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Article

Combination Therapy of Irreversible Electroporation and Cytokine-Induced Killer Cells for Treating Mice Bearing Panc02 Pancreatic-Cancer Xenografts

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Simple Summary: In recent years, irreversible electroporation (IRE) has been used clinically as a novel way to ablate tumors nonthermally and has been approved for LAPC. Previous studies and clinical applications have regarded IRE and CIK cells as two independent types of therapy. In our opinion, further research is necessary to identify possible internal interactions between IRE and CIK cells that might underlie their combined therapeutic effects in PC. In this study, IRE combined with CIK cells showed superior antitumor efficacy in a PC subcutaneous-xenograft model, which we attributed to the promotion of lymphocytic infiltration, as well as to upregulation of chemokine receptor expression and of regulators of CIK cell proliferation.

Abstract: The current study aimed to investigate the antitumor effects and potent mechanism of cytokine-induced killer (CIK) cells combined with irreversible electroporation (IRE) via an *in vitro* function assay in an *in vivo* Panc02 cell–bearing mouse model. We established an *in vitro* culture assay for CIK cells and determined the proportions of different peripheral lymphocytes. The antitumor effect of the combination of IRE and CIK cells in a Panc02 pancreatic-cancer (PC) subcutaneous-xenograft model was investigated; tumor size and mouse survival rates were recorded. We used flow cytometry (FCM) to analyze the proportion of intratumoral lymphocytes, the expression of chemokine receptors, and the proliferative activity of CIK cells. The proportion of cells that were positive for clusters of differentiation 3 and 8 ($CD3^+CD8^+$) and the proportion of $CD3^+CD56^+$ cells were both significantly increased after 21 days of *in vitro* culture. Combined IRE/CIK cell treatment significantly inhibited tumor growth and increased the survival rate of Panc02 cell–bearing mice. Furthermore, infiltration of lymphocytes into tumor tissue was significantly increased by this combination therapy compared with the untreated group or monotherapy group. In addition, IRE significantly enhanced the expression of chemokine receptors elicited by, and the proliferative activity of, CIK cells. In conclusion, IRE combined with CIK cells showed superior antitumor efficacy in a PC subcutaneous-xenograft model, which we attributed to the promotion of lymphocytic infiltration, as well as to upregulation of chemokine receptor expression and of regulators of CIK cell proliferation.

Keywords: Irreversible electroporation**;** Cytokine-induced killer cells; Combination therapy; Pancreatic cancer; Chemokine receptors

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1. Introduction

Pancreatic cancer (PC) is a malignant digestive-system tumor that ranks fourth in lethality among various malignant tumors. Its 5-year survival rate is <5%, and new cases and mortality rates are increasing year by year [1]. The American Joint Committee on Cancer (AJCC) reports that ~40% of PC patients are diagnosed with locally advanced PC (LAPC) and either cannot undergo surgical resection or have poor prognoses after this procedure [2, 3].

For LAPC patients, gemcitabine-based regimens with or without radiation have been the standard treatment, yielding a median overall survival (OS) rate of 9-11 months [3]. However, chemotherapy's high rates of toxicity and adverse events (AEs) limit its use. Cytokine-induced killer (CIK) cells are a heterogeneous group of cells acquired from human peripheral blood mononuclear cells (PBMCs) cultured *in vitro* with a variety of cytokines for a certain period [4, 5]. They have been extensively used in clinical studies and treatment of various malignant tumors, but results in PC have been disappointing [5]. It is reported that pancreatic ductal adenocarcinoma (PDAC) contains a large number of immunosuppressive cells [6]. Such an immunosuppressive microenvironment (ISM) can inhibit the activity of tumor-infiltrating lymphocytes [7, 8]. Therefore, novel, effective therapies for LAPC patients are needed.

In recent years, irreversible electroporation (IRE) has been used clinically as a novel way to ablate tumors nonthermally and has been approved for LAPC by the US and the Chinese Food & Drug Administrations (USFDA and CFDA) [9, 10]. IRE has certain unique advantages: it is quick, controllable, visible, selective, and nonthermal in its mechanism, meaning that it can be used to treat tumors adjacent to vital organs and tissues such as blood vessels and nerves [11]. It reportedly might also cause systemic tumor control by changing the structure and composition of the tumor microenvironment (TME) or by priming tumor-specific immunity [12]. Scheffer *et al.* demonstrated that IRE in LAPC transiently alleviates immunosuppression, creating a window for antitumor T-cell activation [13]. Therefore, IRE could be a potential immunomodulatory treatment for PC.

Previous studies and clinical applications have regarded IRE and CIK cells as two independent types of therapy. In our opinion, further research is necessary to identify possible internal interactions between IRE and CIK cells that might underlie their combined therapeutic effects in PC. In this study, we established Panc02 tumor–bearing mouse models to investigate the antitumor efficacy of IRE combined with CIK cells in the management of PC, as well as IRE's effect on the migratory and killing activities of CIK cells.

2. Materials and methods

2.1. Materials and animals

We purchased a CNP-III Steep Pulse Therapy System from Zhejiang Curaway Medical Technology Co., Ltd. (Hangzhou, China). Human PBMCs were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 culture medium were obtained from Merck KGaA (Darmstadt, Germany). We purchased antibodies (aBs) against mouse chemokines (C-X3-C motif) 3 and 4 (CXCR3, CXCR4) and C-C chemokine receptor type 5 (CCR5) from Corning (Corning, New York, USA). Monoclonal antibodies (maBs) for CD3, CD4, CD8, and CD56 determination assays were purchased from Sigma-Aldrich (Taufkirchen, Germany). Anti-mouse Ki-67-FITC, ICOS-PE, Granzyme B-APC were purchased from Thermo Fisher Scientific (USA). Unless otherwise indicated, all other reagents were also obtained from Sigma-Aldrich.

C57BL/6 mice (male, ~6 weeks old, and body weight [BW]: ~18 g) of specific pathogen–free (SPF) grade were provided by Shanghai SL Biotechnology Co., Ltd. (Shanghai,

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China). Six mice were kept in a cage in an SPF environment at a relative humidity of 40–60% and temperature of 25 ± 1°C. All animal experiments in this study were approved by the Zhejiang Medical Laboratory Animal Center (ZJCLA; Hangzhou, China) and conducted following its guidelines. The animal study was approved by the Institutional Animal Care and Use Committee of ZJCLA (Approval No. ZJCLA-IACUC-20040042).

2.2. Cell culture

Panc02 cells (murine PC cells) were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). We cultured them in RPMI 1640 containing 10% inactivated FBS. Briefly, after the recovery of the Panc02 cells, the culture medium was removed from the dish, and 0.15% trypsin digestion solution (containing 0.02% ethylenediaminetetraacetic acid [EDTA]) was added. Subsequently, we removed the digestive juice, added new RPMI 1640 culture medium, and repeatedly centrifuged the mixture. The cells were then maintained in an incubator at 37°C and 5% CO_2.

2.3. Generation of CIK cells

The mice were sacrificed by cervical dislocation and were immediately immersed in the volume fraction of 75% ethanol for 5 min. The spleen was removed under sterile surgical conditions and the spleen cells were isolated. Then, we resuspended ~1.0×10^6 cells with 1 mL CIK cell culture medium (100 U/mL interleukin-1 [IL-1] + 300 U/mL interleukin-2 [IL-2] + 1000 U/mL interferon gamma [IFN-γ]), placed them into 75-cm^2 culture flasks coated with 50 ng/mL mouse anti-human CD3 mAb, and maintained them in an incubator at 37°C and 5% CO_2. Cultures were supplemented with fresh complete medium and 1000 U/mL IL-2 every 3 days for 24 days[4].

2.4. Evaluation of antitumor efficacy in the xenograft tumor model

We established a Panc02 subcutaneous-xenograft model as described previously [14]. Briefly, Panc02 cells in the logarithmic-growth phase were diluted to 2×10^7/mL with sterile PBS. Then, we subcutaneously injected 0.1 mL Panc02 cells into the right axillae of the BALB/c mice. A Vernier caliper was used to measure the longest diameter (length) and vertical diameter (width) of each tumor. We calculated tumor size according to the formula length × width (mm^2). The Panc02 tumor–bearing model was considered successfully established when tumor volume = 50 mm^2.

2.5. Treatment procedures

We randomized 40 model mice into four groups (n = 10 each): a combination group (IRE + CIK cells), an IRE-alone group, a CIK–alone group, and a control group receiving no treatment.

On day 0, animals in IRE + CIK and IRE-alone groups were underwent IRE ablation procedure while the mice in CIK-alone and control groups were underwent sham surgery by inserting a needle electrode into the tumor without performing ablation. IRE ablation procedure were performed after the mice were anaesthetized by intraperitoneal injection of 1.5% pentobarbital (30 mg/kg) on day 0 of IRE + CIK and IRE-alone groups. IRE therapy system was used with the following system parameters: two electrodes, with 90 pulses, a pulse length of 70 μs, field strength of 1500 V/cm, 90 pulses, needle distance adjusted by tumor size. We inserted two electrodes into the central part of the tumor along the tumor's longitudinal axis and energized them. At the end of the ablation, the mouse's vital signs were observed. Mice were kept warm until they were awakened and returned to their cages.

Optimal CIK cells were harvested after incubation for 21 days based on experimental results 3.1. On day 3, 0.2 mL CIK cells were infused intravenously to each mouse in IRE + CIK and CIK-alone groups (Fig. 1C).

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Tumor sizes in the mice were recorded once every 3 days. Mice were killed when the tumor the reached 2cm in diameter or tumor ruptured, and xenograft tumors harvested. After tissue mincing and incubation at 37°C for 2 h, we added 2 mL RPMI 1640 digestive juice. Subsequently, the tissue was ground, filtered, and washed, and the cells were resuspended with 1% FBS in PBS to create a cell suspension at a concentration of 5 × 10^6 cells/mL for further flow cytometry analysis.

2.6. Flow cytometry analysis

Flow cytometry phenotyping was performed by four-color labeling of samples with anti-CD3, anti-CD8, anti-CD4, and anti-CD56 monoclonal antibodies respectively conjugated with FITC, PE, PE-Cy 5, and APC at 4°C for 30 min. To analyze all T cell subsets ($CD3^+/CD4^+$ and $CD3^+/CD8^+$), we first gated CD3 + cells, opened CD4 × CD8 dot plots, and after gating all subsets, we performed phenotypic assays. The levels of subpopulations were analyzed FACSCalibur flow cytometer with multiset software (BD Biosciences). Dead cells were excluded from analysis using BD Horizon ™ fixed viability stain 510 (BD Biosciences). Data analysis was performed using CellQuest software version 3.2 (Becton Dickinson). For the detection of CIK cell proliferation ability, samples were stained intracellularly with Ki-67-FITC, inducible costimulator-PE, Granzyme B-APC according to the manufacturer 's protocol. After washing, all samples were stored at 4 °C until read in flow cytometry cytometer.

2.7. Statistical analysis

We analyzed all data using GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA, USA) and one-way analysis of variance (ANOVA). Results are presented as mean ± standard deviation (SD). Degrees of significance are represented as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3. Results

3.1. In vitro expansion of CIK cells and expression of chemokine receptors

When we observed the growth state of CIK cells in suspension under a microscope, the cells presented with clear edges. Total CIK cells were counted twice a week and plotted against culture duration. After 7 days of culture, CIK cells began to rapidly proliferate and gradually reached a proliferative plateau on day 21 (Fig. 1A).

As shown in Figure 1B, we recorded *in vitro* expression of CD3, CD4, CD8, and CD56 on the surfaces of CIK cells for different culture durations. $CD3^+CD4^+$ cells accounted for approximately 40% of the newly isolated peripheral blood lymphocytes, while $CD3^+CD8^+$ cells, $CD3^+CD56^+$ cells, and $CD3^-CD56^+$ cells accounted for approximately 20%, 7%, and 16%, respectively. After 21 days of culture, proportions of $CD3^+CD8^+$ and $CD3^+CD56^+$ cells were significantly increased, while those of $CD3^+CD4^+$ and $CD3^-CD56^+$ cells were significantly decreased. These results collectively demonstrated the successful expansion of CIK cells and chemokine receptors.

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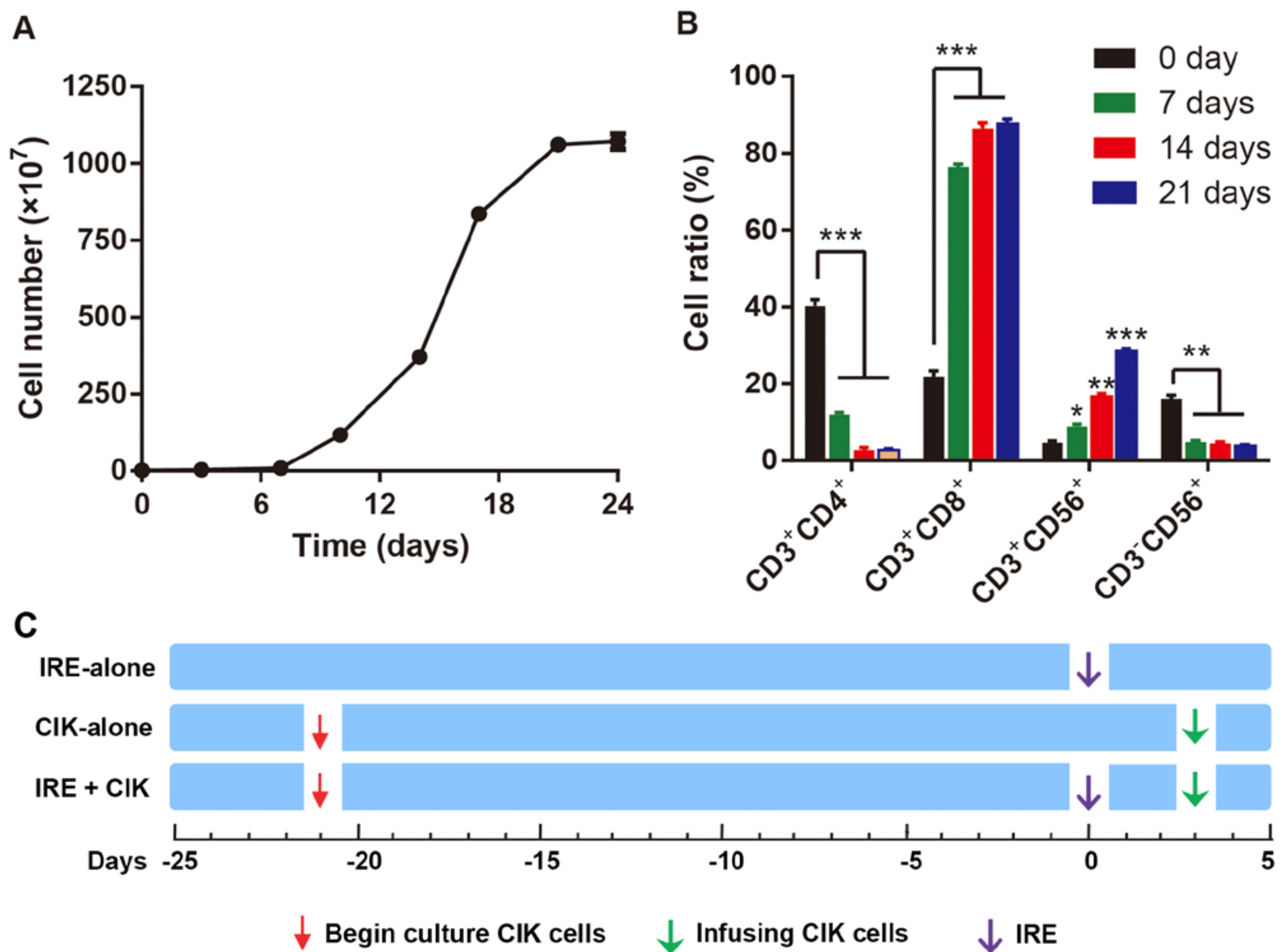

Figure 1. *In vitro* expansion of cytokine-induced killer cells, and treatment schedule. (A) *In vitro* growth curves of CIK cells for 24 days. (B) Percentages of CD3, CD4, CD8, and CD56 expressed on the surfaces of CIK cells on different days of culture. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *versus* 0 days (mean ± SD, n = 6). (C) Mice in IRE-alone group just received IRE on 0 day. And one CIK cells treatment was designed on 3 day in CIK-alone group. Mice in IRE + CIK group were received IRE plus one course of CIK cells.

3.2. Antitumor effect of combination therapy with CIK cells and IRE in Panc02 tumor–bearing mice

To further evaluate the antitumor efficacy of IRE combined with CIK cells, we established Panc02 cell–bearing mice and administered combination therapy to them of IRE and CIK cells (IRE was performed on day 0, followed by injection of 0.2 mL CIK cells on day 3). Tumor lengths and widths in each group of mice were measured every 3 days with the Vernier caliper and recorded. Mice were considered dead when the tumor ruptured or when tumor width > 2 cm. As shown in Figure 2A, treatment with either IRE or CIK cells alone inhibited tumor growth, with a growth arrest time of approximately 6–9 days. Interestingly, combined IRE/CIK cell treatment inhibited tumor growth to a greater extent, for up to ~12 days. Moreover, the survival time of the combination group was significantly longer than those of the untreated and monotherapy groups (Fig. 2B), suggesting that IRE combined with CIK cells could produce a superior antitumor effect in mice with PC.

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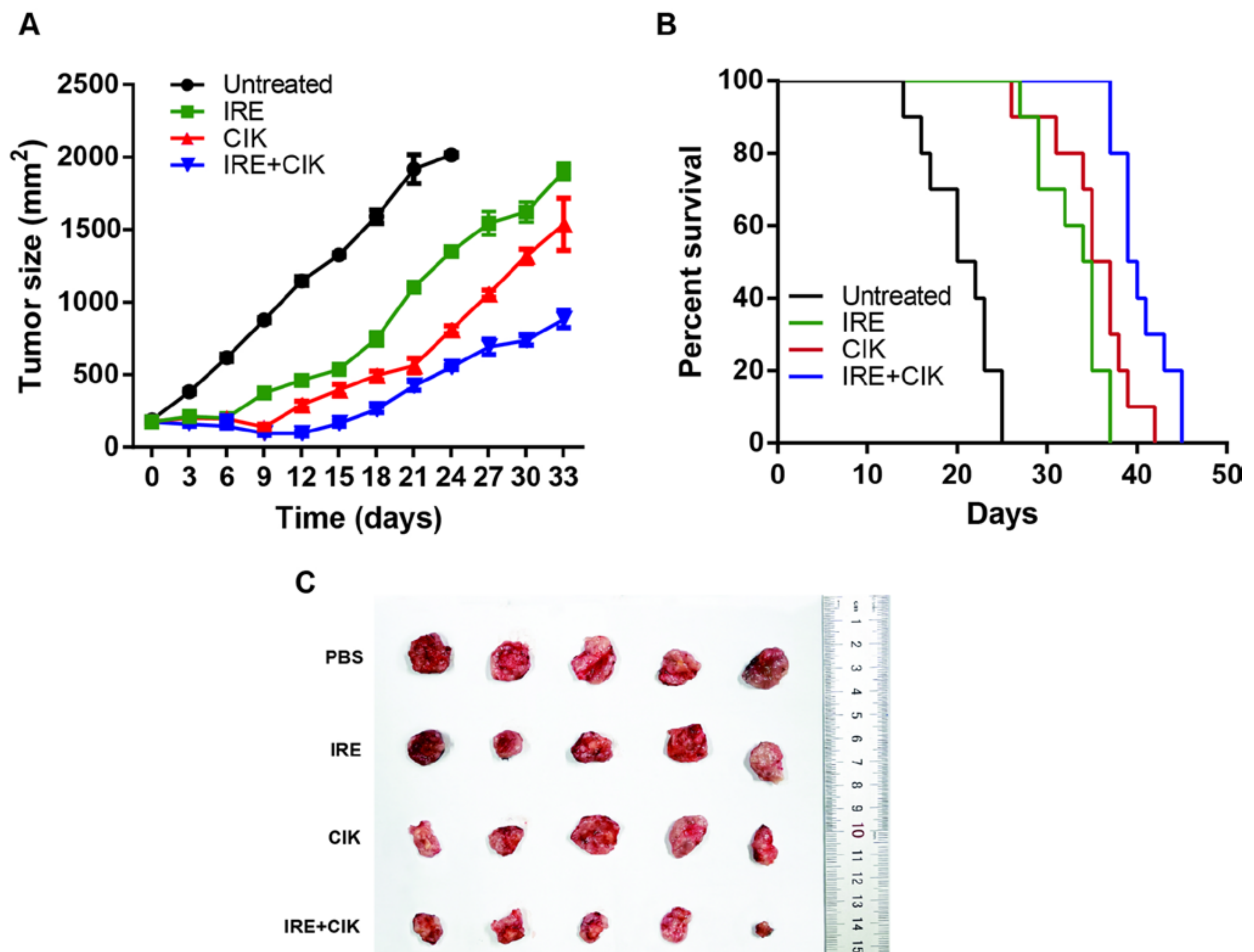

Figure 2. Antitumor effects of irreversible electroporation combined with cytokine-induced killer cells on pancreatic cancer in tumor-bearing mice. (A) Tumor growth, (B) mouse survival, and (C) tumor image per treatment group were monitored (mean ± SD, n = 10).

3.3. Combination IRE/CIK cell therapy increased infiltration of lymphocytes into tumor tissues

We analyzed the number of lymphocytes infiltrating tumor tissue via flow cytometry (FCM). As illustrated in Figure 3, numbers of intratumoral $CD3^-$ cells and $CD3^+$ cells were higher in the IRE-alone and CIK cells-alone treatment groups than in the untreated group. Numbers of $CD8^+$ and $CD4^+$ cells were also increased to varying degrees. What is more, numbers of $CD3^-$, $CD3^+$, $CD4^+$, and $CD8^+$ cells in the combined IRE/CIK cell treatment group were even higher than those in the CIK cells-alone group, and the differences were statistically significant. This suggested that IRE further amplified the immune response in the TME caused by CIK cells.

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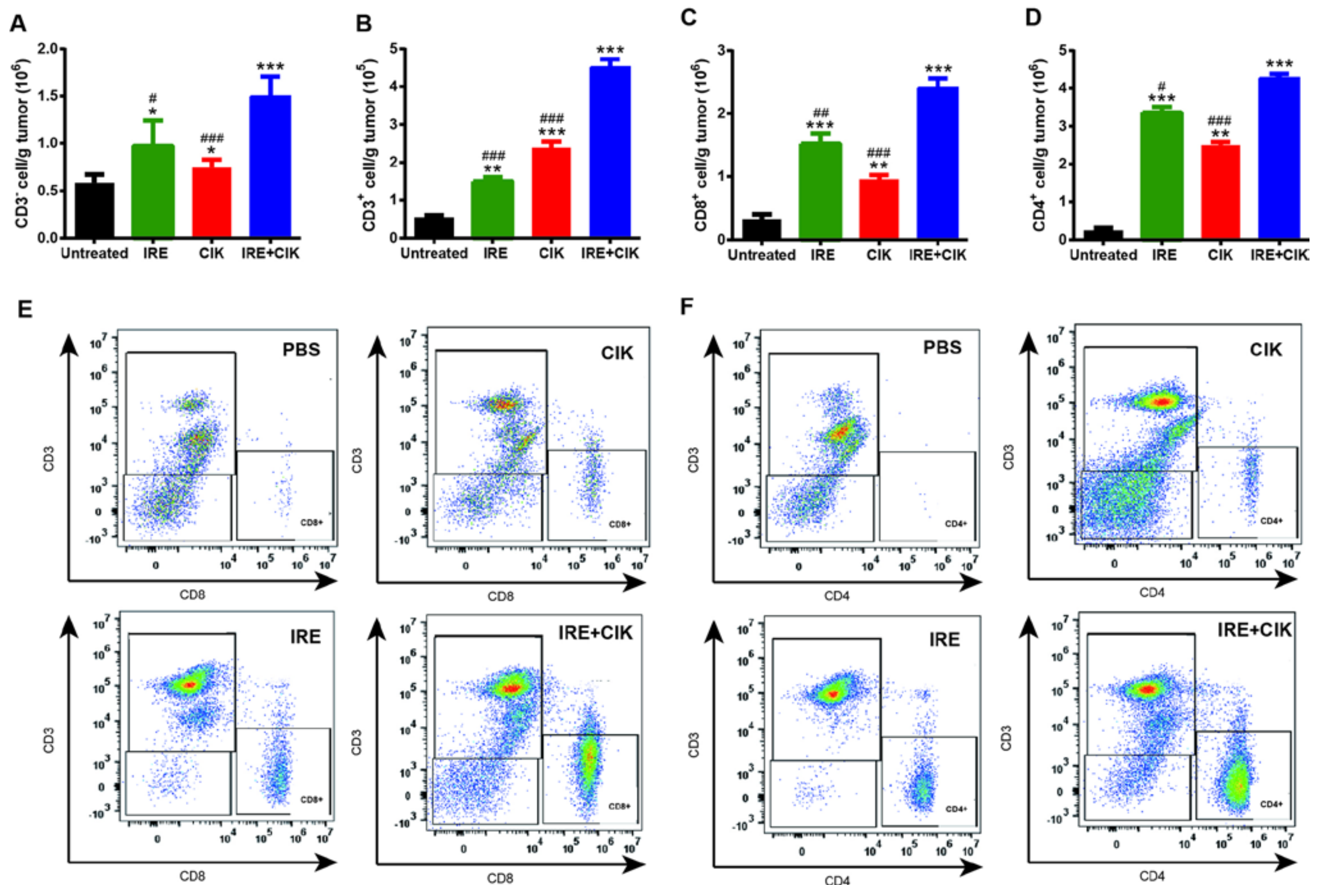

Figure 3. Combination therapy with irreversible electroporation and cytokine-induced killer cells increased the infiltration of lymphocytes into tumor tissue. Numbers of (A) CD3⁻ cells, (B) CD3⁺ cells, (C) CD8⁺ cells, and (D) CD4⁺ cells with tumor infiltration. (E–F) FCM analysis. Numbers of intratumoral CD3⁻ cells and CD3⁺ cells were higher in the IRE-alone and CIK cells-alone treatment groups than in the untreated group. Numbers of CD8⁺ and CD4⁺ cells were also increased to varying degrees. What is more, numbers of CD3⁻, CD3⁺, CD4⁺, and CD8⁺ cells in the combined IRE/CIK cell treatment group were even higher than those in the CIK cells-alone group, and the differences were statistically significant. $^{###}P < 0.001$ *versus* IRE+CIK group (mean ± SD, n = 5); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* untreated group (mean ± SD, n = 5).

Subsequently, we examined levels of chemokine receptors (CXCR3, CCR5, CXCR4) expressed by CIK cells. As shown in Figure 4, CD3⁺ cells in the CIK cell treatment group highly expressed all three of these receptors, with the positive rate of CXCR4 exceeding 80%. Interestingly, all three chemokine receptors were significantly elevated in the combined IRE/CIK cell treatment group compared with the CIK cells–alone group, indicating that IRE could significantly promote the antitumor immune response elicited by CIK cells.

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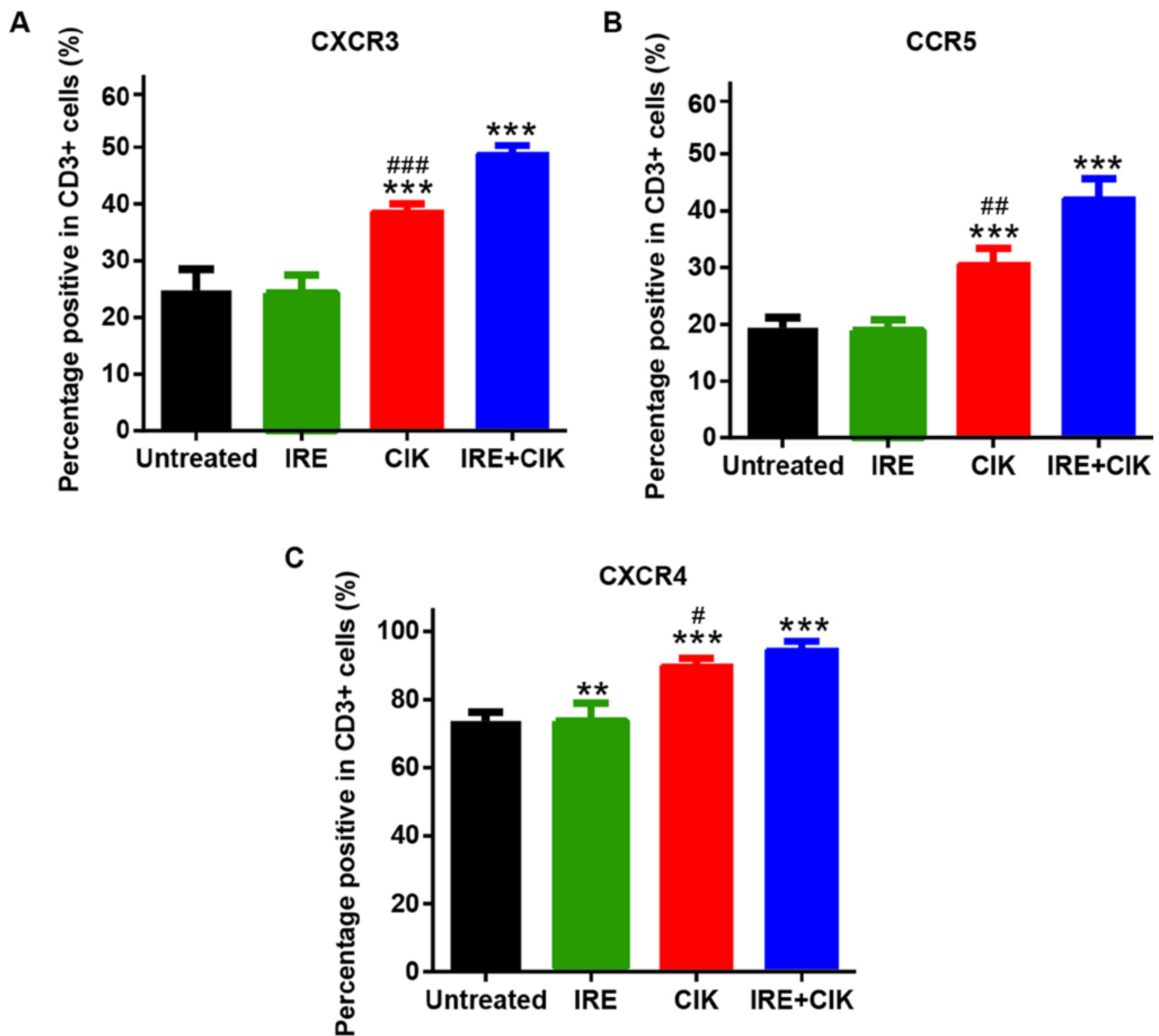

Figure 4. Percentages of chemokine receptor positivity in CD3⁺ cells. (A) CXCR3, (B) CCR5, and (C) CXCR4. CD3⁺ cells in the CIK cell treatment group highly expressed all three of these receptors, with the positive rate of CXCR4 exceeding 80%. All three chemokine receptors were significantly elevated in the combined IRE/CIK cell treatment group compared with the CIK cells-alone group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* untreated group (mean ± SD, n = 5); ###$P < 0.001$ *versus* IRE+CIK group (mean ± SD, n = 5).

3.4. IRE enhanced the proliferative ability of CIK cells in pancreatic tumors

Because they are markers of immune cell proliferation, we detected expression levels of Ki-67, inducible costimulator (ICOS), and granzyme B (GrB) in exogenous CIK cells in tumors using FCM to analyze IRE's effect on the proliferative ability of CIK cells. As shown in Figure 5, treatment with IRE alone only slightly increased expression levels of Ki-67 and ICOS but had no significant effect on expression levels of IFN-γ, tumor necrosis factor-alpha (TNF-α), GrB, or perforin compared with the untreated group. By contrast, treatment with CIK cells alone greatly increased expression levels of Ki-67, ICOS, and GrB. In addition, expression levels of these three factors were significantly increased in the combination treatment group *versus* the CIK cells–alone group, indicating that IRE could significantly promote the *in vivo* proliferative capacity and functions of CIK cells to effectively kill tumor cells.

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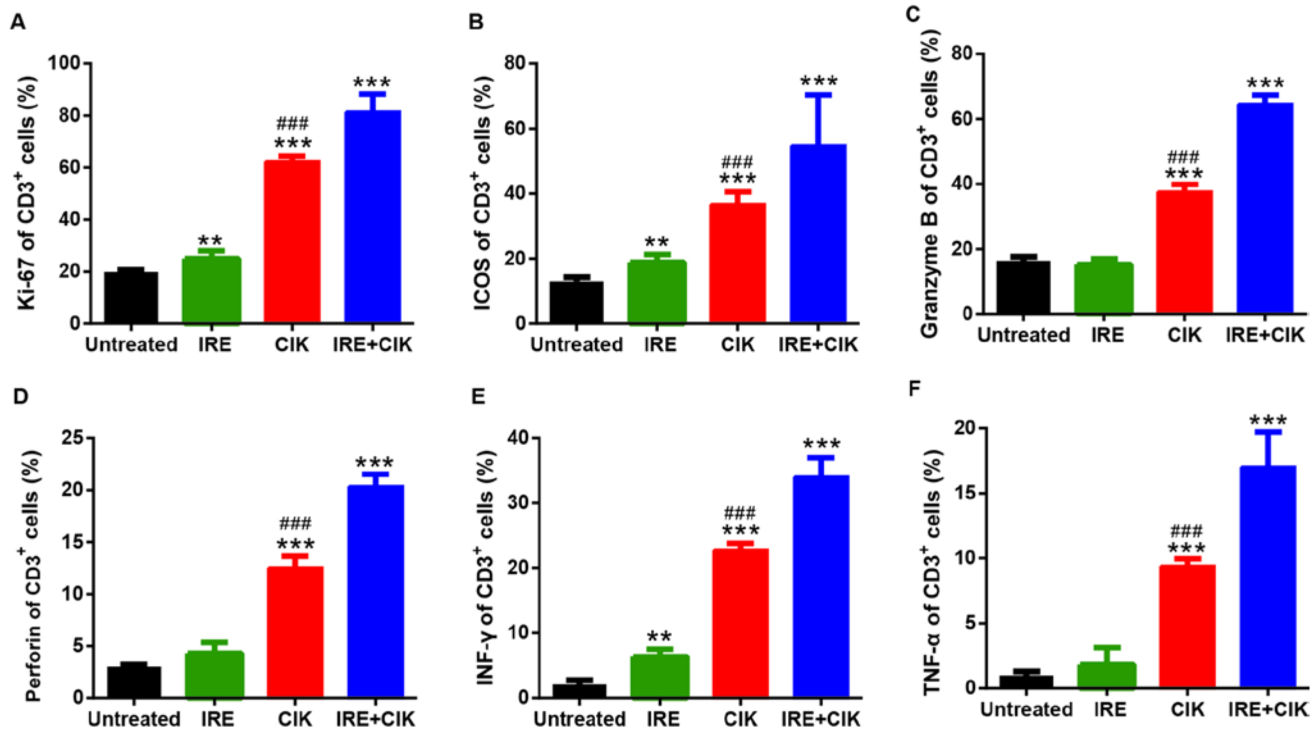

Figure 5. Effect of irreversible electroporation on *in vivo* proliferation of cytokine-induced killer cells in tumor tissues. Proportions of CIK cells expressing (A) Ki-67, (B) ICOS, (C) GrB, (D) perforin, (E) IFN-γ, and (F) TNF-α in tumors were detected via intracellular staining with the corresponding aBs. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ *versus* untreated group (mean ± SD, n = 5); ###$P <$ 0.001 *versus* IRE+CIK group (mean ± SD, n = 5).

4. Discussion

Patients with PC have a high mortality rate due to the strong invasive ability of this cancer and are prone to metastasis and recurrence [14]. Therefore, finding new survival prolongation strategies is critical. Mounting evidence confirms that IRE for LAPC is safe and effective. In recent years, some studies have emerged on IRE combined with chemotherapy for PDAC, which demonstrates an appropriate extension of survival. However, chemotherapy's high rates of toxicity and AEs cannot be ignored. Therefore, novel treatments are needed for LAPC patients [15].

In recent years, immune therapy has become one of the most powerful treatment strategies for malignant tumors. Adaptive cellular immunotherapy is an important part of the comprehensive treatment of PC [16]. The killing effect of CIK cells on tumors is characterized by high efficiency and lack of major histocompatibility complex (MHC) restriction [17]. CIK cells have good synergistic effects with other adjuvant therapies and the advantages of strong antitumor activity, wide antitumor spectrum, and no obvious adverse reactions [18-20]. Studies have shown that adaptive therapy with CIK cells is currently the best cellular-immunotherapy regimen for solid tumors and has a good therapeutic effect on a variety of tumors, especially liver cancer and leukemia [21, 22]. However, due to the presence of an immunosuppressive tumor-associated stroma, adaptive cellular immunotherapy has only limited efficacy against PDAC.

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The ability of CIK cells to migrate and colonize tumor sites *in vivo* is related to their chemotactic-adhesion ability [23]. In addition, whether there are sufficient adaptive immune cells with effector functions to reach target organs and inhibit tumor growth is an important influencing factor in their *in vivo* effects. Therefore, promoting the migration and colonization of CIK cells in the tumor site is a strategy to improve the adaptive immunotherapy of CIK cells [24, 25]. IRE not only kills tumor cells but also promotes the entrance of immune cells into PDAC tumors, demonstrating its regulation of the stroma [26, 27]. Scheffer *et al.* showed a transient decrease in regulatory T cells (Tregs) and a simultaneous transient increase in activated programmed cell death protein 1–positive (PD-1^+) T cells, which was consistent with the transient reduction of tumor-related immune suppression after IRE [13]. Based on these findings, we created a novel treatment combination of IRE plus CIK cells and studied its potential therapeutic mechanisms in mice with PC xenografts.

CIK cells are a heterogeneous group of cells obtained by inducing PBMCs with cytokines *in vitro*; they have been applied in the clinical treatment of cancer patients [28-30]. Different culture methods greatly affect the safety and antitumor activity of CIK cells and are directly related to therapeutic effects in cancer patients. Therefore, it is important to establish a standardized *in vitro* expansion method to obtain therapeutically competent CIK cell populations with high antitumor activity before applying them in clinical cancer therapy. First, we successfully established a method for *in vitro* expansion of CIK cells, enabling their total number to exceed 1×10^{10} (Fig. 1A). In addition, after 21 days of *in vitro* incubation, proportions of $CD3^+CD8^+$ and $CD3^+CD56^+$ cells were significantly increased, while those of $CD3^+CD4^+$ and $CD3^-CD56^+$ cells were significantly decreased (Fig. 1B).

Subsequently, we verified the antitumor effect of IRE plus CIK cells in tumor-bearing mice. Both IRE alone and CIK cells alone effectively suppressed tumor volume and improved survival in these mice (Fig. 2). Meanwhile, IRE combined with CIK cells could inhibit tumor growth to an even greater extent, prolonging mouse survival. We also found that this combination therapy remarkably increased numbers of $CD3^-$, $CD3^+$, $CD4^+$ and $CD8^+$ cells in tumor tissue (Fig. 3), suggesting that the combination of IRE and CIK cells can enhance the intensity of antitumor immune response in the TME outside the ablation zone.

The characteristics of CIK cells that colonize tumor sites *in vivo* are related to their chemotactic-adhesion ability [23]. The chemokine receptor pathway plays a key role in this process. CIK cells have been shown to express a range of chemokine receptors (CXCR3, CCR5, and CXCR4). Chemokines with increased expression in the TME can reach specific sites of inflammatory cells such as neutrophils, mononuclear macrophages, and lymphocytes; at the same time, they bind to chemokine receptors on cell surfaces to play a biological role [31]. In this study, we found that CXCR3, CCR5, and CXCR4 were highly expressed in the treatment groups compared with the untreated group. In addition, IRE increased lymphocytic infiltration, which could promote the expression of chemokine receptors by CIK cells (Fig. 4).

Ki-67 is expressed in all cells that are in a proliferative state and is a marker of cell proliferation [32, 33]. ICOS and GrB are expressed and activated by T cells during the effector phase of the immune response and are hallmarks of immune response generation [34-36]. To evaluate the effect of IRE on *in vivo* proliferation of CIK cells, we examined the expression of Ki-67, ICOS, and GrB after treatments with IRE and CIK cells. As shown in Figure 5, CIK cells–alone treatment significantly increased the expression of Ki-67, ICOS, IFN-γ, TNF-α, GrB, and perforin antigens compared with the untreated group. However, in the combination group the expression of Ki-67, ICOS, and GrB was further increased, indicating that IRE could promote the proliferative activity of CIK cells in the tumor ablation zone.

In conclusion, combination therapy of IRE and CIK cells showed a superior antitumor effect in a tumor-bearing mouse model of PC. This combination treatment could

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significantly inhibit the growth of tumors in the ablation zone and prolong mouse survival time. Moreover, there may be a synergistic effect between IRE and CIK cells in inhibiting tumor growth, and this synergistic effect might be achieved by IRE promoting the proliferation and activation of CIK cells in the tumor ablation zone, promoting the infiltration of effector lymphocytes within the tumor, and amplifying the immune response in the TME. This requires further in-depth study. In the future, IRE combined with CIK cells will provide a new therapeutic strategy for LAPC patients.

Acknowledgments

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Baohua Wang: conceptualization, methodology, data curation, writing—original draft. Huiyang Wang: methodology, data curation, writing—original draft. Lan Yue: formal analysis, writing—reviewing and editing. Qiang Chen: methodology, writing—reviewing and editing. Junjie Dong: writing—reviewing and editing. Tian'an Jiang: conceptualization, funding acquistion, writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Data Availability

All data generated or analyzed during this study are included in this article.

Funding

This study was supported by Development Project of National Major Scientific Research Instrument (82027803), National Natural Science Foundation of China (81971623), and Key Project of Natural Science Foundation of Zhejiang Province (LZ20H180001)

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