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Irreversible Electroporation Combined With Dendritic Cell-based Vaccines for the Treatment of Osteosarcoma

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Abstract

Osteosarcoma is the most common primary bone malignancy, and surgical resection combined with neoadjuvant chemotherapy is the gold-standard treatment for affected patients. Although the overall survival rates for patients with osteosarcoma currently range from 60% to 70%, outcomes remain disappointing for patients with recurrent, metastatic, or unresectable disease. Irreversible electroporation (IRE) is a novel ablation technique with the potential to elicit an immune response in solid tumors. Dendritic cell (DC)-based tumor vaccines have shown promising therapeutic efficacy in preclinical studies focused on osteosarcoma; however, only limited therapeutic efficacy has been observed in clinical trials. Thus, there is considerable potential therapeutic value in developing combination osteosarcoma treatments that involve IRE and DC-based tumor vaccines. In this review, we discuss recent advances in preclinical and clinical DC-based immunotherapies, as well as potential combinations of DC-based vaccines and IRE, that may improve therapeutic outcomes for patients with osteosarcoma.

Keywords

Dendritic cells; vaccine; irreversible electroporation; osteosarcoma; combination therapies; review

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Osteosarcoma, the most common primary bone malignancy, originates from mesenchymal stem cells; it primarily affects adolescents and young adults. Recurrence and metastasis of the primary tumor are the leading causes of death in patients with osteosarcoma, and

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the most common sites of metastasis are the lungs and bones (1). Prior to the widespread use of chemotherapy, prognoses for patients with osteosarcoma were poor, with survival rates of <20% before the 1970s (2). Because of substantial advances in surgical techniques and neoadjuvant chemotherapy, the overall survival rates for patients with osteosarcoma have improved to 60%−70%. However, therapeutic outcomes remain disappointing for patients with recurrent, metastatic, or unresectable disease. The long-term survival rate is approximately 65% for patients with localized osteosarcoma, whereas it is <20% for patients with metastatic disease (3–5). There have been no groundbreaking advances over the past three decades; furthermore, resistance to existing chemotherapeutic agents is often observed among patients with recurrent or metastatic osteosarcoma. Therefore, novel osteosarcoma treatment approaches are needed to improve patient outcomes.

IRE is a relatively novel ablation technique that has been used since 2006 for the focal treatment of solid tumors (6–8). IRE generates brief high-voltage electrical pulses, which can produce a destabilizing electrical potential that causes permanent nanoscale defects in the membranes of tumor cells. The resulting persistent cell membrane permeability leads to altered intracellular homeostasis and eventual cell death (9). Thus, IRE can destroy the cell membrane and cause non-thermal cell death, leading to focal tumor ablation (10). This process leads to the exposure of many autologous tumor antigens *in situ* (11–13). It remains unclear whether IRE elicits an immune response when used in the treatment of solid tumors.

Immunotherapy has been primarily studied in the treatment of osteosarcoma and is generally regarded as an effective therapeutic method. This approach includes (but is not limited to) antibody-mediated cell surface protein targeting, tumor vaccines, oncolytic viruses, adoptive cell therapy, and checkpoint inhibitors (14–21). Tumor vaccines are among the original treatment modalities for cancer immunotherapy; these vaccines were constructed to induce antitumor responses through exposure to tumor antigens (22). Tumor vaccines can target whole cells, lysates, proteins, DNA, RNA, and peptides (23–26). Dendritic cells (DCs) comprise a rare subset of hematopoietic cells, with broad distribution in lymphoid tissues, non-lymphoid tissues, and other tissues (27). The main function of DCs is antigen presentation to T cells. Notably, DCs are the only antigen-presenting cells with the capacity to activate unsensitized naïve T cells (28). Their abilities to internalize, process, and present antigens in the context of major histocompatibility complex class I and class II molecules can be exploited in the development of cancer vaccines (29). With respect to osteosarcoma treatment, the results of DC-based vaccine therapy have considerably differed between animal experiments and clinical trials (29–31). In animal models, DC-based vaccines effectively delayed osteosarcoma progression and caused the regression of established osteosarcoma (22, 32). In two clinical trials, DC-based vaccines established by pulsing with autologous tumor cell lysates showed limited activity in patients with recurrent osteosarcoma (29, 33). Nonetheless, these studies showed that DC-based vaccines are safe and can partially activate the immune system in humans and animals (30, 34). Similarly, studies of other immunotherapies for osteosarcoma have yielded disappointing results (20, 35–38). Considering the complexity of the immune system and the disappointing results of immunotherapy alone, combination treatment may be necessary to improve the efficacy of immunotherapy for osteosarcoma (39).

This review focuses on the potential for combined IRE and DC-based vaccine therapy as a treatment for osteosarcoma. We summarize the results of preclinical studies and clinical trials, then discuss the underlying mechanisms involved in the antitumor effects of IRE and DC-based vaccines, as well as their functional interaction. We hope to provide a theoretical basis for the development of a combination treatment strategy for osteosarcoma.

Mechanistic Explanation of IRE

IRE is a new technique that involves focal ablation of pathological tissue using high-voltage, low-energy electrical pulses (40, 41). The IRE procedure comprises the placement of electrodes inside the target area, followed by the generation of a series of electrical pulses (each with a duration of several to hundreds of microseconds) (42). The pulses can create an electrical field within the tissue, which alters cell status by increasing the resting transmembrane potential. The extent of increase in the transmembrane potential is determined by the electrical pulse properties (*e.g.*, strength, duration, repetition rate, shape, and number) and the physical configuration of the electrodes used to deliver the pulses (43). Depending on the magnitude of the change in transmembrane potential, as well as the pulse duration and rate of repetition, the electrical pulses used in electroporation can have no effect, transiently increase membrane permeability, or cause cell death; thus, the electroporation procedure can be reversible or irreversible (44, 45). Spatially, for a specific set of conditions, the transmembrane potential and the extent of electroporation are dependent on the local electrical field to which each cell is exposed. Because the electrical pulses cause sudden changes in treated cells, those cells can be clearly identified in the target tissue (46).

The exact mechanism of IRE-induced cell death remains unknown, but Lee *et al.* demonstrated that IRE-induced cell death involves apoptosis, whereas thermal ablation involves coagulative necrosis (47). Apoptosis is defined as physiological programmed cell death that can be induced by various internal and external stimuli (48). Multiple molecular, genetic, and protein markers can be used to confirm apoptosis in various pathologic and oncologic processes. IRE-mediated induction of apoptotic cell death enables the utilization of immune-mediated cell death processes whereby phagocytic cells remove post-ablation debris; the activation of these processes leads to more rapid recovery and regeneration by some organs because apoptosis is a natural stage of growth and development in all cells (49).

The earliest studies of IRE used electrical currents to explore the potential for cellular destruction. Using mathematical and *in vitro* models (43), Davalos *et al.* confirmed that IRE could induce cell death without the use of thermal energy. In subsequent efforts to optimize tissue ablation *in vivo*, Edd *et al.* demonstrated that the ablation area is clearly visible after IRE; ablated and nonablated areas can be identified at the cellular level (50).

IRE-mediated Immunoregulation

In the past decade, cancer treatment has increasingly involved focal thermal therapy (Heat), cryosurgery (Cryo), and IRE (51–53). However, the inability of focal therapies to completely destroy tumors leads to local recurrence or systemic metastasis, with poor outcomes (54–

57). The combination of novel immunotherapy techniques (*i.e.*, checkpoint blockade or DC-based vaccination) with focal therapies may help to improve patient outcomes (58–64). To explore the role of focal therapy in stimulating the immune system to facilitate immunotherapy, Qi Shao *et al.* (65) designed an *in vitro* model of the T-cell response, which involved stimulation with the lysates of B16 melanoma cells that had been subjected to Heat (50°C, 30 min), Cryo (−80°C, 30 min), or IRE (1250 V/cm, 99 pulses, 50-ns pulses with 1-μz intervals). After assessment of viability using cell counting kit-8 assays, cell lysates were collected and protein release was assessed *via* bicinchoninic acid assays.

Other assessments included protein denaturation by Fourier-transform infrared spectroscopy, tyrosinase-related protein 2 antigen release by western blotting, and T-cell activation by antigen-specific cluster of differentiation (CD) T-cell proliferation (Figure 1 reproduced with permission from Int J Hyperthermia) (65). The results showed that the release of protein and antigen (*i.e.*, tyrosinase-related protein 2) was greatest in the B16 melanoma cells subjected to IRE, followed by the cells subjected to Cryo and then by the cells subjected to Heat. However, cells subjected to Cryo released more native (*i.e.*, not denatured) protein, compared with cells subjected to IRE or Heat. Importantly, IRE was substantially better than Cryo or Heat in terms of T-cell activation, and Cryo was slightly better than Heat. These findings support the establishment of protein-based metrics upon which focal therapies can be designed to stimulate the immune system; these focal therapies could be implemented in conjunction with immunotherapies to ultimately achieve improved and durable cancer treatments *in vivo.*

Al-Sakere *et al.* used a sarcoma mouse model for immunohistochemistry-based analysis of immune cell recruitment during IRE (66). Notably, they did not observe infiltration by immune cells (CD4$^+$ and CD8$^+$ T cells, macrophages, activated antigen-presenting cells, and DCs) at 72 h after ablation. They attributed these findings to vascular destruction. Conversely, Li *et al.* reported prominent immune cell infiltration in the ablation area after IRE, consistent with findings in previous studies (67–71). Li *et al.* explored whether tumor ablation by IRE could activate the immune response in a rat model of osteosarcoma. They analyzed changes in T-cell subsets, as well as soluble interleukin (IL)-2 receptor and IL-10 levels, in peripheral blood. They also used intracellular cytokine staining to analyze splenocyte-mediated production of interferon-gamma and IL-4. The results showed that tumor cells were completely ablated by direct IRE. Moreover, there were significant increases in the proportions of CD3$^+$ and CD4$^+$ cells among peripheral lymphocytes, as well as an increase in the ratio of CD4$^+$ cells to CD8$^+$ cells, at 7 days after treatment in rats that underwent IRE and rats that underwent surgical resection. The cellular response was stronger in the IRE group than in the surgical resection group. In the peripheral blood, the level of soluble IL-2 receptor significantly differed between the IRE and surgical resection groups; the level in the surgical resection group decreased over time. Additionally, the proportion of interferon-gamma-positive splenocytes significantly increased after IRE. Overall, these findings indicate that IRE can cause local tumor destruction and changes in the cellular immunity of osteosarcoma-bearing rats, providing experimental evidence to support the use of IRE in the clinical treatment of osteosarcoma. The findings by Li *et al.* and Al-Sakere *et al.* may have differed because of other types of immune cells that were not tested in these studies (*e.g.*, neutrophils and plasma cells) (72–75). Given the result of

another study, the damaged vessels caused by IRE returned to normal after three weeks (76). Another possibility is that the mice were sacrificed before the infiltration of immune cells in the study of Al-Sakere *et al.*

Other experimental studies focusing on different types of tumors have confirmed the phenomenon of IRE-mediated immunoregulation (77–86). Dai *et al.* showed that IRE significantly suppressed tumor growth in a mouse model of hepatocellular carcinoma. Treated mice remained tumor-free after injection of a secondary tumor (87). Additionally, the population of splenic interferon-gamma-positive CD8^{+} T cells significantly increased after IRE, while increasing numbers of CD8^{+} T cells and DCs infiltrated into the area around each treatment site. Furthermore, the depletion of CD8^{+} T cells by anti-CD8α blocking antibodies led to local tumor regrowth and distant metastasis after IRE, suggesting that CD8^{+} T cells are essential for IRE-mediated antitumor immunity. The above findings indicate that IRE can activate a CD8^{+} T-cell–mediated immune response to hepatocellular carcinoma. The immunosuppressive tumor microenvironment plays a key role in impairing the antitumor response, and reversal of this immunosuppression can promote ablation-induced antitumor effects. For example, significant decreases in the numbers of regulatory T cells and programmed cell death protein 1-positive lymphocytes were observed in local tumor sites and the spleen after IRE in a model of hepatocellular carcinoma, which led to the reversal of immunosuppression (88). The mechanism underlying the effects of IRE involves the induction of cell necrosis and substantial release of danger-associated molecular patterns (*e.g.*, adenosine triphosphate, high mobility group box 1, and calreticulin) that are important for CD8^{+} T-cell–mediated immunity (89, 90). The findings in recent studies suggest IRE can alleviate the immunosuppressive tumor microenvironment (79, 85, 91), presumably by reducing tumor burden and physically eliminating cells that contribute to immunosuppression (78, 92). Other factors, such as IRE-induced cell necrosis and the release of danger-associated molecular patterns, may also contribute to antitumor immunity. In addition to the findings in models of hepatocellular carcinoma, three recent studies revealed that IRE promotes CD8^{+} T-cell–mediated immunity in animal models of pancreatic cancer. Zhao *et al.* reported that IRE causes immunogenic cell death, activates DCs, and reduces stroma-induced immunosuppression. The combination of IRE and anti-programmed cell death protein 1 immune checkpoint blockade facilitated tumor infiltration by CD8^{+} T cells and prolonged survival time, while promoting the formation of long-term immune memory (91). Narayanan *et al.* demonstrated that the IRE treatment could act as an "*in situ*" vaccine, because it successfully induced prophylactic immunity in an immunocompetent murine pancreatic cancer model. Immunohistochemical staining found significant increase of CD8^{+} T cell infiltration inside the tumor after IRE. Meanwhile, the depletion of CD8^{+} T cell blocked the anti-tumor response induced by IRE. The combination of IRE, intratumoral toll-like receptor-7 agonist, and anti-programmed death-1 receptor checkpoint blockade effectively improved the therapeutic effect (93). He *et al.* described a similar long-term protective effect of IRE in terms of suppressing pancreatic cancer (79).

DC-based Immunotherapy for Osteosarcoma

DCs are potent antigen-presenting cells that can effectively induce T-cell responses (94). Multiple recent studies have shown that DCs can also activate innate immune cells with

robust antitumor activity, such as $\gamma\delta$ T cells and cytokine-induced killer cells (95–97). However, tumors attempt to reduce the availability of antigen for subsequent display by antigen-presenting cells, which results in immunosuppression and interferes with the production of an effective antitumor response (98, 99). Accordingly, DC-based vaccines have been developed to bypass this mechanism. The process of DC-based vaccine therapy can be summarized as follows. First, DCs are isolated from peripheral blood mononuclear cells and cultured until maturation. Next, DCs are exposed to a specific cocktail of tumor antigens *in vitro.* Finally, the antigen-activated DCs are infused into the patient (Figure 2) (28). These antigen-activated DCs are expected to enhance the immune response. Related preclinical studies focusing on osteosarcoma DC-based vaccines are listed in Table I (15, 24, 25, 31, 32, 100–104). These studies can be divided into three categories according to their antigen-loading protocol (105): 1) co-culture of DCs with tumor-specific peptides/proteins or tumor cell lysates; 2) transfection of DCs with tumor cell DNA, antigen-encoding RNA, or total RNA; and 3) fusion of DCs with inactivated tumor cells. Several preclinical trials focusing on the treatment of osteosarcoma with DC-based vaccines have yielded encouraging results. Using a rat model of osteosarcoma, Chauvin *et al.* demonstrated that a distinct subset of splenic CD4— rat DCs, known as killer DCs, induces rapid and caspase-independent apoptosis-like cell death in a large number of tumor cells *in vitro.* They confirmed killer DCs could kill and engulf tumor cells while maintaining the ability to efficiently cross-present tumor cell-derived antigens *in vivo*; thus, the killer DCs induced an antitumor adaptive immune response (22). Yu *et al.* investigated the therapeutic efficacy of osteosarcoma DC-based vaccines generated by the fusion of DCs with whole tumor cells or transduction with tumor total RNA. Most immunized tumor-free rats exhibited partial or complete protection from tumor challenge. Additionally, vaccination induced tumor inhibition in tumor-bearing rats (24, 32). Similarly, osteosarcoma immunotherapy using a DC-fused tumor vaccine successfully stimulated T-cell proliferation and induced tumor cytotoxic activity in cytotoxic T cells of Wistar rats and Sprague–Dawley rats (31). However, clinical trials of DC-based vaccines have generally shown weak effects on osteosarcoma (29, 30, 33). In a phase I clinical trial, 12 patients with recurrent osteosarcoma were treated with an autologous DC-based vaccine, which had matured in the presence of autologous tumor lysate and keyhole limpet hemocyanin. Feasibility and safety were evaluated, along with the tumor-specific immune response (*i.e.*, levels of interferon-gamma, IL-2, and granzyme B). The results showed that DC-based vaccines are safe and feasible for patients with recurrent osteosarcoma. However, only 2 of the 12 vaccinated patients had a robust antitumor response, and there was no evidence of clinical benefit. The findings suggest that monocyte-derived DCs from many patients with osteosarcoma are non-functional or inhibitory (29). There are three explanations for the lack of clinical benefit in these patients. First, these patients may have comparatively few and generally low-quality immune effector cells. Patients with osteosarcoma usually receive a complete course of prophase chemotherapy, which can damage innate and adaptive immune effector cells; this damage limits their availability and effectiveness in terms of responding to increased antigen presentation. Second, the migration of effector cells to the tumor site is ineffective, potentially because of reduced chemokine expression. Third, other powerful immunosuppressive mechanisms (*e.g.*, immune checkpoints) may influence immune cell activity (106). Therefore – when combined with the administration of DC-based vaccines

– measures that increase the ratio of active effector cells to tumor target cells, enhance the infiltration of effector cells, or remodel the tumor microenvironment may help to enhance antigen presentation, immune response, and clinical efficacy.

Combination of IRE and DC-based Vaccines as Treatment for Osteosarcoma

Although DCs have an important role in the immunosurveillance of osteosarcoma, the overall efficacy of DC-based therapeutic strategies alone is poor. The results of previous research suggest that IRE has considerable potential for use in the treatment of osteosarcoma (67, 107). Additionally, IRE causes the formation of nanopores, which results in a robust release of immunostimulatory cytokines. There is evidence that IRE can overcome immunosuppression by modifying the tumor microenvironment, thereby mediating antitumor responses (66, 108, 109). IRE and DC-based vaccines are expected to have synergistic effects. A recent preclinical study showed that IRE could overcome tumor-associated immunosuppression in a mouse model of pancreatic cancer, thereby improving the efficacy of DC-based vaccines (110). In that study, IRE combined with DC-based vaccines induced immunogenic cell death and the reduction of immunosuppressive components in the pancreatic ductal adenocarcinoma tumor microenvironment; the effects also included increased infiltration of $CD8^+$ T cells and granzyme B^+ cells into the tumors. The combination of IRE and DC-based vaccines significantly prolonged the overall survival of immunocompetent tumor-bearing mice. Both osteosarcoma and pancreatic ductal adenocarcinoma tumors are difficult for $CD8^+$ T cells to infiltrate; thus, they are insensitive to single-agent immunotherapy. Considering the improved efficacy of combined IRE and DC-based vaccine therapy in the management of pancreatic ductal adenocarcinoma, we hypothesized that this combination therapy would be useful in the treatment of osteosarcoma; the findings of our pilot study supported this hypothesis (Figure 3).

The main strength of this article is that we have proposed a potential combination therapy as well as its theoretical basis for the treatment of osteosarcoma, and it may bring a breakthrough in the treatment of such disease. Meanwhile, there may be a potential limitation in this study. Until now, there have been relatively few research papers focused on related fields, which may potentially affect the theoretical basis of this combination therapy. However, we have analyzed the related papers as much as possible in this study.

Conclusion

Recent preclinical studies have shown that IRE can alleviate the immunosuppressive tumor microenvironment produced by some solid tumors, suggesting that it may be useful in tumor immunotherapy. Research in a model of pancreatic ductal adenocarcinoma indicates that IRE has the potential for synergistic effects when administered in conjunction with immunotherapies, such as DC-based vaccines. Thus, we suspect that a similar synergistic effect can be achieved in the context of osteosarcoma. Additional studies focused on the mechanisms and efficacies of IRE and DC-based vaccine combination therapy for osteosarcoma may support further progress in the treatment of osteosarcoma.

Acknowledgements

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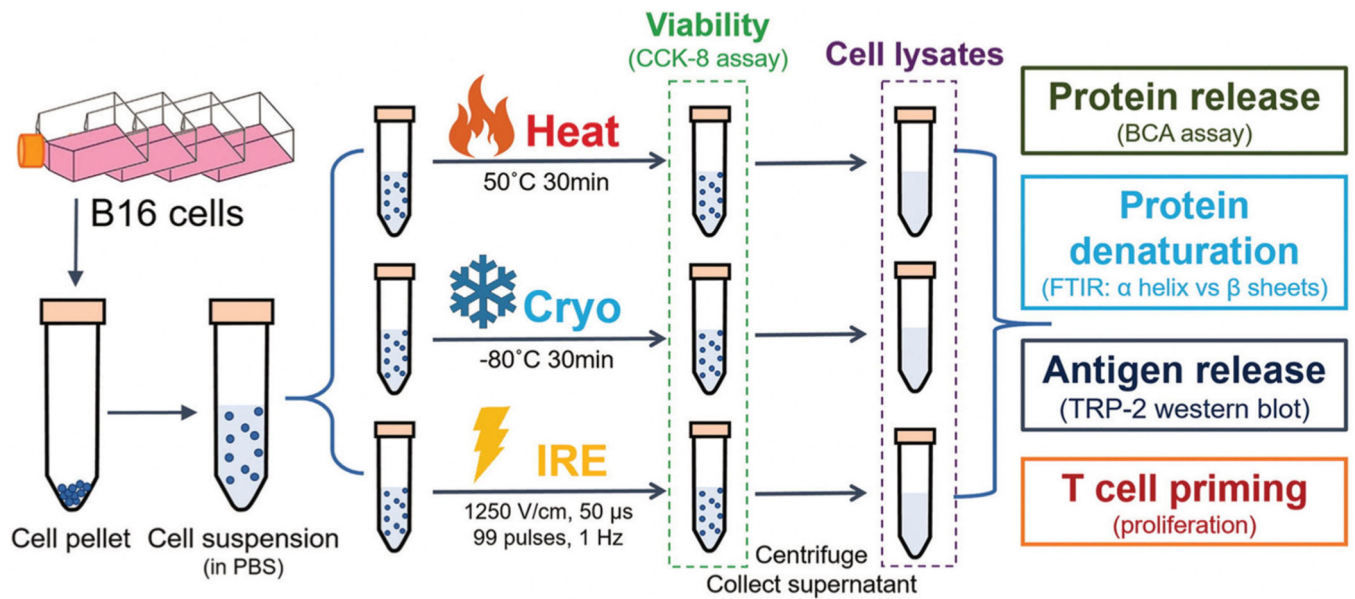
Figure 1.
Schematic diagram of in vitro cell treatments and assessments. B16 cell suspensions were generated and treated with heat, freezing, or IRE. Cell viabilities were measured immediately after treatment. The lysates of treated cells were collected and subjected to assessments of protein release, protein denaturation, antigen release, and T-cell stimulation. Adapted from Shao et al. (65), Copyright 2019 by Taylor & Francis Group, LLC. BCA, Bicinchoninic acid; CCK-8, cell counting kit-8; FTIR, Fourier-transform infrared; IRE, irreversible electroporation; PBS, phosphate-buffered saline; TRP-2, tyrosinase-related protein 2.
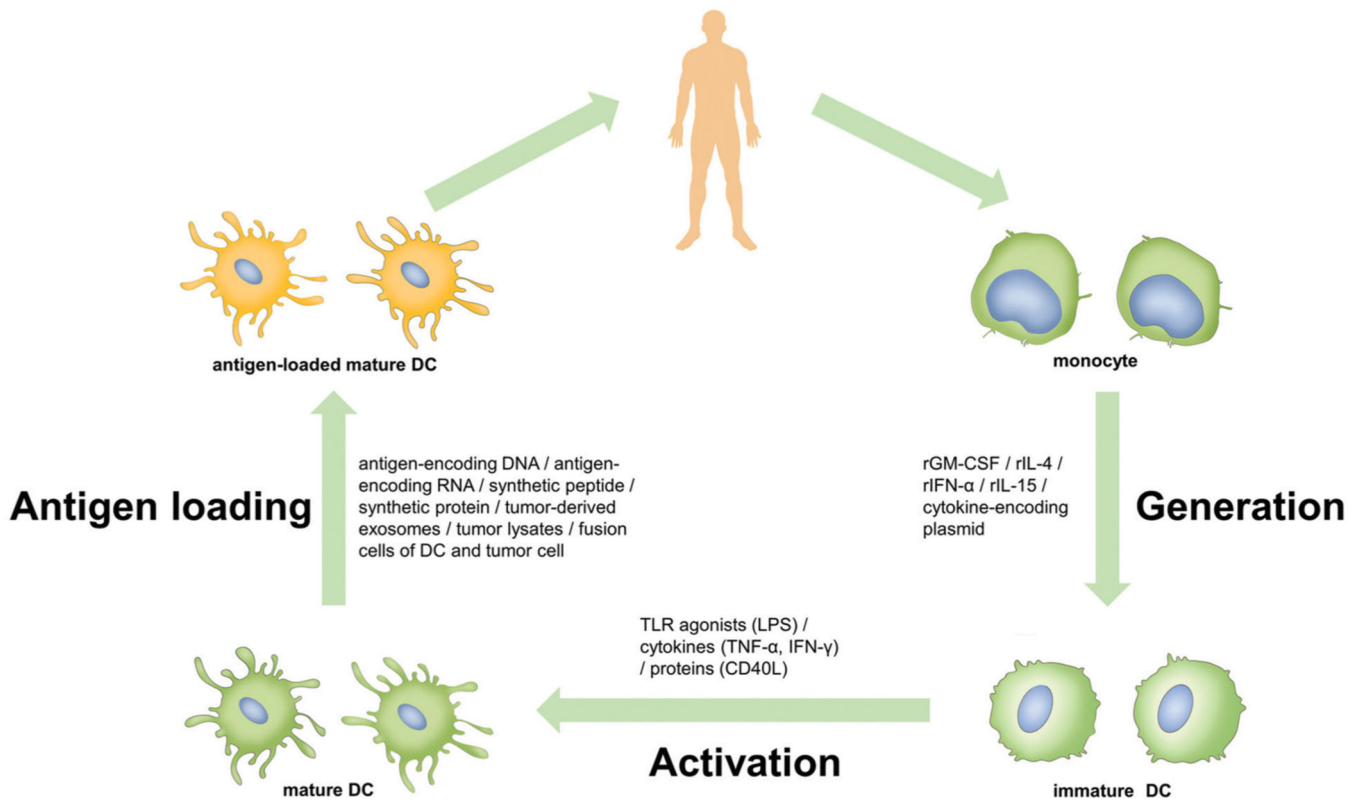
Figure 2.
Strategy for vaccination with monocyte-derived DCs. 1) Monocytes are obtained from the patient's peripheral blood and differentiated into DCs. This differentiation can be achieved by using different recombinant cytokines (most commonly rGM-CSF + rIL-4) or by transfecting monocytes with plasmids that encode the necessary cytokines. 2) After DCs have been differentiated, they can be activated using various stimuli, generally associated with tissue damage, inflammation, or pathogen presence. 3) Finally, DCs are loaded with selected or total tumor antigens, then infused into the patient, where they are expected to elicit a tumor-specific adaptive immune response that will eradicate the tumor. CD40L, Cluster of differentiation 40 ligand; DC, dendritic cell; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; rGM-CSF, recombinant granulocyte-macrophage colony-stimulating factor; rIFN-α, recombinant interferon-alpha; rIL-4, recombinant interleukin-4; rIL-15, recombinant interleukin-15; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha.
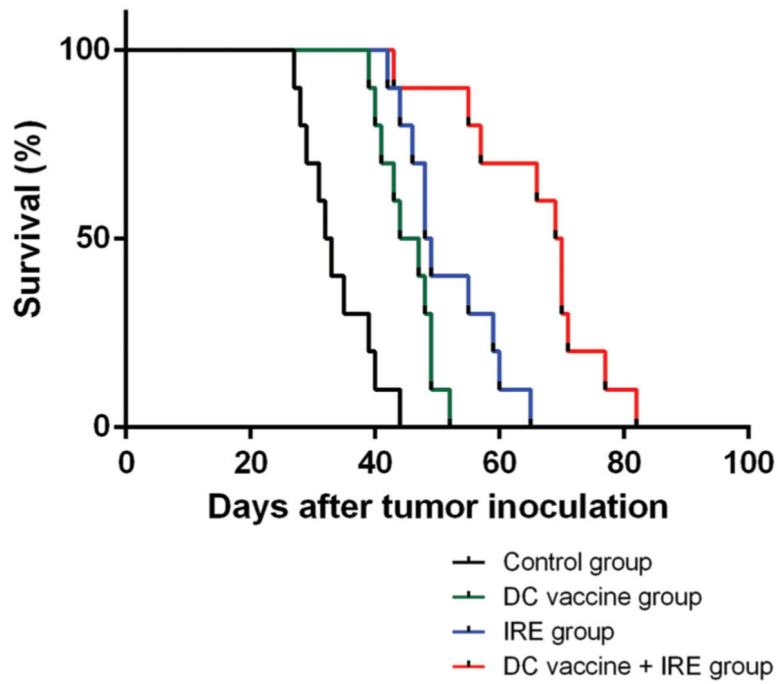
Figure 3.
Survival of osteosarcoma mice after IRE and/or DC-based vaccine treatment. Kaplan-Meier survival analysis of tumor-bearing mice treated with control (n=10), IRE (n=10), DC-based vaccine (n=10), or IRE + DC-based vaccine (n=10). p<0.0001 using the log-rank test. DC, Dendritic cell; IRE, irreversible electroporation.

Table I.

The characteristics of DC vaccine treatment in preclinical in vivo studies of osteosarcoma.

Species and tumor model	Vaccine preparation	Ancillary therapy	Route of vaccine administration	Injection site	Time of injection	Treatment effect	The changes in survival of tumor bearing animals	Reference
Rat subcutaneous OS	Allogeneic and autologous DC fused with OS cell	None	Intradermal injection	The groin region	4, 8, and 16 days after the injection of OS cells	Significantly higher cytotoxicity; tumor atrophy or disappearance	Longer survival times	(31)
Mouse subcutaneous OS	Autologous DC loaded with OS cell lysate	Anti-GITR antibody	Intradermal injection	The gluteal region	Twice a week	Higher CD8+ T lymphocytes infiltration and lower activity of tumor-infiltrating Tregs in the tumor; higher IFN-γ levels and lower IL-10 levels in serum; inhibition of primary tumor	Survival was significantly prolonged	(100)
Mouse subcutaneous OS	Autologous DC loaded with OS cell lysate	Cryotherapy	NA	The gluteal region	Twice a week	Greater serum IFN-γ level; lower serum IL-4 level; decrease in metastatic lesions; increase in the number of CD8+ T lymphocytes in the pulmonary metastatic lesion	NA	(15)
Rat subcutaneous OS	Allogeneic and autologous DC fused with OS cell	None	Intradermal injection	The groin region	The pretreatment model: day 0 and day 14; the therapeutic model: days 3,7 and 14	The pretreatment model: 70% of the rats immunized with electrofused cells rejected tumor challenge, remained tumor-free, and developed a long-term memory response	The therapeutic model: 60% long term survivors when a higher dose of the vaccine was used	(24)
Mouse lung metastatic OS	Autologous DC loaded with OS cell lysate; Autologous DC pulsed with irradiated whole OS cell	None	Subcutaneous injection	The inguinal region	Twice a week	Lower number and area of metastatic lesions; fewer large metastatic areas (>0.1 mm^2)	Longer median survival	(101)
Mouse subcutaneous OS	Autologous DC loaded with OS cell lysate	Anti-TGF-β antibody	Subcutaneous injection	The gluteal region	Twice a week	Greater number of CD8+ T lymphocytes per unit area in metastatic tumor lesions; smaller volume of the metastatic lesion; higher serum IFN-7 levels; lower serum IL-10 levels	NA	(102)
Rat orthotopic OS (femur)	Autologous DC transfected with total OS mRNA	None	Intradermal injection	The groin region	The pretreatment model: days 0 and 14; the therapeutic model: days 3,7, and 14	Effective in inducing cytotoxicity of UMR106 tumor cells; the pretreatment model: 70% of the rats rejected tumor challenge and remained tumor-free	The therapeutic model: 70% survivors were obtained with a higher dose of the vaccine	(25)
Rat orthotopic OS	Autologous DC transfected with total OS mRNA	None	Intradermal injection	The groin region	The pretreatment model: days 0 and 14;	The pretreatment model: 80% of the rats rejected tumor challenge and remained tumor-free	The therapeutic model: 70% long-term survivors	(32)

Species and tumor model	Vaccine preparation	Ancillary therapy	Route of vaccine administration	Injection site	Time of injection	Treatment effect	The changes in survival of tumor bearing animals	Reference
					the therapeutic model: days 3,7, and 14		were obtained with a higher dose of the vaccine	
Mouse subcutaneous OS	Autologous DC loaded with OS cell lysate	Anti-CTLA-4 antibody	Subcutaneous injection	The gluteal region	Twice a week	Higher number of CD8$^+$ T lymphocytes and lower number of Tregs inside the tumor; lower volume of the lung metastatic lesion; reduction of Tregs in the spleen; higher serum IFN-γ levels and lower serum IL-10 levels	Longer survival time	(103)
Mouse subcutaneous/ metastatic OS	Autologous DC loaded with OS cell lysate	Anti-CTLA-4 antibody/anti-PD-1 antibody	Intratumoral/ intravenous injection	The abdomen region/vein	One or two times 4–7 days following tumor implantation; metastasis assays: approximately 30 days prior to metastasis challenge	Inhibition of OS growth; inducing long-lasting protection against OS; increasing of IFN-γ$^+$ CD8$^+$ T cell and Th1 infiltration into OS tumors; lower lung weights; lower total tumor and necrotic areas in lung	Longer survival time	(104)

IRE: Irreversible electroporation; OS: osteosarcoma; DC: dendritic cell; CTLs: cytotoxic T lymphocytes; GITR: glucocorticoid-induced tumor necrosis factor receptor; NA: not available; Tregs: regulatory T cells; Th1: T helper 1; IFN-γ: interferon-γ; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; TGF-β: transforming growth factor-β; IL-4: interleukin-4; IL-10: interleukin-10; PD-1: programmed cell death protein 1.