Original Article Irreversible electroporation enhances immunotherapeutic effect in the off-target tumor in a murine model of orthotopic HCC

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Abstract: Irreversible electroporation (IRE) has been postulated to have an off-target effect on lesions not in the tumor-ablative field, possibly through heightened immunologic response. In this study, we evaluated whether combination IRE and immunotherapy would lead to increased tumor necrosis and T cell recruitment to both the treated tumors and tumors outside the local ablative field. An *in vitro* cell-IRE model was established to evaluate the ability of T lymphocytes (EL4 cell and HH cells) migration in response to Hepatocellular carcinoma (HCC) cells (Hepa1-6 and HepG2) with IRE treatment. An orthotopic HCC mouse model was established by implantation of 1mm^3 sections of Hepa1-6 tumor tissues into the right and left lobes of the liver. The Hepa1-6 cells and HepG2 cells with IRE treatment increased the migration ability of EL4 cell and HH cells, specifically when they were pretreated with immunotherapeutic agents in vitro. In the orthotopic HCC mouse model, IRE+immunotherapy treatment enhanced the necrosis and subpopulation of infiltrated CD8 positive cells, but attenuated the tumor associated inflammatory cells in both IRE target tumor tissues and IRE off-target tumor tissues from the mice with 4 weeks of immunotherapy following IRE. This study provided the evidence that combination of IRE and immunotherapy enhances tumor necrosis and immune responses, not only in the IRE-treated tumor but also in the off-target tumor.

Keywords: Irreversible electroporation, immunotherapy, tumor microenvironment, hepatocellular carcinoma, tumor-associated neutrophils, tumor-associated macrophages

Introduction

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and is a leading cause of cancer-related death worldwide [1-3]. Over 500,000 deaths related to HCC are observed annually, with the vast majority occurring in low- and middle-resource countries as a result of endemic viral hepatitis [1, 4]. However, with the rising prevalence of obesity, the incidence of HCC is rapidly increasing even in nations with low viral hepatitis rates and available hepatitis treatment. In the U.S., HCC has tripled from 1.6 to 4.9 per 100,000 individuals annually in the past 30 years largely as a result of obesity-related cirrhosis [5, 6]. The 1year overall survival (OS) for HCC remains 47% [5] and improvements in therapeutic options are needed to improve survival outcomes.

Few effective systemic therapies exist for hepatocellular carcinoma. Multikinase inhibitors, such as sorafenib and lenvatinib, recently remained the only effective first-line systemic therapies in advanced HCC. In the SHARP trial, sorafenib demonstrated moderate therapeutic benefit in patients with advanced HCC versus placebo (median OS 10.7 months vs 7.9 months, respectively) [7]. The relative immunosuppression within the local tumor microenvironment of HCC has made immune checkpoints attractive targets for therapy [8], however these therapies have failed to improve OS as first-line monotherapy compared to sorafenib [9]. To improve survival in patients with unresectable tumors/disease, resistance to systemic immunotherapy must be overcome. Indeed, recent trial data suggest pre-treatment with sorafenib significantly improved response rates and OS [10, 11]. The combination of the CTLA-4 inhibitor ipilimumab with the PD-1 inhibitor nivolumab produced a two-fold improvement in response rates compared to monotherapy with nivolumab alone and demonstrated a median OS of 23 months after pre-treatment [11]. It has been postulated that pre-treatment may enhance the efficacy of immunotherapy by disrupting the HCC tumor microenvironment [12].

Irreversible electroporation (IRE) is a wellestablished non-thermal tumor ablative therapy that disrupts tumors through high electrical voltage. Unlike thermal ablation, IRE induces permanent cell membrane pores which leads to cellular apoptosis rather than thermal necrosis [13]. In vivo murine studies have shown that IRE disrupts the tumor microenvironment [14] and may assist in neoantigen recognition. In clinical practice, IRE modulates circulating immunophenotypes following treatment for patients with locally advanced pancreatic cancer [15].

In this study, we utilized a murine model of multifocal HCC to evaluate the local tumor milieu following IRE and immunotherapy as well as the response to treatment of tumors outside of the electroporated field (non-ablated tumors). We hypothesize that IRE generates a pro-inflammatory milieu that enhances the efficacy of immunotherapy activity to non-ablated/off-target tumors and may create an adaptive immune response.

Methods

Cell lines and immunotherapeutic agents

A murine hepatoma cell line Hepa1-6 (ATCC[®] CRL-1830), a murine T lymphocyte (lymphoma) line EL4 (ATCC[®] TIB-39[™]), a human T lymphocyte (lymphoma) line HH (ATCC[®] CRL-2105[™]), and a human HCC cell line HepG2 (ATCC[®] HB-8065), were purchased from American Type Culture Collection (ATCC, Manassas, VA). The EL4, Hepa1-6 and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning Cellgro) with 10% FBS (SigmaAldrich, MO) and Pen/Strep (Corning Cellgro). HH cells were cultured in RPMI-1640 medium (ATCC 30-2001) with 10% FBS (SigmaAldrich, MO) and Pen/Strep (Corning Cellgro). Nivolumab and ipilimumab (Bristol-Meyers Squibb, NY) were provided through support by the James Graham Brown Cancer Center (Louisville, KY) and the Price Surgical Research Institute (Louisville, KY). Murine anti-PD-L1 was obtained from BioXcell (Item No. BE0101).

In vitro IRE and cell migration

An *in vitro* IRE assay was performed on the HCC cells, hepa1-6 or HepG2, using an ECM 830 system (Harvard BioScience, MA). In brief, the cultured cells in the IRE-cuvettes were electroporated at 280 V (duration 0.2 ms, 10 pulses at pulse interval of 1.0 seconds with electrode gap 4.0 mm). Following electroporation, the HCC cells were seeded into 24-well plates at 1×10^5/well and cultured in DMEM for 12 hours. EL4 cells were treated with murine anti-PD-L1 (5 µg/ml) while HH cells were treated with ipilimumab (5 μ g/ml) and nivolumab (10 µg/ml) for 12 hours. The cells were then counted at 1×10^5 and transferred into BD Biocoat 8 µm membrane inserts (BD Biosciences, 354480) which have been pre-coated with collagen I at 50 µg/ml to perform co-culture and migration assay. For co-culture, the inserts containing EL4 or HH were placed in wells containing hepa1-6 or HepG2 in the DMEM media with 10% FBS. After 24 hours, the inserts were removed, washed with PBS, fixed in methanol and stained with crystal violet (0.05% w/v in methanol). The bottom surfaces of the stained inserts were then observed under a light microscope, and the numbers of stained cells were counted in 5 fields/high power filed (HPF). The cell migration capacity was calculated based on the numbers of crystal violet stained cells.

Establishment of orthotopic HCC model

Male eight-week old C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) were utilized for orthotopic tumor inoculation. The animals were housed four per cage, given rodent chow and tap water, and maintained at 22°C and on a 12-hour light/dark cycle. To establish orthotopic HCC model, tumor cell inoculation was first performed in 2 mice. In brief, a 1.5-cm midline laparotomy incision was made under anesthesia. Hepa1-6 cells derived from murine hepatoma were injected at a concentration of 1×10⁶ cells/injection into the small bowel mesentery. The abdominal wall was closed in two layers using 5-0 Vicryl suture (Ethicon, Incorporated, New Brunswick, NJ). Tumor growth was monitored weekly by ultrasound with a small linear probe array [16, 17]. When the mesenteric tumors reached 5 mm³ in size, the tumor tissue was harvested for the HCC orthotopic model which was established by orthotopic implantation of tumor tissue in the liver lobes of male eight-week old C57/BL6 mice. Briefly, a 1.5-cm midline laparotomy incision was made to expose the liver. A 0.5-0.7 cm tunnel was created at the inferior margin of liver towards the central lobe using a blunt bore IV needle (1" 22 G). Through this tunnel, a 1 mm³ portion of the previously sectioned tumor tissue was directly implanted in both the right I left lobes.

Irreversible electroporation (IRE), treatment and group assignment

Tumor growth was monitored weekly by ultrasound. When tumor size reached 1.5-2.0 cm³, IRE was performed on the tumor at the left liver lobe. In brief, under anesthesia and sterile conditions, a 1.5-cm midline laparotomy incision was made on the tumor-burden animals to expose the tumor on the left liver lobe. Surgical tweezer-type electrodes, an ECM 830 system (Harvard BioScience, MA), were placed around the tumor. IRE was delivered through 10-20 microsecond electrical pulses, with pulse duration of 100 µsec and a pulse interval of 100 ms at 1000 V. The tumor in the right lobe of liver was not treated and used as our off-target tumor. Following IRE, the animals were treated with immunotherapeutic (IT) agent using 10 mg/kg anti-PD-L1 monoclonal antibody via 250 µL, intraperitoneal injection every 3 days up to four weeks, while an equal volume of saline was used as an immunotherapeutic treatment control. The schematic diagram for the IRE off-target tumor and IRE target tumor, as well as the surgical operation for IRE on a tumor in right liver lobe, are shown in Figure 1. The treatment algorithm following tumor implantation/IRE by grouping is shown in Table 1. One week following the last IT treatment, the animals were euthanized and the tissues were harvested for further analysis. All animal studies comply with the regulatory provisions for animal testing and research as approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Louisville.

Histopathological examination and scoring

The harvested liver tissues were fixed in 10% neutral phosphate buffered formalin further embedded in paraffin and sectioned to a thickness of 5 µm for histological and immunohistochemical examinations. Hematoxylin-and-eosin (H&E) staining for histology and chloroacetate esterase (CAE) staining for neutrophil detection were performed in paraffin-embedded frozen tissue. The images were reviewed and analyzed microscopically for determination of necrotic index and inflammatory infiltration index based on histopathological scoring. This scoring system for the indexes of necrosis and inflammatory infiltration calculated by the percentage of either necrotic area or inflammatory infiltration area. The scoring was conducted as follows: <25%/(low-power field, 100×); 25-50% (lowpower field, 100×); 50-75% (low-power field, 100×); >75% (low-power field, 100×). A total of 5 images for each slide were selected randomly to calculate the indexes of necrosis and inflammatory infiltration. All the slides were reviewed and scored by 2 pathologists blind to the experiment groups.

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed on 20-µm, paraffin-embedded sections of specimen using DAKO EnVision + System Kit (DAKO EnVision + System, HRP, Carpinteria, CA) as previously reported [18]. In brief, sections were deparaffinized and hydrated, washed with TRIS-buffer, and peroxidase blocking was performed. After re-washing, antibodies of interest were applied and incubated with labeled polymer for 30 minutes at room temperature. The substrate-chromogen solution (diaminobenzidine) was added as a visualization reagent; 0.25% bovine serum albumin in phosphate-buffered saline without antibody was used as a negative control. Haematoxylin and eosin staining were performed in standard fashion. Digital images were acquired with the Olympus 1×51 microscope (Olympus, Pittsburgh, PA) at 20× magnification using the Olympus DP72 digital camera and measured via the cellSense Dimension imaging system (Olympus Life Science, Tokyo, Japan). The procedure for computer image analysis of staining intensity was performed, and the acquired color images from IHC staining were defined and quantified per software



Figure 1. A: Schematic diagram for the IRE off-target tumor and IRE target tumor. B: Surgical operation for IRE on the tumor in right liver lobe. Arrow: tumor.

specifications with the antibody expression defined as percent of threshold area represented in acquired color.

Isolation of murine macrophages

Thioglycollate medium was used to obtain activated inflammatory macrophages being recruited to the peritoneal cavity of mice. In brief, 1 mL of 3% thioglycollate medium was injected into the peritoneal cavity of the mouse. After 4

days, the mice were sacrificed and an incision was made with scissors to expose all peritoneum. The peritoneal membrane was carefully pulled up using forceps, 6 mL of ice-cold PBS was injected into the peritoneal cavity, and then the mouse was shaked gently for 10 seconds. The macrophages suspension was collected using a 10 mL syringe. The collected cells were plated in a concentration 1×10^6 cells/mL in the RPMI 1640 medium with 5% FCS for further Flow Cytometry analysis.

| Treatment Arm (n = 6 per arm) | Day 0 | Weekly Treatment | Duration of Therapy |
|-------------------------------|--|--|---------------------|
| Control | No Treatment | Saline injection | 4 weeks |
| IRE Alone | Voltage: 1000 V Duration: 0.1 ms Pulses: 10 Pulse Interval: 0.1 s | Saline injection | 4 weeks |
| IT | No Treatment | anti-PD-L1 monoclonal antibody (10 mg/kg) | 4 weeks |
| IRE+IT | Voltage: 1000 V Duration: 0.1 ms Pulses: 10 Pulse Interval: 0.1s | anti-PD-L1 monoclonal antibody (10 mg/kg) | 4 weeks |

Table 1. Treatment algorithm following tumor implantation/IRE by grouping

Flow cytometry

Macrophage analysis with M1/M2 differentiation was performed using fluorochromes F4/80 PE, CD11b FITC, CD206 APC, and MHC II Percp/ cy5.5 (Biolegend, San Diego, CA) at 5 μ l of fluorochrome per 1×10⁶ cells. Frozen cells were rapidly thawed and assessed for viability with Trypan blue. Cells were stained after Fc block to eliminate nonspecific binding. Macrophage flow cytometry was analyzed using FlowJo (Ashland, OR) to gate F4/80⁺/CD11b⁺ followed by CD206 for M2 polarization and Percp/cy5.5 for M1 polarization. Gating controls were utilized with single fluorochrome and fluorescence minus one (FMO) staining.

Statistical analysis

Standard error of mean (SEM) is indicated on all figures, with P<0.05 considered significant. Experiments were performed with a minimum of triplicate samples and triplicate repetition of experiments. Paired or unpaired student t-test, Wilcoxon rank sum tests were utilized depending on data distribution and experimental design. Microsoft Office Excel 2018 (Microsoft, USA) was utilized for statistical calculations.

Results

Combination of IRE and immunotherapeutic agents enhances immune cell migration in vitro

Substantial evidence is accumulating that curative intent IRE treatment of solid tumors can enhance immune cell recruitment in tumor tissue and may overcome resistance to immunotherapy [19]. Therefore, we first established an *in vitro* cell-IRE model to evaluate the ability of T lymphocytes migration in response to the cancer cells treated with IRE. The cells of T lymphocytes (EL4 and HH) were pretreated with immunetherapeutic (IT) agents, and then cocultured with the HCC cells (Hepa1-6 and HepG2) which had been exposed to the in vitro cell-IRE treatment. The results indicated that co-culture with HCC cells could increase the cell migration ability of both EL4 and HH cells. compared to the EL4 and HH cells without HCC cells co-culture (Figure 2A and 2B). IT pretreatment significantly increased the migration ability of EL4 cells co-cultured with Hepa1-6 cells without IRE, compared to untreated control (P<0.05). Interestingly, the migration ability of the co-cultured EL4 cells treated with in vitro cell-IRE showed 3-fold higher (versus co-culture alone, P<0.01) and 1.2-fold higher (versus coculture and IT treatment, P<0.05) (Figure 2A). IT pretreatment further increased the migration ability of EL4 cells when they were co-cultured with Hepa1-6 cells with in vitro cell-IRE treatment, compared to the EL4 cells without IT treatment (P<0.05) (Figure 2A). The migration ability of human HH cells showed a similar pattern in response of either with the IT pretreatment or the Hepa1-6 cells with in vitro cell-IRE (Figure 2B). Collectively, the cultured HCC cells with IRE treatment could increase T lymphocytes migration ability, specifically the T lymphocytes which were pretreated with immunotherapeutic agents in vitro. To further study the synergistic effects of IRE and IT in vivo, we established an orthotopic HCC model to evaluate the tumor microenvironments for both IRE target and off-target.

Enhanced off-target necrosis and inflammatory infiltration in mice with IRE and IT treatment

Successful establishment of the orthotopic HCC model was demonstrated by formation of



Figure 2. Representative images of cell migration capacity by a trans-well assay with crystal violet staining. The numbers of crystal violet stained cells were analyzed in all 4 treatment groups as well as the controls. A: Cell migration capacity of EL4 cells co-cultured with Hepa1-6 cells. B: Cell migration capacity of HH cells co-cultured with HepG2 cells. HPF: high power field. Scale bar = 100 μ m. *, P<0.05; **, P<0.01.

macroscopic HCC nodules (Figure 1B) which were further diagnosed in micro-sections with

H&E staining, as evident by numerous HCC cells growing in the hepatic parenchyma.



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Figure 3. Representative histological images by H&E staining. A: Successfully established HCC orthotopic model showing an abnormal hepatic architecture occupied by tumor nodules, compared to normal hepatic architecture. B: Twenty-four hours Post-IRE treatment showing massive haemorrhage and extensive tumor tissue damage as well as hepatic architecture damages. C: Necrosis and inflammatory cells infiltrated were detected in all the tissues of treatment groups (IRE, IT treatment, direct IRE with IT treatment, and IRE-off target with IT treatment). Necrotic index and index of inflammatory cells infiltrated were calculated based on the histology. LPF: low power field. Scale bar = 200 µm. Dash circle, necrotic area; Arrow, area of inflammatory infiltration. *, P<0.05.

On the H&E sections of tumor tissues, pathological changes of HCC was defined by the cytological features of cancerous cells; ranging from well to poorly differentiated; distributed in the parenchyma showing an abnormal hepatic architecture occupied by tumor nodules (Figure 3A). Twenty-four hours Post-IRE treatment, massive haemorrhage and extensive hepatic architecture damages were found in tumor tissues as well as in hepatic parenchyma (Figure **3B**). When the mice finished the treatments (Table 1), the indexes of necrosis and inflammatory infiltration were analyzed in the liver tissue of all study arms. For the IRE+IT treatment arm, both direct IRE exposure tissues (left liver lobe) and off-target tissues (right liver lobe) were analyzed. As shown in Figure 3C, index of necrosis was significantly increased in all the tissues of treatment groups (IRE, IT treatment, direct IRE with IT treatment, and IRE-off target with IT treatment) compared to the untreated control tissues (no-IRE, no-IT; P<0.05). Although IT treatment was showed an increase of necrotic index, it was about 3-fold lower compared to the groups (IRE only, direct IRE with IT treatment, IRE-off target with IT treatment). Interestingly, the necrotic index of IRE-off target tissues with IT treatment showed 2.5-fold higher than that in the IT treatment arm (P<0.05). There were no statistical significances between the groups [IRE only versus direct IRE+IT (P>0.05); IRE only versus IRE-off target+IT (P>0.05); and direct IRE+IT versus IRE-off target+IT]. The index of inflammatory infiltration significantly increased in the IRE treatment groups (IRE only, direct IRE with IT, and IRE-off target with IT treatment) compared to the IT only (P<0.05). Notably, numerous inflammatory cells infiltrated surrounding the tumor tissues from IRE-off target+IT, rendered its highest index of inflammatory infiltration compared to the other IRE groups (IRE only, direct IRE with IT). Taken together, IRE+IT induced necrosis and inflammatory infiltration not only in the direct-IRE treated tumor tissues, but also in the IRE off-target tumor tissues.

IRE+IT increases peritumoral CD8 positive cell infiltration in off-target lesions

Ipilimumab activates T cells by blocking CTLA-4, while nivolumab blocks PD-1 to turn on the immune response, allowing T cells to attack tumor cells. To better characterize the synergistic effect of IRE and immunotherapeutic modulation on the tumor microenvironment, we first performed IHC to detect CD8 positive cells in both IRE target and IRE off-target tissues. There were markedly fewer CD8 positive T cells detected in the normal liver tissues and in the untreated (UT: no-IRE and no-IT) tumor tissues (Figure 4). However, there was an increase of CD8 positive cells distributed into the tumor tissue with IT, while the IRE-induced increase of CD8 positive cells was distributed peritumorally into the tissues (Figure 4). There was about a 1-fold increase of CD8 positive cells in the animals with IRE only compared to that in the animals with IT only, with statistical significance (P<0.05). Importantly, after 4 weeks of IT following IRE, there were about 3-fold increases of CD8 positive cells in either IRE target tissues or IRE off-target tissues, compared to that in the animals with IRE treatment only (P<0.01). When compared to the animals with IT only, there were about 6-fold increases of CD8 positive cells in either IRE target or IRE off-target tissues (P<0.01). Notably, the peritumoral CD8 positive cell infiltration in the IRE off-target tissues was more than that in the IRE target tissues, even though the statistical significance was not reached (P>0.05). This result indicated that IRE+IT contributed CD8 positive cell recruitment in not only IRE target tumor tissue but also IRE off-target tissue, and this synergistic effect of IRE and IT could benefit the immunotherapeutic modulation of the tumor microenvironment for cancer therapy.

IRE+IT decreases tumor associated inflammatory infiltration in off-target lesions

In addition to the tumor cells, the tumor mass contains a complex group of myeloid cells,



Figure 4. Representative histological images of CD8 staining by IHC staining. CD8 positive cells extensively distributed into the direct-IRE tumor tissues and distributed peritumorally into the IRE off-target tumor tissues in the mice with 4 weeks of IT following IRE. HPF: high power field. Scale bar = $200 \mu m$. UT: untreated control. *, P<0.05; **, P<0.01.

which are associated with tumor progression and response to therapy [20]. In correlation with clinical outcomes being reported for human malignancies, the tumor-associated neutrophils (TANs) and tumor-associated macrophages (TAMs) have been considered to associate with disease progression [20, 21]. Cancer cells can influence the 'distant' hematopoietic compartment contributing to recruitment of TANs and TAMs to promote tumor growth [21, 22]. To evaluate the effects of IRE and IT on the tumor-associated proinflammatory infiltration, we performed CAE staining to detect neutrophils, which are derived from a common myeloid series progenitor in the bone marrow. The results indicated that the levels of neutrophils were significantly increased in the untreated tumor tissues, while IT following IRE significantly attenuated neutrophil infiltration in both IRE target tumor tissues (P<0.05) and IRE off-target tumor tissues

(P<0.001) (Figure 5A). We further performed dual immunofluorescent staining using the antibodies of anti-F4/80 and anti-CD206 to detect TAMs in tumor tissues. The results indicated that there were numerous F4/80⁺ cells as well as F4/80⁺CD206⁺ cells distributed into the untreated tumor tissues (Figure 5B). However, in the animals with 4 weeks of IT following IRE, the F4/80⁺CD206⁺ cells as well as the ratio of F4/80⁺CD206⁺ cells to F4/80⁺ cells showed significant decreases in both IRE target tumor tissues (P<0.001) and IRE off-target tumor tissues (P<0.001), compared to the untreated animals. Although there were also many F4/80 positive cells distributed into the IRE target tumor tissues, the level of F4/80 positive cells in the IRE target tumor tissues was lower than that in the tumor tissues without treatment, while most F4/80 positive cells did not showed co-expression of CD206, resulting in the lowest level of the ratio of F4/80⁺CD206⁺



Figure 5. A: Representative images of CAE staining for neutrophil detection in the IRE target tumor tissues and IRE off-target tumor tissues from the animals with 4 weeks of IT following IRE, in comparison with the tumor tissues without treatment (no-IRE, no-IT). B: Dual immunofluorescent staining using the antibodies of anti-F4/80 and anti-CD206 to detect TAMs in the IRE target tumor tissues and IRE off-target tumor tissues from the animals with 4 weeks of IT following IRE, in comparison with the tumor tissues and IRE off-target tumor tissues from the animals with 4 weeks of IT following IRE, in comparison with the tumor tissues and IRE off-target tumor tissues from the animals with 4 weeks of IT following IRE, in comparison with the tumor tissues without treatment (no-IRE, no-IT). LPF: Low power field. Scale bar = 100 μ m. *, P<0.05; ***, P<0.001.

cells to F4/80⁺ cells. Taken together, IRE and IT attenuated the neutrophils infiltration and

TAMs infiltration in both the IRE target tumor tissues and the IRE off-target tumor tissues.

IRE+IT decreased phenotypic plasticity of TAMs in off-target lesions

As reported previously, the cancer conditions could perturb the steady state of leucocyte supply resulting in aberrant myelopoiesis, which was accepted as the main resource for the TAMs development [23]. The published data indicate that myeloid cells with the phenotype of inflammatory monocytes (CD11b+ Ly6C^{hi}Ly6G⁻) in tumors have potent immunesuppressive activity, while the apparent phenotypic plasticity of TAMs can be distinguished by increased relative expression of F4/80, low-tointermediate expression of Ly6C [24]. To study whether the synergistic effect of IRE and IT could block the communication between cancer and 'distant' hematopoietic compartment for development of TAMs, We performed dual immunofluorescent staining using the antibodies of anti-CD11b and anti-Ly6C to detect the monocytic myeloid-derived suppressor cells (M-MDSCs) derived TAMs in tumor tissues. The results indicated that the CD11b⁺ cells extensively distributed into the untreated tumor tissues, while the levels of CD11b⁺ cells were significantly increased (P<0.01) in the animals with 4 weeks of IT following IRE (Figure 6). However, levels of CD11b+Ly6C+ cells were much lower with statistical significance (P< 0.001) in either IRE target tumor tissues or IRE off-target tumor tissues from the animals with 4 weeks of IT following IRE (Figure 6). To further confirm the plastic potential of TAMs from M-MDSCs, a Flow Cytometry assay was performed in the isolated peritoneal inflammatory cells from the mice with IRE+IT and untreated controls. The results indicated that there was no statistical significance for the subpopulation of F4/80⁺CD11b⁺ cells between with IRE+IT and controls, however the subpopulation of F4/80⁺CD11b⁺CD206⁺ cells in the IRE+IT was lower than that in the controls with statistical significance (P<0.05), implying that the combination of IRE and IT could block the aberrant myelopoiesis that contributed to the TAMs infiltration in both IRE target tumor tissues and IRE off-target tumor tissues (Figure 7).

Discussion

Combination immunotherapy is rapidly becoming an attractive therapeutic option for unresectable and metastatic solid organ tumors. Dual checkpoint inhibition, sequential immunotherapy, or immunotherapy in combination with other forms of therapy are all therapeutic options in practice or under investigation in multiple tumor types [25]. Recently, atezolizumab and bevacizumab combination therapy improved 12-month overall survival in advanced HCC by 12.6% and in progression free survival by 2.5 months versus sorafenib alone [26].

Local therapy, in combination with systemic treatment is redefining the landscape of advanced solid organ tumors. Talimogene laherparepvec, a herpes simplex virus-derived oncolytic therapy, when combined with systemic ipilimumab resulted in 2.9 higher odds of objective response versus ipilimumab monotherapy in metastatic melanoma [27]. Importantly, 52% of patients demonstrated objective responses in 'off-target' lesions compared to 23% on monotherapy alone. However, Pexa-vec, a vaccinia virus similarly designed to express GM-CSF, failed to demonstrate benefit versus sorafenib monotherapy in advanced HCC [7]. Though trial design may have hindered outcomes, the liver remains relatively immune guiescent and tolerant to immunologic signals. Tumor disruptive therapy, such as irreversible electroporation, may overcome immune tolerance and enhance responses in advanced HCC especially when combined with immunotherapy.

In this study, we utilized combination immunotherapy and IRE to observe the in vitro T-cell migration and immunotherapeutic responses in tumor microenvironment in the locally IREtreated and off-target lesions in a murine model of multi-focal HCC. Consistent with the enhanced in vitro T-cell migration, we observed the enhanced T-cells recruitment not only in the IRE-treated tumor but also in the off-target tumor in the animals' combination of IRE and immunotherapy. The reason for the alterations of T-cell and TAMs in the off-target tumor could be the IRE mediated immune-regulation which had been reported previously [28]. We speculate that, 1) the IRE-targeted tumor cells released tumor antigens which presented to T cells by dendritic cells/antigen presenting cells (DCs/APCs), resulting in expansion of tumorspecific T cell populations to enhance systemic anti-tumor effects for the off-target tumor; 2) the alteration of the physical peritumoral milieu by IRE might also act as a "reset" switch for eliminating the immunosuppressive factors such as TAMs and Tregs [15]. Further study is

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Figure 6. Dual immunofluorescent staining using the antibodies of anti-CD11b and anti-Ly6C to detect the M-MDSC derived TAMs in the IRE target tumor tissues and IRE off-target tumor tissues from the animals with 4 weeks of IT following IRE, in comparison with the tumor tissues without treatment (no-IRE, no-IT). LPF: low power field. Scale bar = $100 \mu m. **, P<0.001; ***, P<0.001.$



Figure 7. Flow Cytometry to detect F4/80⁺CD11b⁺ cells and F4/80⁺CD11b⁺CD206⁺ cells for potential M-MDSC derived TAMs in the isolated peritoneal inflammatory cells from the HCC mice with 4 weeks of IT following IRE and the untreated HCC controls. Ctrl, control. n.s, no statistical significance. *, P<0.05.

needed to explore the potential mechanism (s) of IRE induced alteration of tumor immune microenvironment. In addition, the combination of IRE and immunotherapy attenuated the tumor associated inflammatory infiltration and TAMs in the IRE-treated tumor as well as in the off-target tumor. Although there was no previous report for the IRE mediated attenuation of TAMs, this result was indirectly supported by a study in which an aspect of IRE mediated a switch of Th2 status back to Th1 status [29]. The anti-tumor activity of Th1 cells was well accepted, and the IRE mediated switch was accompanied by an increase of macrophage infiltration [29]. Collectively, tumor-disruptive IRE may synergistically enhance systemic effects of immunotherapeutic checkpoint blockade, but also inhibit the tumor-related aberrant myelopoiesis. Further research is required to develop mechanistic insight for clinical trial initiation.

This presents a novel tumor disruptive therapy that is well tolerated in animal models and rapidly deployable for clinical study. Further investigations are warranted to compare the efficacy of other ablative therapies such as microwave ablation to IRE. While these therapies have never been directly compared in a study to our knowledge, we suspect that the non-thermal electroporation of IRE may prove more efficacious given the preserved tissue architecture and possibility for enhanced antigen recognition over thermal tissue destruction. The findings here should support phase I clinical study.

In conclusion, the combination of irreversible electroporation and immunotherapy enhances tumor necrosis in treated and 'off-target' lesions in multi-focal hepatocellular carcinoma. This combination also enhances T cell recruitment, but attenuates the tumor associated inflammatory infiltration and TAMs, not only in the IRE-treated tumor but also in the offtarget tumor. These findings support early phase trial initiation for multi-focal hepatocellular carcinoma.

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Disclosure of conflict of interest

None.

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