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Electroporation of 3D-Cultured Breast Cancer Cells Elicits T Lymphocyte-Mediated Killing

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Abstract

Background: Electrochemotherapy (ECT) is emerging as a powerful strategy to induce immunogenic cell death and profoundly reshape the tumor microenvironment. However, the specific immunomodulatory role of electric pulses, independently of chemotherapeutic agents, remains to be fully elucidated. This study investigates the potential of high-voltage electroporation, as used in ECT protocols, to stimulate immune responses in breast cancer.

Materials and Methods: A 3D hydrogel-based culture system was used to generate uniform spheroids from HCC1954 breast cancer cells. Electroporation was applied with increasing electric field strengths (0–1000 V/cm) to modulate membrane permeability. Spheroids were then co-cultured with phytohemagglutinin-stimulated Jurkat cells or primary human $CD4^+/CD8^+$ T lymphocytes. Electroporation efficiency was assessed using propidium iodide (PI) staining. T cell-mediated cancer cell mortality was assessed through cancer cell viability assay via flow cytometry. Expression of Interleukin-2 (IL-2), Tumour Necrosis Factor alpha (TNF-α), and Interferon gamma (IFN-γ) was evaluated to assess T cell immune activation.

Results: Electroporation at 600 and 1000 V/cm caused marked structural changes in the spheroids. At 1000 V/cm, a necrotic core was observed, surrounded by a layer of viable but stressed cells, whereas 600 V/cm resulted in a favorable proportion of successfully electroporated cells while preserving overall cell viability. These heterogeneous responses promoted differential immune recognition and cytotoxic targeting of cancer cells in co-culture experiments with immune cells. In co-culture, spheroids treated at 600 V/cm increased T cell activation and promoted targeted tumor cell killing. These effects were confirmed using both Jurkat cells and primary human lymphocytes. Gene expression analysis revealed upregulation of pro-inflammatory cytokines, indicating enhanced immunogenicity following high-voltage electroporation.

Conclusions: High-voltage electroporation alone can increase the immunogenic potential of breast cancer cells and promote T cell-mediated anti-tumor activity. These findings support its application as a standalone immunomodulatory strategy and lay the groundwork for its integration into combined immunotherapeutic approaches.

Keywords: electroporation, immunogenic cell death, anti-tumor immunity, cytotoxic T cell immune response, 3D cell culture

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Introduction

Among various locally applied chemical, physical, and physicochemical methods for achieving effective oncolysis, electrochemotherapy (ECT) stands out as a powerful approach that combines the benefits of chemotherapy with electroporation (EP).[1,2] This technique involves the application of precisely controlled electric pulses to temporarily increase the permeability of the tumor cell membrane, facilitating the uptake of chemotherapeutic agents such as bleomycin, cisplatin, or oxaliplatin. By significantly improving drug delivery, ECT maximizes the cytotoxic effects of these agents, leading to localized tumor destruction.

Beyond its direct cytotoxic action, ECT can trigger immunogenic cell death (ICD), a process that enhances the body's anti-tumor immune response, potentially contributing to localized tumor control.[3] The tumor microenvironment is also influenced by ECT, as the treatment not only reduces tumor-associated immunosuppression but also promotes the infiltration of immune cells into the tumor.[4] This dual effect can enhance the body's natural immune response against cancer, potentially improving therapeutic outcomes.

So far, ECT has demonstrated efficacy in treating solid tumors, including those resistant to conventional therapies. It is primarily used for superficial tumors, such as melanoma, squamous cell carcinoma, and breast cancer, and has shown potential for treating deep-seated tumors like colorectal cancer.[5,6] ECT is also widely used in veterinary oncology.[7,8] Studies have shown that ECT not only induces localized tumor regression but also promotes systemic immune activation, potentially leading to the abscopal effect—where untreated metastases regress due to an enhanced immune response.[9] This high immunomodulatory potential positions ECT as a promising adjuvant therapy for cancers where immune activation plays critical roles in disease control. When combined with other immunotherapeutic approaches, like immune checkpoint inhibitors (ICIs) or cancer vaccines, ECT has the potential to improve patient outcomes by enhancing systemic anti-tumor immunity, paving the way for combined strategies to boost treatment efficacy and long-term patient outcomes.

While ECT is known to induce ICD, thereby stimulating an anti-tumor immune response, the precise contribution of the electric pulses themselves—independent of the cytotoxic effects of the chemotherapeutic agents—remains unclear. Some studies suggest that electric fields may influence immune activation by modulating cellular stress responses, altering tumor microenvironment dynamics, or affecting antigen presentation.[4] However, further research is needed to elucidate the exact mechanisms by which EP contributes to immunostimulation and to determine whether it can be optimized to enhance the systemic anti-tumor response in ECT-treated patients. To this end, 3D cultures, commonly based on hydrogels, natural and synthetic polymer-based scaffolds, and decellularized extracellular matrices, serve as a reliable model for mimicking the *in vivo* tumor tissue architecture. They possess the unique ability to generate an extracellular matrix (ECM) and facilitate cell-to-cell interactions in a more physiologically relevant manner—both of which are absent in traditional 2D cell cultures.[10,11]

These models not only offer more predictive results for treatment response but also play a crucial role in reducing the reliance on animal models in cancer research. By providing an accurate simulation of human tumor biology and immune interactions, 3D *in vitro* systems allow early-stage testing of therapeutic strategies, including ECT, in a controlled environment. This contributes to the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal experimentation, helping to minimize animal use while ensuring the scientific validity and translational relevance of the findings.[12]

Here, we applied high-voltage electroporation, usually used in ECT protocols, on HCC1954 breast cancer cells embedded in 3D scaffolds to investigate whether this treatment triggers immunogenic mechanisms capable of inducing T cell migration and activation. To this end, we performed co-cultures with Jurkat cells, a human T lymphocyte cell line commonly used as a model for studying T cell signaling, activation, and immune responses. This system allowed us to assess whether electroporated cancer cells can potentially lead to the targeted cytotoxic elimination of cancer cells.

Materials and Methods

Scaffold preparation

The synthetic self-assembling peptide EAbuK-IKVAV (sequence: amide-terminal BEBEBKBKBEBEBKBKIKVAV, where B denotes α-aminobutyric acid and IKVAV is a laminin-derived peptide) was dissolved in MilliQ water under continuous stirring. The peptide was 5% w/w with respect to hyaluronic acid (HA). HA (1000–1250 kDa; Contipro Biotech s.r.o., Dolní Dobrouč, Czech Republic) was gradually added to the peptide solution until reaching a final concentration of 12 mg/mL. The resulting dense solution was aliquoted by weight into the wells of a culture plate, frozen in liquid nitrogen, and subsequently lyophilized.

Scaffold cross-linking was carried out using 60 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 95% ethanol for 24 hours. Following cross-linking, the scaffold was washed six times with ethanol and six times with MilliQ water using an ultrasound bath—each wash involving 1 minute of sonication followed by 2 minutes without sonication. A final wash with MilliQ water was performed overnight at 4°C. Excess water was then removed, and the scaffolds were frozen in liquid nitrogen and lyophilized.

3D breast cancer HCC1954 cell culture and EP treatment

Breast cancer HCC1954 cells were cultured in RPMI medium supplemented with 1% penicillin/streptomycin, 1% L-glutamine, and 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. The HA-EAbuK-IKVAV-based scaffolds, previously sterilized under UV light, were inserted in 8-well LabTek chambers (9 × 10 mm) and hydrated in 200 μL of culture medium for 1 hour at 37°C in a humidified 5% CO_2 atmosphere. HCC1954 cells were labeled with CellTracker™ Green CMFDA Dye (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. This dye is retained in daughter cells upon division due to its covalent bonds with intracellular molecules. A total of 2×10^5 cells were seeded onto the scaffolds and cultured for 3 days.[13] Scaffolds were placed in rectangular chamber slides.

Electroporation of cells embedded in the scaffolds was performed 3 days after seeding using the Cliniporator™

EPS-02 voltage pulse generator (Igea S.p.A., Carpi [MO], Italy) in culture medium. Eight voltage pulses at 1 Hz were applied using a stainless-steel two-plate electrode, 30 mm in length, with a 10 mm side and a 7 mm gap (pulse length: 100 μs, period: 200 μs). The pulse amplitude was adjusted from 0 to 700 V to generate an electric field of up to 1000 V/cm.[14–16] This pulse protocol was applied to all cell cultures.

Breast cancer HCC1954 cells and Jurkat cell co-culture

Jurkat cells were cultured under conditions similar to those used for HCC1954 breast cancer cells. Following electroporation of HCC1954 cells, phytohemagglutinin-M (PHA-M, Roche Diagnostics, Basel, Switzerland)-activated and nonactivated Jurkat cells were added to both electroporated and non-electroporated control HCC1954 cells at a 1:5 ratio, in accordance with the manufacturer's instructions, and incubated for 24 hours. Cells were then treated with Accutase (Merck Millipore, Darmstadt, Germany) for 5 minutes at 37°C, centrifuged at 1200 rpm, and resuspended in 1× PBS to perform the cell viability assay.

Human T lymphocyte isolation and 3D co-culture with breast cancer HCC1954 cells

The isolation of T cells from the peripheral blood of healthy donors following the acquisition of informed consent was approved by the Ethics Committee of the University of Perugia, Italy (no. 3384198, registered on 18/12/2023, granted to Dr. Vincenzo Maria Perriello). The research was completed in accordance with the Declaration of Helsinki as revised in 2013. Peripheral blood mononuclear cells (PBMCs) were isolated through gradient centrifugation using Ficoll-Paque™ PLUS (Cytiva, Marlborough, MA), followed by multiple washes in PBS containing 0.5 M EDTA (pH 8) and 20% HSA. T lymphocytes were enriched using anti-CD4 and anti-CD8 MACS® MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and LS separation columns (MACS® LS Columns; Miltenyi Biotech, Bergisch Gladbach, Germany), in accordance with the manufacturer's instructions. Isolated human T lymphocytes ($CD4^+/CD8^+$) were then cultured in RPMI medium supplemented with 1% penicillin/streptomycin, 1% L-glutamine, and 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.

Following 24 hours of incubation after electroporation of HCC1954 cells, $CD4^+/CD8^+$ T lymphocytes were added to both electroporated and non-electroporated control HCC1954 cells at a 2:1 $CD4^+/CD8^+$ ratio and at a 1:1 effector-to-target (E:T) ratio with HCC1954 cells, in accordance with the manufacturer's protocol. The cell co-cultures were then incubated for an additional 24 hours. Subsequently, cells were treated with Accutase (Merck Millipore, Darmstadt, Germany) for 5 minutes at 37°C, centrifuged at 1200 rpm, and resuspended in 1× PBS to perform the cell viability assay.

Flow cytometry and cell viability assay

Both electroporated and non-electroporated control HCC1954 cells, previously stained with CellTracker™ Green CMFDA Dye for long-term tracing of live cells (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) and co-cultured with Jurkat cells or human T lymphocytes, were resuspended in 1× PBS and stained with SYTOX™ Blue Dead Cell Stain (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Cells were then analyzed by flow cytometry using a CytoFLEX instrument (Beckman Coulter, Brea, CA). Green fluorescence intensity (FL1) was detected using a 525/40 nm filter, while violet fluorescence intensity (Pacific Blue, FL9) was detected using a 450/45 nm filter. Cell viability was quantified by calculating the percentage change in dead cells using the following formula:

$$\{[(X_f - X_i)/X_i] \times 100\}\%$$

X_f = final value (electroporated cells) of % HCC1954 cells positive for SYTOX™ Blue and CellTracker™ Green CMFDA Dye.

X_i = initial value (non-electroporated control HCC1954 cells) of % HCC1954 cells positive for SYTOX™ Blue and CellTracker™ Green CMFDA Dye.

The results were analyzed using FlowJo software (Version 10.6.1). Scatter plots were gated for live singlet lymphocytes that were positive for CellTracker™ Green CMFDA Dye. At least three replicates were analyzed for each treatment group.

Fluorescence microscopy imaging

To visualize cell membrane electroporation, a set of cultures was treated with 15 μL of PI solution (1 mg/mL in PBS; Sigma Aldrich, Saint Louis, MO) prior to electroporation. PI is a fluorescent dye that is taken up by electroporated cells.[17] After electroporation, 5 µL of Hoechst 33342 (HOE) solution (1 mg/mL in PBS; Thermo Fisher, Waltham, MA) was added to stain all cell nuclei blue. Samples were observed under an inverted fluorescence microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Amsterdam, The Netherlands), equipped with an ANDOR Neo sCMOS camera and DAPI, FITC, and TRITC filter sets. Fluorescence was detected at 538 nm excitation/619 nm emission for PI (red) and 352 nm excitation/ ~455 nm emission for HOE (blue). Blue and red fluorescence images were overlaid onto brightfield images using ImageJ software (NIH; http://imagej.nih.gov/ij). Multiple images per well were acquired for each cell culture.

Morphological analysis

For morphological evaluation, the HCC1954 3D cell cultures were fixed with 4% formaldehyde on microscope slides, air-dried at room temperature, and stained using Masson's trichrome (MT) (Bio-Optica, Milan, Italy) staining, following the supplier's protocol (Bio-Optica, Milan, Italy). MT was used as a specific stain for collagen and connective tissues. In particular, MT stains the ECM in red and the cells in a red-brown color. The stained slides were observed under a Leica DMR optical microscope (Leitz, Wetzlar, Germany) at 20× magnification, and the image data were acquired with a digital Nikon DSU-1 camera (Amsterdam, The Netherlands).

Quantification of IL-2, IFN-γ, and TNF-α expression

IL-2, IFN-γ, and TNF-α expression levels were quantified by real-time PCR. Total RNA was purified from HCC1954 cells using TRI Reagent (Merck, Darmstadt, Germany), followed by DNase treatment with the RNase-Free DNase Set (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. For reverse transcription, 2 µg of

RNA was incubated for 1 hour at 37°C using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). The reaction was terminated by heating at 95°C for 5 minutes. Real-time PCR reactions were performed using a QuantStudio 7 Pro Thermal Cycler (Applied Biosystems, Foster City, CA) in 96-well optical plates. For each reaction, 5 μL of a 1:10 dilution of cDNA was used in a final volume of 20 μL. Final concentrations included 400 nM of each primer and 1× SensiFAST SYBR Lo-ROX Kit (Meridian Bioscience, Inc., Cincinnati, OH). PCR amplification was carried out with the following cycling conditions: 40 cycles of 95°C for 5 seconds (denaturation), 60°C or 65°C for 10 seconds (annealing), and 72°C for 5–20 seconds (extension). No-template controls were included in each run to monitor for contamination. The following primers were used: IL-2 (Forward: AGAACTCAAACCTCTGGAGGAAG; Reverse: GCTGTCTCATCAGCATATTCACAC); IFN-γ (Forward: GAGTGTGGAGACCATCAAGGAAG; Reverse: TGCTTTGCGTTGGACATTCAAGTC); TNF-α (Forward: CTCTTCTGCCTGCTGCACTTTG; Reverse: ATGGGCTACAGGCTTGTCACTC). Expression levels of IL-2, IFN-γ, and TNF-α were normalized to the housekeeping genes GAPDH and HPRT1. Relative gene expression was calculated according to the method of fold change [$2^{-(\Delta\Delta Ct)}$]. Each sample was run in triplicate.

Statistical analysis

The statistical significance of differences between sample groups, both for the analysis of the cell viability assay and real-time qPCR, was assessed using a one-tailed homoscedastic *t*-test. All values are expressed as mean ± standard deviation. A p value <0.01 was considered statistically significant.

Results

Establishment of a tumor microenvironment-mimicking model enriched with matrix protein polymers in 3D HCC1954 cell cultures

3D cultures of HCC1954 breast cancer cells have been established using a scaffold composed of a hydrogel made from low molecular weight HA and ionic-complementary self-assembling peptides.[18] These two components are covalently cross-linked via a carbodiimide-mediated reaction, enhancing the stability and structural integrity of the hydrogel. HA, a fundamental component of biological extracellular matrices, plays a crucial role in cell adhesion, proliferation, and migration. Meanwhile, self-assembling peptides have the unique ability to spontaneously form nanofibrous hydrogels, mimicking the architecture of the ECM. This nanostructured environment offers an optimal setting for cellular interactions, promoting cell viability, differentiation, and functional behavior across various tissue types. Providing a biomimetic 3D environment that more accurately represents *in vivo* conditions compared with traditional 2D cultures, this scaffold supported successful cell proliferation, with cells exhibiting a heterogeneous growth pattern by forming both spheroidal structures and dispersed non-spheroidal cells (Fig. 1). Moreover, the cells actively remodeled their surrounding microenvironment by depositing type I collagen, a key component of the ECM, demonstrating their ability to dynamically interact with and "customize" the scaffold (Fig. 1). Importantly, the morphological characteristics of the 3D cultures closely resembled those observed in breast cancer biopsies. As we previously described, in fact, this 3D scaffold effectively mimics key features of the ECM associated with a precancerous state.[19,20] In this model, cells are embedded in a myxoid stroma, characterized by a high content of basic substances such as glycosaminoglycans (HA) and proteoglycans, low levels of collagen fibers formed by self-assembling peptides (SAPs), and a complete absence of elastin. All these characteristics reinforce the potential of this hydrogel-based scaffold as a reliable *in vitro* tumor tissue model for studying breast cancer biology, drug response, and tumor–stroma interactions in a physiologically relevant setting.

Effect of electroporation on the number, size, and viability of 3D HCC1954 cells co-cultured with activated Jurkat cells

3D cultures of HCC1954 cells, embedded in scaffolds, were subjected to electroporation with increasing voltage pulse amplitudes (from 0 to 1000 V/cm) to investigate the optimal conditions for cell treatment. The goal was to determine the most effective electric field intensity that would facilitate electroporation without causing excessive cell death. The electroporation-dependent cell mortality was estimated by staining with PI, a red-fluorescent dye that can enter cells with compromised membranes, such as those undergoing electroporation. Electric field intensities of 600, 800, and 1000 V/cm resulted in cell death rates of 30%, 55%, and 80%, respectively, as determined by image-based cell counting (red-labeled cells) (Fig. 2A). Although we expected the highest intensity of 1000 V/cm to achieve a high electroporation rate,

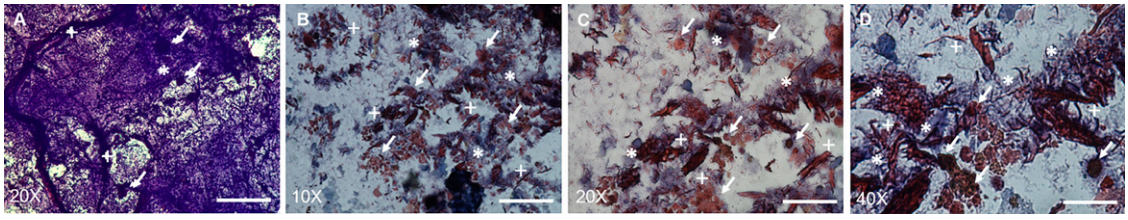

FIG. 1. Cell morphology in 3D cultures of HCC1954 cells. **(A)** Culture images acquired in a bright field. *White arrows* indicate cells, whereas *white asterisks* indicate the extracellular matrix deposited by cells. Data are from triplicate wells. Magnification 20×, scale bar 100 μm. **(B–D)** Masson's trichrome staining of HCC1954 cells cultured for 3 days within the scaffold. The staining highlights collagen (*white asterisks*), along with fiber structures (*white plus*). *White arrows* indicate spheroids. Data are from triplicate wells for each condition in three independent experiments. Magnification 10×, 20×, and 40×, scale bar 200, 100, and 50 μm, respectively.

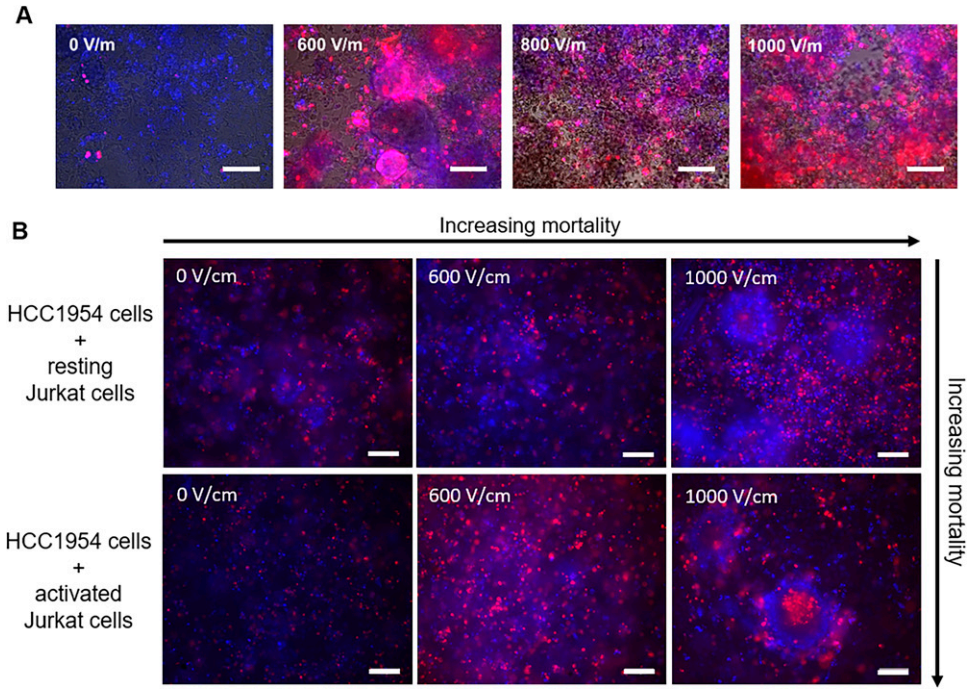

FIG. 2. Cell mortality post-electroporation through propidium iodide and HOE staining in **(A)** 3D HCC1954 cell cultures treated with electroporation at increasing voltage pulse amplitudes; **(B)** 3D co-cultures of HCC1954 and Jurkat cells, both in a steady state and after stimulation with phytohemagglutinin (PHA-M). Data are from triplicate wells for each condition in three independent experiments. Magnification 20×, scale bar 100 μm. HOE, Hoechst 33342.

it also led to increased cell mortality due to more extensive membrane damage. In contrast, a moderate electric field intensity of 600 V/cm allowed a suitable proportion of cells to undergo successful electroporation while maintaining cell viability (Fig. 2A, B). Based on these findings, we selected these two distinct EP conditions—commonly used in ECT protocols—to treat 3D co-cultures of HCC1954 and Jurkat cells. Our controls consisted of cells not exposed to electroporation. The images, captured 24 hours posttreatment and stained with PI, revealed dead cells (in red), with cell mortality increasing in a voltage-dependent manner at 600 and 1000 V/cm (Fig. 2A). Interestingly, the overall inhibitory effect—measured in terms of the number and size of cancer cell-associated 3D structures (spheroids)—was significantly enhanced when stimulated Jurkat cells were used compared with resting Jurkat cells (Fig. 2B).

Based on the observation of an excessive cell death rate in cells treated with 1000 V/cm EP, we decided to employ 600 V/cm for the subsequent experiments to balance treatment efficacy with cell viability. This choice aimed to maximize the therapeutic effect while minimizing unintended cytotoxicity. Electroporation of co-cultures using activated Jurkat cells had a significantly greater impact on reducing cancer spheroids compared with resting Jurkat cells. This suggests that the activation state of immune cells plays a crucial role in modulating the tumor microenvironment, potentially influencing tumor resistance or sensitivity to treatment. The effect of adding PHA-stimulated Jurkat cells on cancer cell viability was further confirmed by cytofluorimetric analysis, which provided quantitative evidence of changes in cell death rates. We observed a clear and statistically significant increase in cancer cell mortality upon the addition of PHA-stimulated Jurkat cells to electroporated HCC1954 cells, with cell death reaching 55.50%, compared with 30.92% in non-electroporated cells (Fig. 3A, B). This substantial difference suggests that the activation of Jurkat cells enhances the susceptibility of cancer cells to electroporation-induced damage, possibly through immune-mediated mechanisms or by altering the tumor microenvironment in a way that makes cancer cells more vulnerable to treatment. Interestingly, no statistically significant differences were observed between the mortality rates of electroporated HCC1954 cells co-cultured with non-stimulated Jurkat cells and electroporated HCC1954 cells alone when compared with their respective non-electroporated controls. This suggests that the presence of Jurkat cells in their resting state does not significantly impact cancer cell survival following electroporation. Overall, these effects were particularly evident after calculating the percentage change in dead cells across the three experimental conditions: HCC1954 cells alone (10.78%), HCC1954 cells co-cultured with Jurkat cells in a steady state (29.60%), and HCC1954 cells co-cultured with activated Jurkat cells (79.75%) (Fig. 3C).

Effect of electroporation on the viability of 3D HCC1954 cells co-cultured with human T lymphocytes

Encouraged by these results, we moved forward with a more clinically relevant experimental model by co-culturing HCC1954 cells with primary human T lymphocytes. To achieve this, we isolated $CD4^+$ and $CD8^+$ T lymphocytes from the PBMCs of two different donors (P1 and P2) and established 3D co-cultures with HCC1954 cells. Electroporation was then performed using the optimized conditions previously established (600 V/cm), ensuring consistency across experiments. Our findings revealed that the presence of primary T cells in the co-cultures significantly enhanced cancer cell mortality following electroporation (Fig. 4A, B). The experiment was replicated using T cells derived from two different human donors, and the same effect of cancer cell mortality was consistently observed in both cases, further reinforcing the robustness of the results. In particular, the

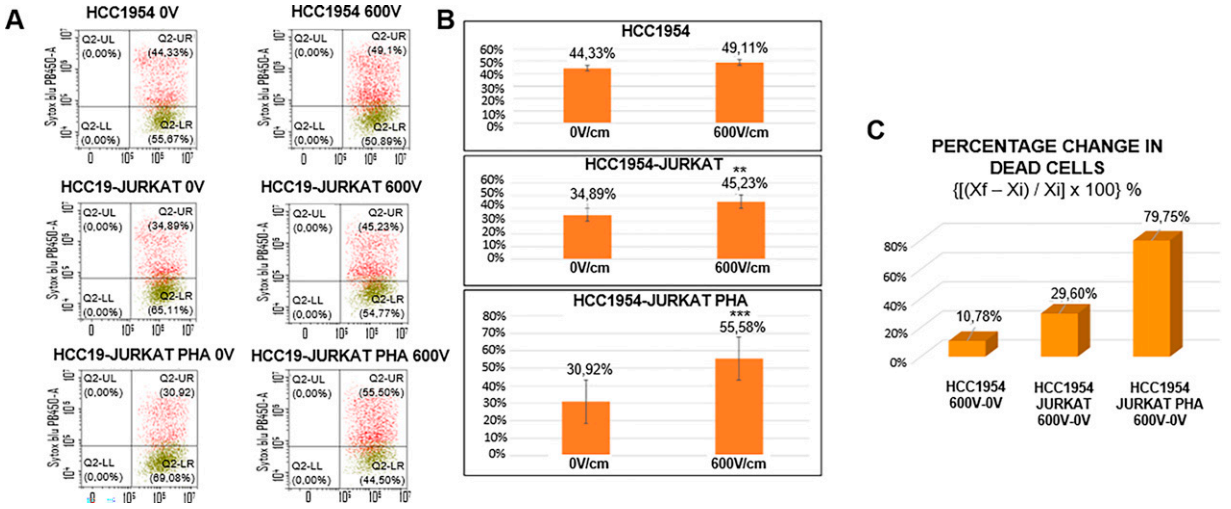

FIG. 3. Cytofluorimetric analysis of cell mortality in electroporated HCC1954 cells alone, HCC1954 cells co-cultured with Jurkat cells in a steady state, and HCC1954 cells co-cultured with activated Jurkat cells. Non-electroporated HCC1954 cells are included as controls. **(A)** Representative density plot of cells after SYTOX blue dead cell staining. **(B)** Percentage of cell mortality in electroporated HCC1954 cells across the three experimental conditions compared with their respective non-electroporated cell groups. **(C)** Data are presented as mean with standard deviation. Percentage change in dead cells. A one-tailed homoscedastic *t*-test was performed (**$p < 0.001$; ***$p < 0.0001$).

percentage of dead HCC1954 cells in co-cultures with P1- and P2-derived T cells was 74% and 112%, respectively, compared with their corresponding controls, which showed 69% and 2% cell death in HCC1954 cells cultured alone (Fig. 4C). Notably, the increase in cell death mirrored the trend previously seen with commercially available Jurkat cells, further supporting the role of T lymphocytes in potentiating the impact of electroporation on tumor cell viability.

Electroporation significantly increases the production of IL-2, IFN-γ, and TNF-α in human T lymphocytes co-cultured with HCC1954 cells

We hypothesized that electroporation, within the context of our co-culture experimental setup, could potentially stimulate ICD in treated cancer cells, thereby enabling T cells to mount an anti-tumor response. Based on this hypothesis, we aimed to investigate whether electroporation of HCC1954 cells could lead to an increased production of key cytokines secreted by T cells, which are responsible for mediating T-cell activation and subsequent anti-tumor functions, namely IL-2, IFN-γ, and TNF-α. IL-2 is a critical cytokine involved in regulating the activation and proliferation of cytotoxic T lymphocytes (CTLs). It not only supports the survival of T cells but also promotes their expansion and differentiation into effector cells capable of targeting tumor cells. Increased IL-2 production is typically associated with heightened T-cell responsiveness, facilitating a more effective immune response against cancer cells.[21] IFN-γ, on the other hand, is a key mediator of T-cell cytotoxicity. It enhances T-cell motility and functions to promote CTL-mediated killing of tumor cells.[22] In addition to its direct cytotoxic effects, IFN-γ also plays a role in blocking tumor cell proliferation by interfering with the mechanisms that normally promote tumor growth and survival.[23] Its production is a strong indicator of the activation of T cells in an anti-tumor context. TNF-α, a pro-inflammatory cytokine, plays a central role in immune responses by inducing the proliferation of both naïve and memory T cells. It is known to be involved in amplifying immune reactions and contributes to the recruitment of additional immune cells to the tumor site. TNF-α also enhances the ability of T cells to mount a stronger response against cancer cells.[24]

To evaluate the influence of electroporation on T cell cytokine production, we performed quantitative reverse transcription PCR (qRT-PCR) to quantify the mRNA expression levels of these three cytokines involved in T cell activation and anti-tumor responses, in the co-cultures of T cells and HCC1954 cells (Fig. 5). We found a statistically significant increase in the mRNA levels of both IL-2 and TNF-α in the EP-treated co-cultures, indicating that electroporation may stimulate T cells to enter an active state, thereby promoting their expansion and enhancing their potential to eliminate cancer cells. Moreover, although our data showed a trend toward increased IFN-γ production, this did not achieve statistical significance, which could be due to the complexity of cytokine regulation or the experimental conditions. It is possible that IFN-γ production requires additional stimulation or a longer duration of treatment to reach detectable levels in our experimental setup. These findings provide compelling evidence that electroporation can enhance the activation of T cells, potentially facilitating their anti-tumor activity through the increased production of key immune mediators.

Discussion

Although the immunostimulatory potential of EP is increasingly supported by evidence,[25] the precise molecular and cellular pathways through which it exerts this effect remain largely undefined. A deeper understanding of these underlying mechanisms is essential, as it could unlock new strategies to harness and refine EP from a supportive technique into a central component of cancer immunotherapy.

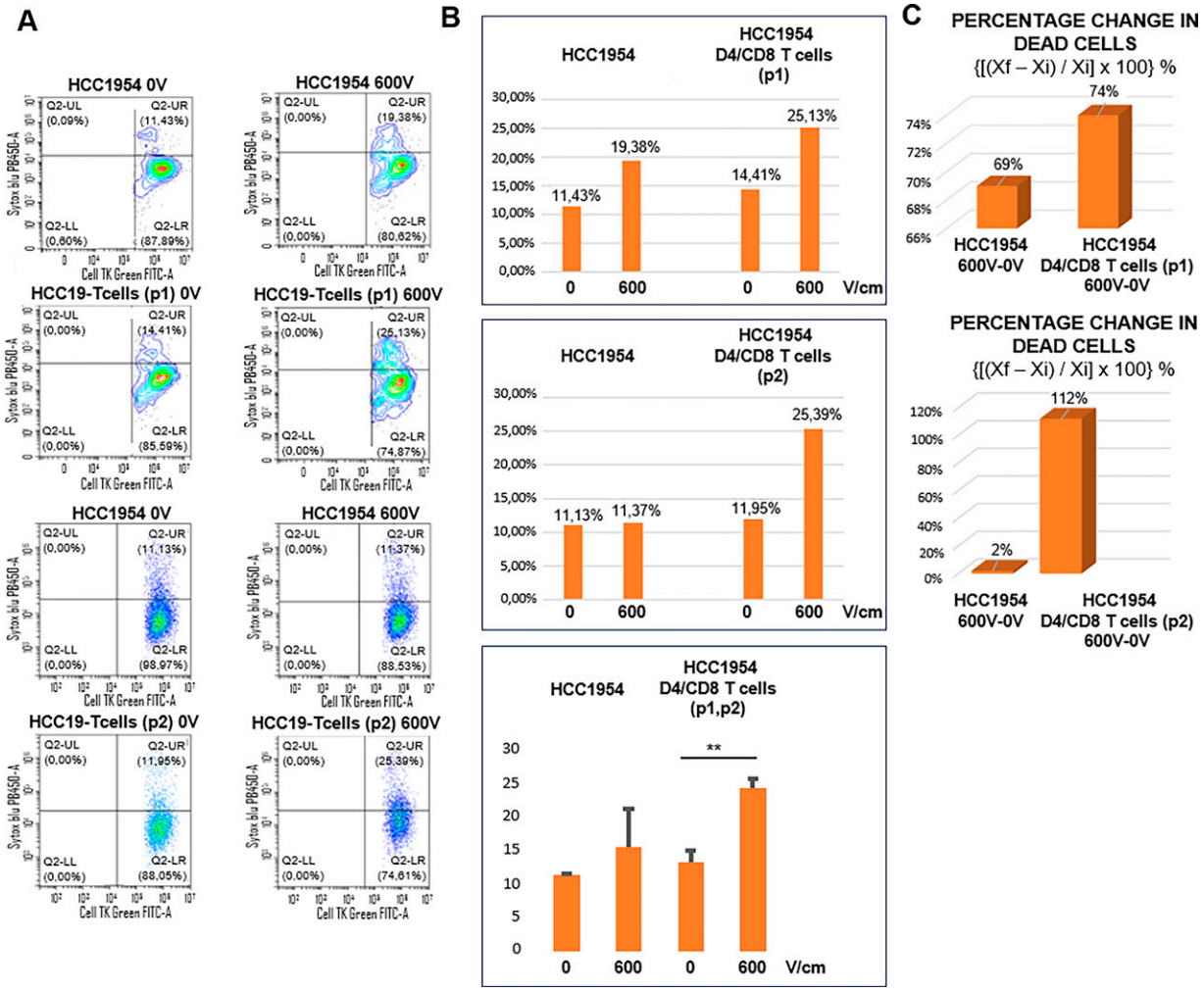

FIG. 4. Cytofluorimetric analysis of cell mortality in electroporated HCC1954 cells alone, and HCC1954 cells co-cultured with human T cells from two different donors (patient 1, P1; patient 2, P2). Non-electroporated HCC1954 cells are included as controls. **(A)** Representative density plot of cells after SYTOX blue dead cell staining. **(B)** Percentage of cell mortality in electroporated HCC1954 cells across the two experimental conditions compared with their respective non-electroporated cell groups. **(C)** Percentage change in dead cells. A one-tailed homoscedastic *t*-test was performed (**$p < 0.001$; ***$p < 0.0001$).

Our findings provide compelling evidence that high-voltage electroporation can effectively modulate the tumor microenvironment and enhance immune-mediated cytotoxicity in 3D breast cancer cell cultures. Specifically, we demonstrated that electroporation alone can increase tumor cell membrane permeability, and when combined with activated immune cells, it significantly boosts cancer cell death.

First and foremost, we validated the reliability of the 3D culture system employed in our study. Our results confirmed the suitability of 3D cell cultures within the hydrogel-based scaffold as an effective model for reproducing the morphological characteristics of the breast cancer "precancerous state" microenvironment, where cells are surrounded by a myxoid stroma mainly composed of abundant ground

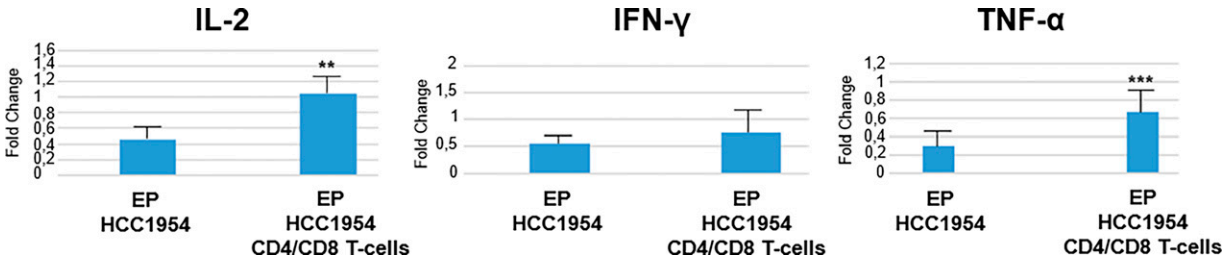

FIG. 5. IL-2, IFN-γ, and TNF-α gene expression levels. A one-tailed homoscedastic *t*-test was performed (**$p < 0.001$; ***$p < 0.0001$).

substances rich in glycosaminoglycans (HA) and proteoglycans, with scarce collagen fibers and no elastin content. Notably, as a third key feature of this novel 3D scaffold, our study also demonstrated that this model enables the observation of matrix remodeling and *de novo* deposition of ECM rich in collagen by cancer cells growing in 3D culture, mirroring what is observed in human mammary tumors (Fig. 1). As previously highlighted in other studies,[13,19,20,26] this system enables the analysis of the effects of electrical impulses on breast cancer biology and tumor–stroma interactions in a physiologically relevant setting, mimicking the complex *in vivo* conditions that influence tumor progression, cell signaling, and response to treatment. Moreover, we observed that electroporated HCC1954 cells within the 3D scaffold exhibit a voltage-dependent increase in permeability to PI. Notably, this increase occurs without compromising the structural integrity of the spheroids embedded in the matrix. These findings underscore the relevance of our experimental conditions and highlight their potential for investigating electroporation responses that may occur *in vivo* within a real tumor microenvironment (Figs. 1 and 2).

Our results indicate that, among the different electric field intensities tested, ranging from 0 to 1000 V/cm, 600 V/cm was effective in maintaining an acceptable level of cell viability, as confirmed by SYTOX staining assay (Figs. 2 and 3). This balance is crucial in therapeutic applications, as excessive cell death due to direct electroporation may limit the potential for immune activation.[27] This is a key consideration in identifying the optimal electric field strength in ECT protocols, as also highlighted by a study, which emphasized that the electric field should not only enhance efficacy against cancer cells but also minimize adverse effects on healthy cells.[28]

A key finding in our study is the synergistic effect between electroporation and immune cell activation. Jurkat cells are a human T lymphocyte cell line originally derived from a patient with acute T cell leukemia, commonly used as a model to study T cell signaling, activation, and immune responses.[29] When co-cultured with PHA-stimulated Jurkat cells, electroporated cancer cells showed a significantly higher mortality rate compared with non-electroporated controls (Fig. 3). This suggests that activated Jurkat cells may enhance the tumor's susceptibility to electroporation-induced damage, potentially through immune-mediated cytotoxicity. Interestingly, the mortality rate of tumor cells remained largely unchanged in the presence of nonactivated Jurkat cells (Fig. 3), further supporting the notion that immune cell activation is essential for enhancing and potentiating the effects of electroporation.

Several studies have demonstrated the crucial role of immune cell activation in significantly enhancing the therapeutic effects of electroporation, particularly in the treatment of immunogenically "cold" or "immune desert" tumors. These tumors, which are characterized by a lack of immune cell infiltration and a suppressive tumor microenvironment, typically exhibit resistance to conventional immunotherapies.[30] In particular, in the context of irreversible electroporation (IRE), its application to pancreatic cancer models not only suppresses tumor growth but also increases $CD8^+$ T cell infiltration, leading to a systemic immune response and the suppression of untreated tumor lesions.[31–33] Similarly, reversible electroporation (RE)-based strategies targeting aggressive cutaneous tumors in both preclinical[34–36] and clinical settings,[37–39] such as ECT or intratumoral electroporation of plasmids encoding immunostimulatory cytokines, not only promote T-cell proliferation and cytotoxicity but also increase cytokine production by T cells while reducing the suppressive environment within the tumor microenvironment. These effects lead to the activation and enhancement of T-cell function, promoting tumor regression and inducing systemic immunity that controls untreated tumors *in vivo*.[37–39] In addition, the treatment restores the function of tumor-infiltrating T cells in the treated patients.[39]

All these observations were confirmed and further extended by our experiments using HCC1954 cells co-cultured with primary human T lymphocytes isolated from two healthy donors. Strikingly, we found that electroporation-induced cancer cell death was significantly higher in cancer cells co-cultured with primary $CD4^+$ and $CD8^+$ T lymphocytes from two healthy donors (Fig. 4), compared with cancer cells cultured alone. Notably, these results were similar to those obtained using activated Jurkat cells, and in one case (patient 2) even exceeded them, underscoring the clinical relevance of these findings.

Given the ability of these T cells to directly recognize and eliminate cancer cells, our data further support the hypothesis that RE alone can serve as an effective strategy to enhance T cell-mediated anti-tumor responses, paralleling the effects of IRE, which is well known for its ability to disrupt tumor cell membranes and facilitate immune activation.[40]

To further validate that electroporation-induced cytotoxic T cell-mediated tumor cell death, we assessed the levels of three key inflammatory cytokines, IL-2, TNF-α, and IFN-γ, in the co-cultured cells. We found that electroporation significantly influences the cytokine profile of co-cultured immune cells. In particular, the observed upregulation of IL-2 and TNF-α gene expression levels suggests that electroporation enhances T-cell activation and proliferation, which are critical components of an effective anti-tumor response. Primarily produced by $CD4^+$ helper cells, IL-2 is known for promoting T cell expansion and differentiation into cytotoxic effectors[41,42] and memory subsets,[43] while TNF-α is involved in amplifying immune responses and recruiting additional immune cells to the tumor site.[44] The observed increase in these cytokines following electroporation (Fig. 5) indicates that high-voltage electroporation can promote an immunogenic tumor microenvironment, potentially enhancing tumor recognition and clearance by the immune system through a pro-inflammatory skewing of T cells. The third cytokine analyzed, IFN-γ, produced by both helper $CD4^+$ and cytotoxic CD8+ T cells, is known to support pro-inflammatory pathways during T cell activation and boost the cytotoxic activity of CTLs.[45,46] Interestingly, although a trend toward increased IFN-γ expression was observed after electroporation, statistical significance was not reached (Fig. 5). This may be attributed to the complexity of cytokine regulation[47] or the requirement for additional stimuli beyond electroporation alone to induce substantial IFN-γ production. Future investigations should explore whether modulating electroporation parameters such as voltage, duration, or frequency, or the combination of electroporation with other immunostimulatory agents, is

able to further enhance IFN-γ secretion, thereby maximizing the overall immune response.

Conclusion

This study highlights the potential of electroporation not only as a tool for enhancing drug delivery but also as a means to stimulate immune responses against cancer cells. By increasing membrane permeability and promoting T-cell activation, electroporation emerges as a promising immunomodulatory strategy that could improve therapeutic outcomes when integrated into multimodal cancer treatments. Future work should focus on refining electroporation protocols to further harness its immunostimulatory capabilities and translate these findings *in vivo* into effective clinical applications. Several parameters, including pulse intensity, duration, and frequency, could be fine-tuned to maximize immune activation while preserving cell viability. In addition, exploring combinatory approaches with cytokine-based adjuvants or ICIs could further enhance treatment efficacy. Investigating the long-term immune memory effects of electroporation-induced T cell activation will also be critical in determining whether this strategy can contribute to sustained anti-tumor immunity.

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Authors' Contributions

Conceptualization: R.M., E.S. (Elisabetta Sieni), M.D.R., and E.S. (Emanuela Signori); Investigation, formal analysis, validation, visualization, and data curation: R.M., E.S. (Elisabetta Sieni), A.Z., M.T.C., N.M., A.M., and M.C.; Resources: M.D., N.M., V.M.P., M.C., E.S. (Emanuela Signori), and M.D.R.; Writing—original draft: M.D.R. and E.S. (Emanuela Signori); Writing—review and editing: R.M., E.S. (Elisabetta Sieni), M.D., A.M., M.C., E.S. (Emanuela Signori), and M.D.R.; Project administration: R.M. and E.S. (Elisabetta Sieni); Supervision: M.D.R., E.S. (Emanuela Signori); Funding acquisition: M.D.R., E.S. (Emanuela Signori), M.C., and A.M. All authors have read and agreed to the published version of the article.

Author Disclosure Statement

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