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Combination therapy of irreversible electroporation and cytokine-induced killer cells for treating mice bearing panc02 pancreatic-cancer xenografts

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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 Panc02 cell-bearing mouse model in vivo. CIK cells were isolated from the spleens of Panc02 pancreatic-cancer (PC) subcutaneous-xenograft model and the proportion of different lymphocytes was also determined. The antitumor effect of the combination of IRE and CIK cells in a PC subcutaneous-xenograft model was also investigated. The proportion of cells that were positive for $CD3^+CD8^+$ and the proportion of $CD3^+CD56^+$ cells were both significantly increased after 21 days of *in vitro* culture. Combined treatment of IRE and CIK cell 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 CIK cells. In conclusion, IRE combined with CIK cells showed superior antitumor efficacy in a PC xenograft model, which we attributed to the promotion of lymphocytic infiltration, as well as to upregulation of chemokine receptor expression and the regulators of CIK cell proliferation.

1. Introduction

Pancreatic cancer (PC) is a malignant digestive-system tumor that ranks fourth in lethality among various malignant tumors [1]. Its 5-year survival rate is <5%, and new cases and mortality rates are increasing year by year in global [2]. 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 [3,4].

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 [4]. 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 [5,6]. These effector cells express both CD3 and CD56 membrane protein molecules, and therefore not only have antitumor activity of T lymphocytes, but also have natural killer cell-like non-histocompatibility complex (MHC)-restricted antitumor characteristics [7]. They have been extensively used in clinical studies and treatment of various malignant tumors, but results in PC have been disappointing [6]. It is reported that pancreatic ductal adenocarcinoma (PDAC) contains a large number of immunosuppressive cells [8]. Such an immunosuppressive microenvironment (ISM) can inhibit the activity of tumor-infiltrating lymphocytes [9,10]. 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) [11,12]. 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 [13]. 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 [14]. Scheffer et al. demonstrated that IRE in

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LAPC transiently alleviates immunosuppression, creating a window for antitumor T-cell activation [15]. 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. Hence, the aim of this study was to observe the synergistic antitumor effect of RFA combined with CIK cells and to investigate the effect of RFA on the migration and tumoricidal activity of CIK cells in vivo and its possible mechanism. 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

CNP-III Steep Pulse Therapy System was provided 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: ~18 g) of specific pathogen–free (SPF) grade were provided by Shanghai SL Biotechnology Co., Ltd. (Shanghai, China). Sixty mice were kept in a cage in an SPF environment at a relative humidity of 40–60% and temperature of 25 ± 1 °C. This work was approved by the Animal Ethics Committee of the School of Medicine, Zhejiang University.

2.2. Cell culture

Panc02 cells (murine PC cells) were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). Panc02 cells were cultured in RPMI. 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, the digestive juice was removed, and new RPMI 1640 culture medium containing 10% heat-inactivated FBS, 1% penicillin and streptomycin were added. The cells were then maintained in an incubator at 37 °C and 5% CO_2.

2.3. Generation of CIK cells

The Panc02 cell bearing mice were sacrificed by cervical dislocation and were immediately immersed in the volume fraction of 75% ethanol for 5 min. The spleen of the mice was removed in a sterile environment. Cell suspensions of spleen were made by grinding the spleen on nylon nets (200 mesh) in 35 mm petri dishes containing 5 mL of mouse lymphocyte isolation liquid (Dakewe, Shenzhen, China). The splenic cell suspensions were then immediately transferred to a 15 mL centrifuge tube and covered with 1 mL RPMI 1640 medium, then centrifuged at room temperature (800 g, 30 min). After that, the lymphocyte layer was sucked out and 10 mL RPMI 1640 medium was added. At room temperature, cells were collected after centrifugation at 250*g* for 10 min. 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 300 U/mL IL-2 every 3 days for 24 days [5].

2.4. Evaluation of antitumor efficacy in the xenograft tumor model

We established a Panc02 subcutaneous-xenograft model as described previously [16]. Briefly, Panc02 cells in the logarithmic-growth phase (Passaged: 8 times; Cell confluency: ~70%) were diluted to 2 × 10^7/mL with sterile PBS. Then, we subcutaneously injected 0.1 mL Panc02 cells (2 × 10^6 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 size = 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. IRE ablation procedure was 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. In addition, a needle electrode were inserted into tumors from mice in CIK-alone and control groups without performing ablation.

Optimal CIK cells were harvested after incubation for 21 days based on above experiments. On day 3, 0.2 mL CIK (2 × 10^5) cells were infused intravenously to each mouse in IRE + CIK and CIK-alone groups (Fig. 1C).

Tumor sizes in the mice were recorded once every 3 days. Mice were killed when the tumor reached 2 cm in diameter or tumor ruptured, and xenograft tumors harvested. The tumor tissue was finely chopped and mixed with 2 mL RPMI1640 digestive medium and incubated at 37 °C for 2 h. Subsequently, the tumor tissue was ground and filtered by nylon nets (200 mesh) and washed by PBS. 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. In addition, 5 μL PE-CXCR3, 5 μL PE-CXCR4 Ab and 5 μL APC-CCR5 Ab were added to the cell samples. Mix well and label for 20 min at room temperature protected from light. Centrifuge at 500 rpm for 10 min and discard the supernatant. 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,

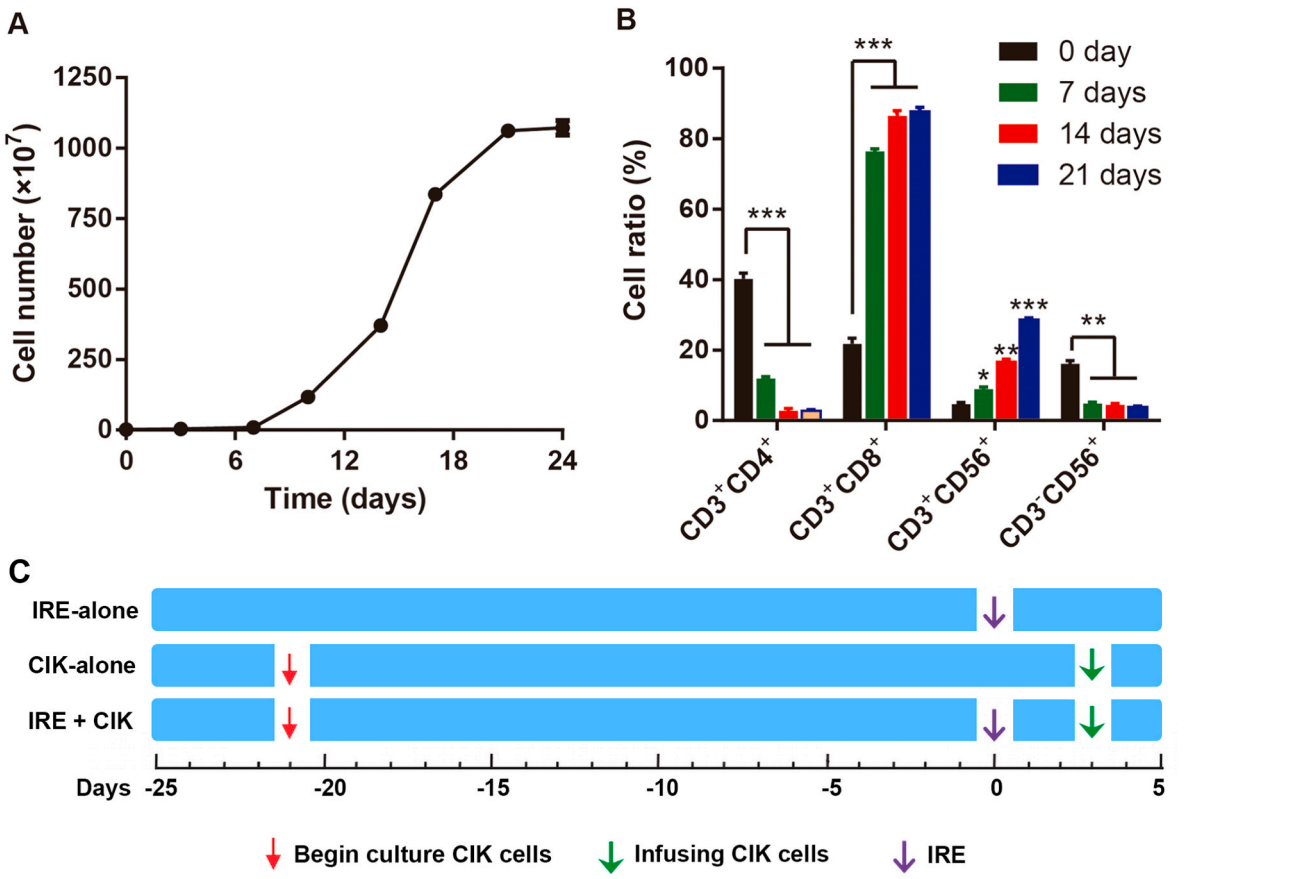

Fig. 1. In vitro expansion of cytokine-induced killer cells, and treatment schedule. (**A**) In vitro growth curves of CIK cells for 24 days. (**B**) Percentages of CD3, CD4, CD8, and CD56 expressed on the surfaces of CIK cells on different days of culture. *P < 0.05; **P < 0.01; ***P < 0.001 versus 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.

inducible costimulator-PE, Granzyme B-APC according to the manufacturer's protocol. All samples were washed and were analyzed by flow cytometry.

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 Fig. 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 cells, 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.

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 Fig. 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.

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 Fig. 3, numbers of intratumoral $CD3^-$ cells and $CD3^+$ cells were higher in the IRE-alone and CIK cells-

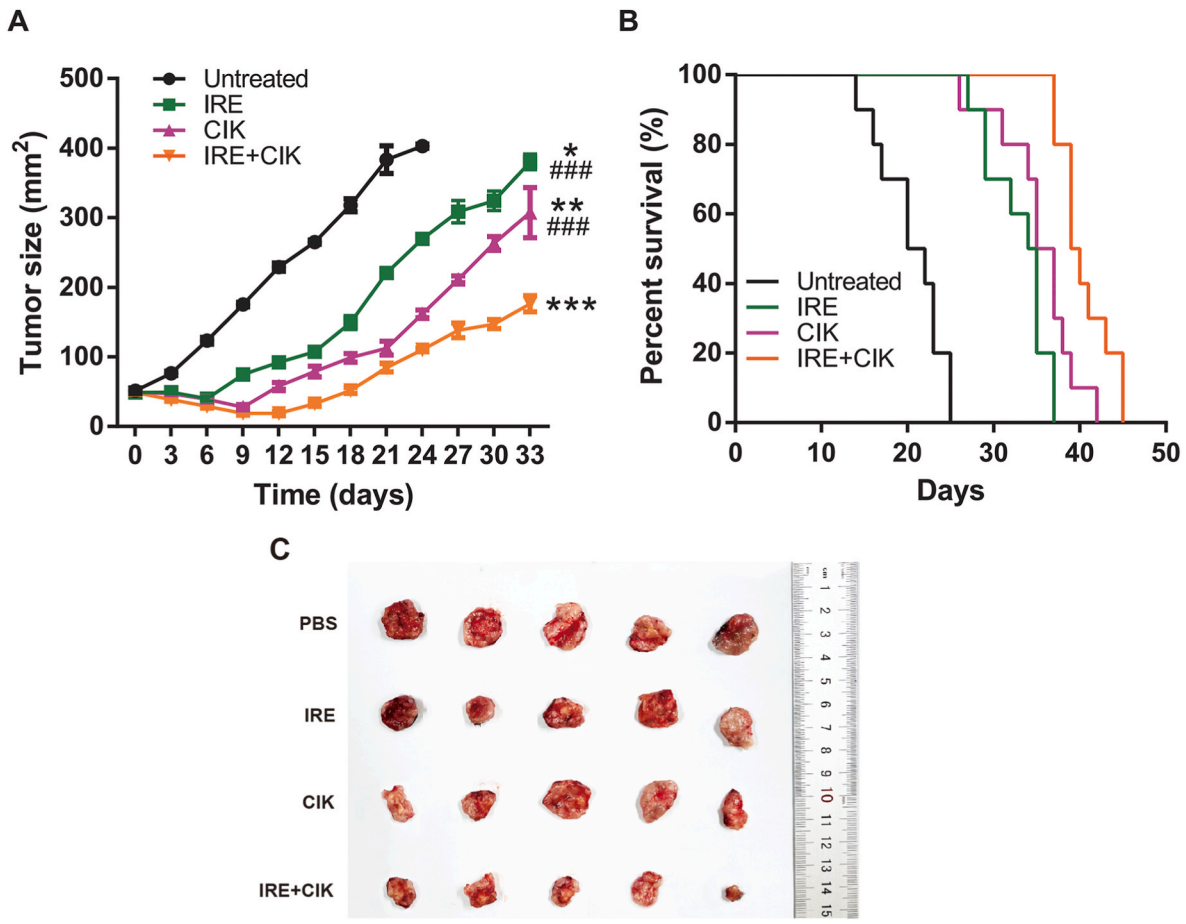

Fig. 2. Antitumor effects of irreversible electroporation combined with cytokine-induced killer cells on pancreatic cancer in tumor-bearing mice. (**A**) Tumor growth, (**B**) mouse survival, and (**C**) tumor image per treatment group were monitored. $^{###}P < 0.001$ *versus* IRE + CIK group (mean ± SD, n = 10); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* untreated group (mean ± SD, n = 10).

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.

Subsequently, we examined levels of chemokine receptors (CXCR3, CCR5, CXCR4) expressed by CIK cells. As shown in Fig. 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.

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 Fig. 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.

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 [17]. 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 [18].

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 [19]. The killing effect of CIK cells on tumors is characterized by high efficiency and lack of major histocompatibility complex (MHC) restriction [20]. 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 [21–23]. 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 [5,24]. However, due to the presence of an immunosuppressive

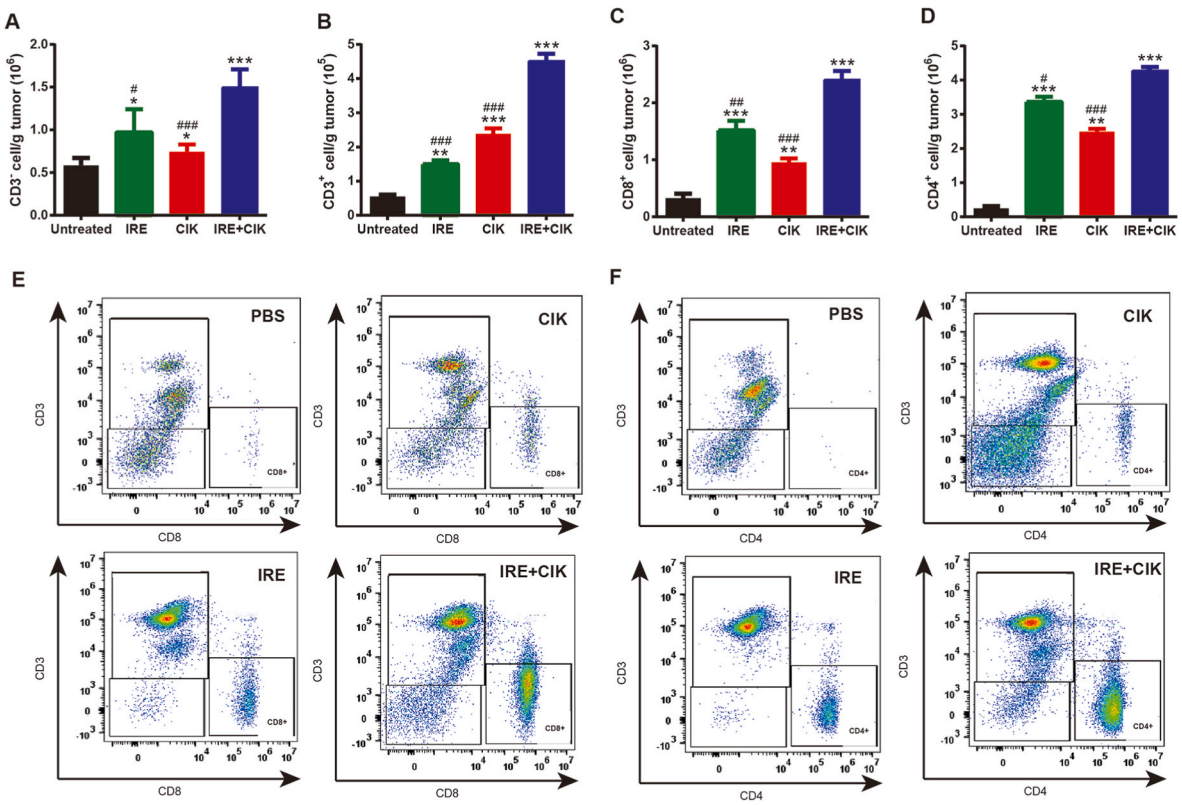

Fig. 3. Combination therapy with irreversible electroporation and cytokine-induced killer cells increased the infiltration of lymphocytes into tumor tissue. Numbers of (**A**) $CD3^-$ cells, (**B**) $CD3^+$ cells, (**C**) $CD8^+$ cells, and (**D**) $CD4^+$ cells with tumor infiltration. (**E–F**) FCM 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).

tumor-associated stroma, adaptive cellular immunotherapy has only limited efficacy against PDAC.

The ability of CIK cells to migrate and colonize tumor sites in vivo is related to their chemotactic-adhesion ability [25]. 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 [26,27]. IRE not only kills tumor cells but also promotes the entrance of immune cells into PDAC tumors, demonstrating its regulation of the stroma [15,28]. 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 ($PD\text{-}1^+$) T cells, which was consistent with the transient reduction of tumor-related immune suppression after IRE [15]. 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 [29–31]. 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 [25]. 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 [32]. 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). CXCR3 axis is a key pathway for recruitment of immune cells in solid tumors, and CXCR3 ligands include CXCL9, CXCL10 and CXCL11. After activation, CXCR3 is induced and highly expressed on effector Th1-polarized CD4+T cells, CD8+T cells, NK cells, and NKT cells [33]. CXCR3 ligand expression in tumors, elevated serum levels of CXCR3 ligand, and CXCR3 on T cells enhance recruitment of T cells [34]. In current study,

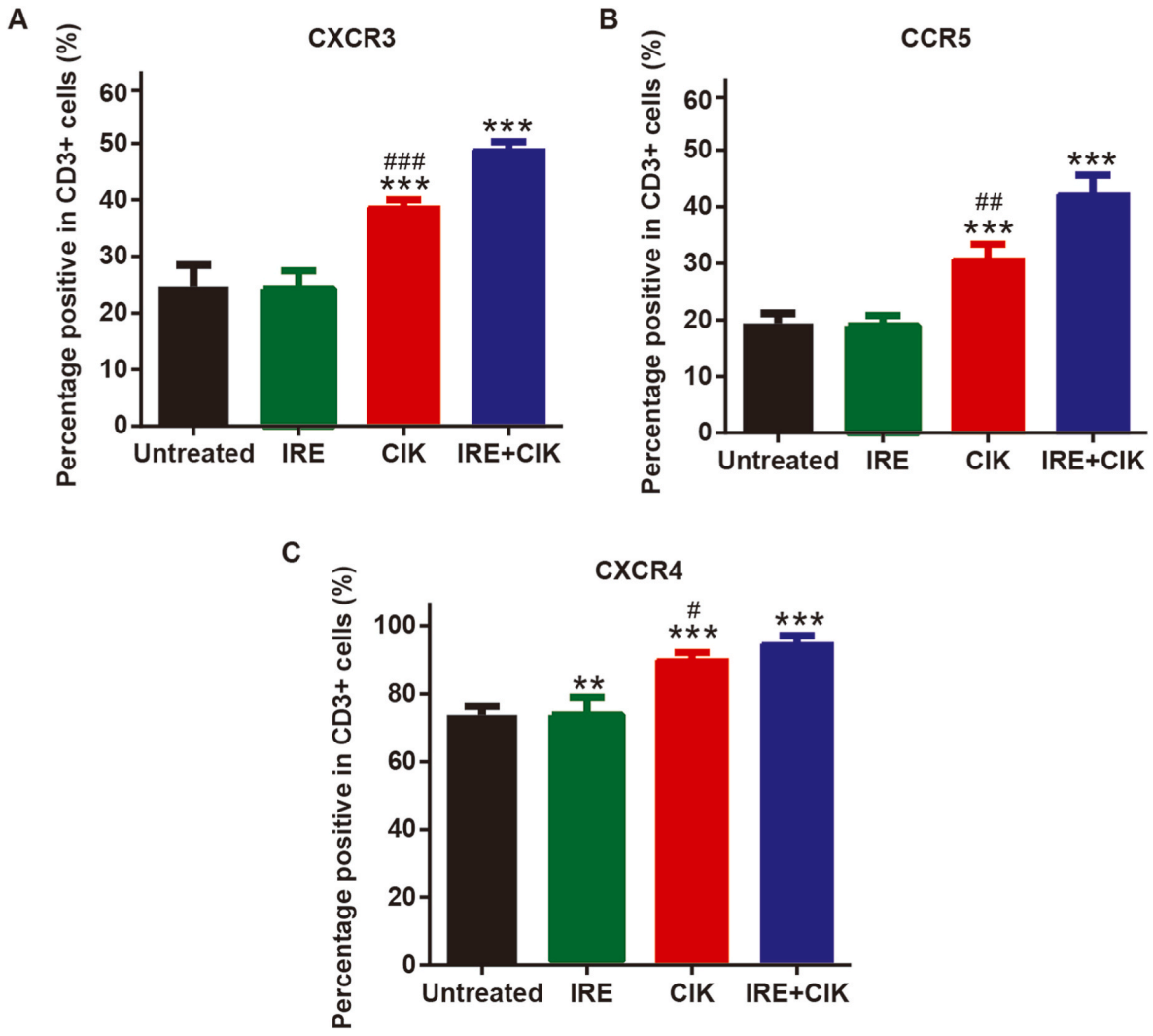

Fig. 4. Percentages of chemokine receptor positivity in $CD3^+$ cells. (**A**) CXCR3, (**B**) CCR5, and (**C**) CXCR4. $CD3^+$ cells in the CIK cell treatment group highly expressed all three of these receptors, with the positive rate of CXCR4 exceeding 80%. All three chemokine receptors were significantly 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).

the increase of ligand level in IRE may be related to the increase of inflammatory cytokines stimulated by IRE, the activation of granulocyte/macrophage/colony-stimulating factor genes, and the invasion of immune cells.

As is well known, Ki-67 is expressed in all cells that are in a proliferative state and is a marker of cell proliferation [35,36]. 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 [37–39]. 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 Fig. 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.

Panc02 pancreatic-cancer is well-known for its resistance to many immunotherapies, in part because of its distinctive fibrotic stroma and infiltration by immunosuppressive cell types. It is not clear that a single subcutaneous implant of Panc02 cells recapitulates these adverse factors, and we will consider testing CIK and/or IRE in a model based on orthotopic implantation. Furthermore, even if a primary tumor of Panc02 pancreatic-cancer has been resected, occult spread and subsequent development of metastatic disease is frequent and deadly. Because of the need for a solution to this problem, and to further elucidate the immunologic consequences of therapy with CIK ± IRE, we will consider investigating the antitumor effects of RFA and/or CIK in metastatic disease models inoculated with Panc02 cells.

5. Conclusion

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

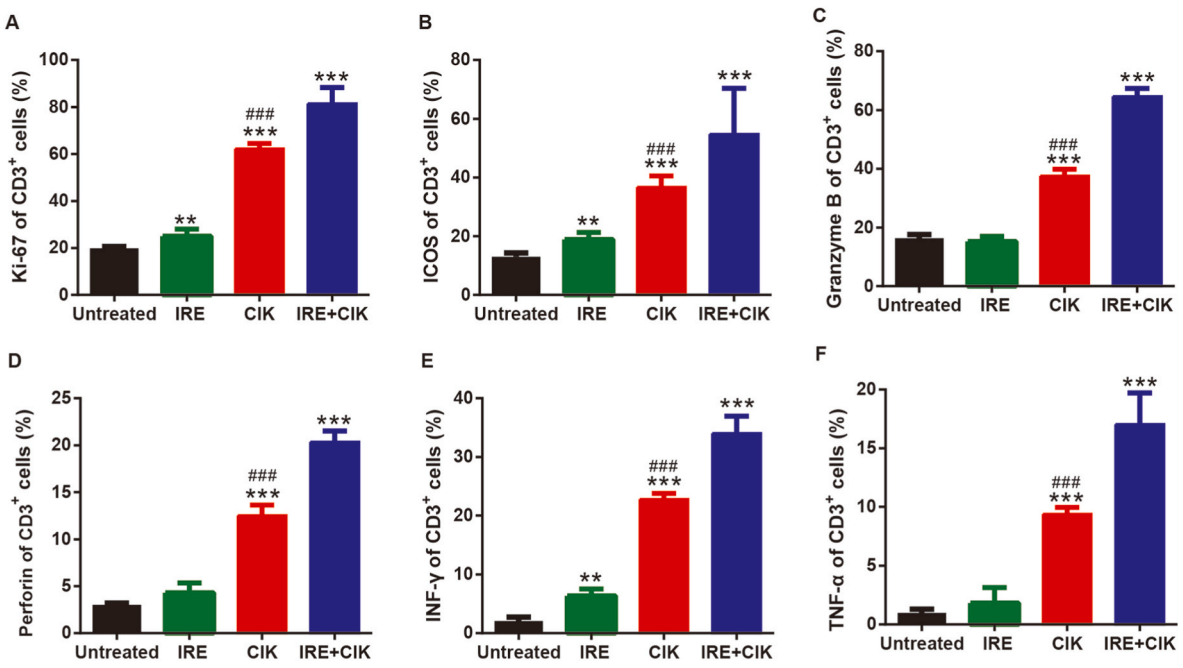

Fig. 5. Effect of irreversible electroporation on in vivo proliferation of cytokine-induced killer cells in tumor tissues. Proportions of CIK cells expressing (**A**) Ki-67, (**B**) ICOS, (**C**) GrB, (**D**) perforin, (**E**) IFN-γ, and (**F**) TNF-α in tumors were detected via intracellular staining 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).

Author contributions

Baobab 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.

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Ethics statement

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).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

[1] L. Perkhofer, T.J. Ettrich, T. Seufferlein, Pancreatic cancer: progress in systemic therapy, Gastrointest. Tumors 1 (2015) 167–179.
[2] A.P. Klein, Pancreatic cancer epidemiology: understanding the role of lifestyle and inherited risk factors, Nat. Rev. Gastroenterol. Hepatol. 18 (2021) 493–502.
[3] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA A Cancer J. Clin. 71 (2021) 209–249.
[4] A.H. Ruarus, L. Vroomen, B. Geboers, E. Van Veldhuisen, R.S. Puijk, S. Nieuwenhuizen, M.G. Besselink, B.M. Zonderhuis, G. Kazemier, T.D. De Gruijl, K.P. Van Lienden, J.J.J. De Vries, H.J. Scheffer, M.R. Meijerink, Percutaneous irreversible electroporation in locally advanced and recurrent pancreatic cancer (PANFIRE-2): a multicenter, prospective, single-arm, phase II study, Radiology 294 (2020) 212–220.
[5] C.C. Shan, L.R. Shi, M.Q. Ding, Y.B. Zhu, X.D. Li, B. Xu, J.T. Jiang, C.P. Wu, Cytokine-induced killer cells co-cultured with dendritic cells loaded with the protein lysate produced by radiofrequency ablation induce a specific antitumor response, Oncol. Lett. 9 (2015) 1549–1556.
[6] A. Heinze, B. Grebe, M. Bremm, S. Huenecke, T. Munir, L. Graafen, J. Frueh, M. Merker, E. Rettinger, J. Soerensen, T. Klingebiel, P. Bader, E. Ullrich, C. Cappel, The synergistic use of IL-15 and IL-21 for the generation of NK cells from CD3/CD19-depleted grafts improves their ex vivo expansion and cytotoxic potential against neuroblastoma: perspective for optimized immunotherapy post haploidentical stem cell transplantation, Front. Immunol. 10 (2019) 2816.
[7] Y.C. Linn, K.M. Hui, Cytokine-induced NK-like T cells: from bench to bedside, J. Biomed. Biotechnol. 2010 (2010), 435745.
[8] Shih-Wei, Chia-Hsun Lai, Tsai, Lin Cheng-Li, Kuan-Fu, Liao, Association between pancreatic cancer and metformin use in patients with type 2 diabetes, Postgrad. Med. 95 (2019) 291.
[9] Y. Chen, M.A.J. Moser, Y. Luo, W. Zhang, B. Zhang, Chemical enhancement of irreversible electroporation: a review and future suggestions, Technol. Cancer Res. Treat. 18 (2019), 1533033819874128.
[10] M.F. Rashid, E.M. Hecht, J.A. Steinman, M.D. Kluger, Irreversible electroporation of pancreatic adenocarcinoma: a primer for the radiologist, Abdom Radiol (NY) 43 (2018) 457–466.

[11] J. Moir, S.A. White, J.J. French, P. Littler, D.M. Manas, Systematic review of irreversible electroporation in the treatment of advanced pancreatic cancer, Eur. J. Surg. Oncol. 40 (2014) 1598–1604.
[12] R.C. Martin 2nd, D. Kwon, S. Chalikonda, M. Sellers, E. Kotz, C. Scoggins, K. M. Mcmasters, K. Watkins, Treatment of 200 locally advanced (stage III) pancreatic adenocarcinoma patients with irreversible electroporation: safety and efficacy, Ann. Surg. 262 (2015) 486–494, discussion 492-4.
[13] B. Geboers, H.J. Scheffer, P.M. Graybill, A.H. Ruarus, S. Nieuwenhuizen, R.S. Puijk, P.M. Van Den Tol, R.V. Davalos, B. Rubinsky, T.D. De Gruijl, D. Miklavcic, M. R. Meijerink, High-voltage electrical pulses in oncology: irreversible electroporation, electrochemotherapy, gene electrotransfer, electrofusion, and electroimmunotherapy, Radiology 295 (2020) 254–272.
[14] B.E. Bulvik, N. Rozenblum, S. Gourevich, M. Ahmed, A.V. Andriyanov, E. Galun, S. N. Goldberg, Irreversible electroporation versus radiofrequency ablation: a comparison of local and systemic effects in a small-animal model, Radiology 280 (2016) 413–424.
[15] H.J. Scheffer, A.G.M. Stam, B. Geboers, L. Vroomen, A. Ruarus, B. De Bruijn, M. P. Van Den Tol, G. Kazemier, M.R. Meijerink, T.D. De Gruijl, Irreversible electroporation of locally advanced pancreatic cancer transiently alleviates immune suppression and creates a window for antitumor T cell activation, OncoImmunology 8 (2019), 1652532.
[16] X. Wang, X. Xie, J. Guo, H. Wang, C. Zhou, L. Zhao, Inhibition activity of BF-30 against murine pancreatic carcinoma cell Panc02 in vitro, Pharmaceut. Biotechnol. 24 (2017) 115–119.
[17] J. Zhao, S. Chen, L. Zhu, L. Zhang, J. Liu, D. Xu, G. Tian, T. Jiang, Antitumor effect and immune response of nanosecond pulsed electric fields in pancreatic cancer, Front. Oncol. 10 (2020), 621092.
[18] A. Gyftopoulos, I.A. Ziogas, A.S. Barbas, D. Moris, The synergistic role of irreversible electroporation and chemotherapy for locally advanced pancreatic cancer, Front. Oncol. 12 (2022), 843769.
[19] S.M. Crusz, F.R. Balkwill, Inflammation and cancer: advances and new agents, Nat. Rev. Clin. Oncol. 12 (2015) 584–596.
[20] J. Jiang, C. Wu, B. Lu, Cytokine-induced killer cells promote antitumor immunity, J. Transl. Med. 11 (2013) 83.
[21] J.J. Mata-Molanes, M. Sureda Gonzalez, B. Valenzuela Jimenez, E.M. Martinez Navarro, A. Brugarolas Masllorens, Cancer immunotherapy with cytokine-induced killer cells, Targeted Oncol. 12 (2017) 289–299.
[22] Q.Z. Pan, J.M. Gu, J.J. Zhao, Y. Tang, Q.J. Wang, Q. Zhu, M.J. Song, Y.Q. Li, J. He, S.P. Chen, D.S. Weng, J.C. Xia, Retrospective analysis of the efficacy of cytokine-induced killer cell immunotherapy combined with first-line chemotherapy in patients with metastatic colorectal cancer, Clin Transl Immunology 9 (2020) e1113.
[23] E. Fiorino, A. Merlini, L. D'ambrosio, I. Cerviere, E. Berrino, C. Marchio, L. Giraudo, M. Basirico, A. Massa, C. Donini, V. Leuci, R. Rotolo, F. Galvagno, L. Vitali, A. Proment, S. Ferrone, A. Pisacane, Y. Pignochino, M. Aglietta, G. Grignani, G. Mesiano, D. Sangiolo, Integrated antitumor activities of cellular immunotherapy with CIK lymphocytes and interferons against KIT/PDGFRA wild type GIST, Int. J. Mol. Sci. 23 (2022).
[24] Z.M. Huang, W. Li, S. Li, F. Gao, Q.M. Zhou, F.M. Wu, N. He, C.C. Pan, J.C. Xia, P. H. Wu, M. Zhao, Cytokine-induced killer cells in combination with transcatheter arterial chemoembolization and radiofrequency ablation for hepatocellular carcinoma patients, J. Immunother. 36 (2013) 287–293.
[25] Y. Dong, S. Gao, X. Zhang, J. Kou, J. Liu, T. Ye, H. Shen, CCL17 and CCL22 induce CCR4 receptor expression and promote cytokine-induced killer cells migration, Anti Cancer Drugs 33 (2022) 149–157.
[26] S. Liang, M. Sun, Y. Lu, S. Shi, Y. Yang, Y. Lin, C. Feng, J. Liu, C. Dong, Cytokine-induced killer cells-assisted tumor-targeting delivery of Her-2 monoclonal antibody-conjugated gold nanostars with NIR photosensitizer for enhanced therapy of cancer, J. Mater. Chem. B 8 (2020) 8368–8382.
[27] H. Liu, Y. Xu, K. Liang, R. Liu, Immune cells combined with NLRP3 inflammasome inhibitor exert better antitumor effect on pancreatic ductal adenocarcinoma, Front. Oncol. 10 (2020) 1378.
[28] M. Lin, X. Zhang, S. Liang, H. Luo, M. Alnaggar, A. Liu, Z. Yin, J. Chen, L. Niu, Y. Jiang, Irreversible electroporation plus allogenic Vgamma9Vdelta2 T cells enhances antitumor effect for locally advanced pancreatic cancer patients, Signal Transduct. Targeted Ther. 5 (2020) 215.
[29] P.K. Haber, M. Puigvehi, F. Castet, V. Lourdusamy, R. Montal, P. Tabrizian, M. Buckstein, E. Kim, A. Villanueva, M. Schwartz, J.M. Llovet, Evidence-based management of hepatocellular carcinoma: systematic review and meta-analysis of randomized controlled trials (2002-2020), Gastroenterology 161 (2021) 879–898.
[30] L. Zhang, J. Ding, H.-Y. Li, Z.-H. Wang, J. Wu, Immunotherapy for advanced hepatocellular carcinoma, where are we? Biochim. Biophys. Acta Rev. Canc 1874 (2020), 188441.
[31] J.H. Lee, J.H. Lee, Y.S. Lim, J.E. Yeon, T.J. Song, S.J. Yu, G.Y. Gwak, K.M. Kim, Y. J. Kim, J.W. Lee, J.H. Yoon, Adjuvant immunotherapy with autologous cytokine-induced killer cells for hepatocellular carcinoma, Gastroenterology 148 (2015) 1383–13891 e6.
[32] D.C. Hinshaw, L.A. Shevde, The tumor microenvironment innately modulates cancer progression, Cancer Res. 79 (2019) 4557–4566.
[33] R. Bonecchi, G. Bianchi, P.P. Bordignon, D. D'ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, F. Sinigaglia, Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s, J. Exp. Med. 187 (1998) 129–134.
[34] Y. Zou, F. Li, W. Hou, P. Sampath, Y. Zhang, S.H. Thorne, Manipulating the expression of chemokine receptors enhances delivery and activity of cytokine-induced killer cells, Br. J. Cancer 110 (2014) 1992–1999.
[35] Q. Qu, Y. Xin, Y. Xu, Y. Yuan, K. Deng, Imaging and clinicopathological features of acinar cell carcinoma, Front. Oncol. 12 (2022), 888679.
[36] S.S. Menon, Chandrasekharansakthivel, Kunnathur Murugesanrasmi, Rajan Radha, Ki-67 protein as a tumour proliferation marker, Clinica chimica acta; international journal of clinical chemistry 491 (2019) 39–45.
[37] O. Jimenez, T. Mangiaterra, S. Colli, M.G. Lombardi, M. Preciado, E.D. Matteo, P. Chabay, PD-1 and LAG-3 expression in EBV-associated pediatric Hodgkin lymphoma has influence on survival, Front. Oncol. 12 (2022), 957208.
[38] C. Peng, M.A. Huggins, K.M. Wanhainen, T.P. Knutson, H. Lu, H. Georgiev, K. L. Mittelsteadt, N.N. Jarjour, H. Wang, K.A. Hogquist, D.J. Campbell, H. Borges Da Silva, S.C. Jameson, Engagement of the costimulatory molecule ICOS in tissues promotes establishment of CD8(+) tissue-resident memory T cells, Immunity 55 (2022) 98–114 e5.
[39] D. Christiansen, E. Mouhtouris, R. Hodgson, V.R. Sutton, J.A. Trapani, F.L. Ierino, M.S. Sandrin, Antigen-specific CD4(+) CD25(+) T cells induced by locally expressed ICOS-Ig: the role of Foxp3, Perforin, Granzyme B and IL-10 - an experimental study, Transpl. Int. 32 (2019) 1203–1215.