1:** Immunohistochemistry study of the immune effector cells infiltrating the LPB tumors before, 2 h and 6 h after the treatment of the tumors by irreversible electroporation. Panels A, D, and G (the left panels) correspond to tumors before the IRE procedure. Panels B, E, and H (the central panels) show the tumors 2 h after IRE. Panels C, F, and I (the right panels) show the tumors 6 h after IRE. Panels A-C (the upper row) are controls corresponding to tumor slices completely processed but with no primary antibody. Panels D-F (the middle row) are stained with an anti-CD4 to reveal the presence of the T CD4+ lymphocytes. Panels G-I (the lower row) are stained with an anti-CD8 to reveal the presence of the T CD8+ lymphocytes. The results show no recruitment of either T CD4+ or CD8+ lymphocytes after the IRE procedure.

### Results and Discussion

To detect the generation of potential immune reactions in the treated tissue, frozen tumor sections were stained with anti-Mac1, anti-CD86, anti-CD80, anti-CD11c, anti-CD4, and anti-CD8 antibodies, which map the evolution of specific cell populations. The Mac 1 stain mainly reveals the presence of macrophages, the CD 86, CD80 and CD11c stains reveal the presence of activated antigen presenting cells and dendritic cells, and the CD4 and CD8 are used to detect the presence of the T4 and T8 lymphocytes. The specificity of the staining is confirmed by the absence of background in the controls completely processed but without a specific first antibody.

In the case of the effector cells of the antitumor response, the T lymphocytes, a rapid decrease in the content of both CD4+ and CD8+ T lymphocytes was observed two hours after the delivery of the electric pulses. An even more pronounced decline was evident six hours after the treatment, particularly in the case of the CD8+ cells (Fig. 1), and even at times longer than 6 hours (data not shown).

All the potential antigen presenting cells (macrophages and dendritic cells) showed no major change two hours after



**Figure 2:** Immunohistochemistry study of the immune antigen presenting cells infiltrating the LPB tumors before, 2 h and 6 h after the treatment of the tumors by irreversible electroporation. The results show no infiltration of these cells in the treated tissue after the IRE procedure. Panels A, D, G, and J (the left panels) correspond to tumors before the IRE procedure, panels B, E, H, and K (the central panels) to tumors 2 h after IRE, and panels C, F, I, and L (the right panels) to tumors 6 h after IRE. Panels A-C (the upper row) are stained with an anti-mac1 to reveal the presence of macrophages. Panels D-F are stained with an anti-CD86 and panels G-I are stained with an anti-CD80 to reveal the presence of antigen-presenting cells (dendritic cells, macrophages, and B lymphocytes). Panels J-L (the lower row) are stained with an anti-CD11c to reveal the presence of dendritic cells and of a subset of NK cells (controls are displayed in Fig. 1).

the electric pulses delivery, neither in the amount nor in the distribution of the stained cells (Fig. 2). Again, at 6 hours after the treatment, a decline in the content of all these cell types was easily noticeable: this decrease in the staining of the antigen presenting cells was important, even for those populations present in large amounts in the tumors before the electric pulses delivery (controls before the IRE) (Fig. 2). The staining controls were identical to those displayed in Figure 1 (not reported). No further changes were observed at times longer than 6 hours (data not shown).

The results are also summarized in Table I. All in all these results show that there is no immune cells infiltration in this tumor model. This is in agreement with previous studies which have shown that the microvasculature architecture of the tissue treated with IRE is destroyed and tumor is occluded to blood flow, while larger blood vessels are unperturbed

Table I

Summary of the immunohistochemistry study of the tumor infiltrating cells after the treatment of the tumors by irreversible electroporation: the specificity of the staining is confirmed by the absence of background in the controls completely processed but without a specific first antibody. The Mac1 stain reveals the presence of macrophages, while the CD86, CD80, and CD11c stain reveal the presence of antigen presenting cells and dendritic cells. The presence of the T lymphocytes is detected by the CD4 and CD8 stains. It can be seen that in no case there is an increase in any of these stains at 6 h after the IRE (a moment where the destruction of the tumor cells and of the endothelial vascular cells is revealed by a simple haematoxylin-eosin-saffron staining, as well as by a specific anti-CD31 staining). (++++) to (-): relative scale in terms of average staining.

Time	Control negative	Mac1	CD86	CD80	CD11c	CD4+	CD8+
0h	-	++++	+++	+++	++	++	++
2h	-	++++	++	+++	++	+	+
6h	-	++	+	+	+	+	+

during the procedure (11, 12). The results suggest that immune cells already inside the tumor are probably destroyed by the IRE pulses, while new infiltration may be restricted by the tumor vasculature disorganization and disruption. T4 (CD4+) and T8 (CD8+) lymphocytes staining rapidly decreases while macrophages and dendritic cells staining remains for longer periods. It is important to note that similar to the case of the CD31 antigen, it may be normal that diffused (or semi-diffused) staining of these markers subsist for a while even though the corresponding cells have been killed by the treatment, explaining the persistence of the various stains, reported in Table I.

The results show that the populations of T cells rapidly decreased, the decrease being detectable within two hours after the IRE. The antigen-presenting cells stayed in the treated volumes at least for two hours, before declining at six hours. Actually, the immune cells present in the tumor at the time of the electric pulses delivery should also be irreversibly electroporated and thus killed. Thus the staining found at two hours after the treatment should correspond to the same cells as those present in the tumor before the IRE. Indeed, the disaggregation of the membrane of the tumor cells does not yet result in morphological changes at two hours after the IRE as shown in Al-Sakere *et al.* (submitted). In particular we found that the staining of the vascular endothelial cells, still delineating the tumor vessels at two hours after the IRE, becomes diffuse at six hours after the treatment, corresponding to the destruction of these cells and to the diffusion of the epitopes in the necrotic tissue. The same evolution should concern the tumor infiltrating cells: the decrease in the staining observed at six hours after the IRE is, thus, not linked to the exit of the immune cells from the treated volume, but to their destruction and dissolution in the necrotic tissue. This evolution, marked by the lack of exchanges between the treated volume and the rest of the organism, reinforces our previous conclusion on the lack of major infiltration of the treated tumor by the cells of the immune system. This conclusion is in agreement with our previous results (Al Sakere *et al.*, submitted) showing that IRE was similarly effective on the same

tumor type transplanted either in immunocompetent or in immunodeficient mice.

The use of electric fields to ablate unwanted tissue has been studied previously. Most methods employ electric fields as a form of thermal ablation (*e.g.*, radiofrequency ablation) and heat the tissue to coagulation (15-18). Methods that employ electric field but do not rely on Joule heating to kill tissue include Electrochemotherapy (ECT) (2) and supra-electroporation (11, 19, 20).

Electrochemotherapy (ECT), the use of reversible electroporation to introduce non-permeable cancer drugs into malignant cells (2), has been shown to be a safe and highly efficient method for drug treatment of cancer and is currently used to treat cutaneous and subcutaneous tumors in humans (21-26). The standard operating procedures (27) have been established after a clinical multicenter study (28) and several previous single center clinical trials, as reviewed by G. Sersa (29).

Supra-electroporation is the killing of tissue using nanosecond pulses or pulsed electric fields (PEFs) to kill cells (20, 30, 31). As opposed to ECT or IRE, supra-electroporation does not electroporate the cells. The pulses are orders of magnitudes shorter (nanoseconds) and much larger in field strength compared to the duration and strength of the pulses used for IRE (our pulses last 100  $\mu$ s). The nanopulses are believed to affect the interior of the cell and kill the cell by apoptosis induction, while IRE kills the cells by a necrotic process since cell death starts by the destruction of the cell membrane (19, 20, 30). Even though each of the promising techniques to destroy tissue are different, it is quite likely that each will find uses in modern medicine separately or in combination (10).

In conclusion, our results indicate that IRE did not induce a substantial infiltration of immune cells into the treated tissue. Since we employed parameters that have been shown to cause the tumor ablation, IRE does not require an immunological response to produce the ablation. Therefore, the efficacy of the IRE will be the same whether the patient is immunodepressed

or not. Although our results are preliminary and there are many other factors to consider such as patient's reaction to the procedure itself, our results suggest that IRE is a potential treatment to consider for local treatment of tumors in immunodepressed patients. The treatment could, therefore, be used to treat cancer patients who suffer from depression of their immune system, caused either by the disease itself or by previous treatment, such as chemotherapies.

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