Chapter

In Situ Vaccine: Breaking the Traditional Vaccine Paradigm

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Abstract

In the pursuit of optimal anti-tumor immune effects, both "passive" and "active" immunotherapies have made significant progress recently. In situ vaccines offer a promising solution by using intratumoral administration of immunomodulators or other local treatments, to scientifically combine active and passive immunotherapies. It forms a repetitive cycle of immune initiation-immune effect-tumor cell death-antigen release, leading to immune re-initiation-immune re-effect. This cycle maximizes the anti-tumor immune effect. In this chapter, we highlight the specific strategies and promising preclinical results of in situ vaccine, along with ongoing clinical trials. We also discuss the advantages, challenges, and perspectives of this novel approach. Overall, in situ vaccine shows great promise in tumor inhibition and could be a valuable addition to the cancer immunotherapy armamentarium.

Keywords: in situ vaccine, tumor, immune adjuvant, probiotic, biomaterial

1. Introduction

In the past decade, "passive" immunotherapies such as adoptive cell therapy and genetically engineered T cells, and "active" immunotherapies such as cytokines, tumor vaccines and immune checkpoint inhibitors, have reshaped the landscape of cancer treatment. Among them, therapeutic cancer vaccines aimed at modulating antigen-presenting cells, such as dendritic cells (DCs), and subsequent T cell activation processes, have been the first batch of cancer immunotherapies approved by the FDA. However, despite demonstrating good safety and the ability to elicit antigen-specific humoral cellular responses, the therapeutic efficacy of cancer vaccines is limited, particularly for immunologically low solid tumors. One key challenge lies in the identification of tumor-specific antigens, which involves a lengthy process of tumor cell isolation, DNA/RNA extraction, sequencing, mutation analysis, epitope prediction, peptide synthesis, and antigen screening. In order to address these issues, in situ cancer vaccines have been actively researched to generate endogenous antigens directly from the tumor and induce effective cytotoxic T lymphocyte (CTL) responses.

In situ vaccines have been reintroduced as a new concept based on old methods, allowing the tumor itself to become a vaccine and stimulate a systemic anti-tumor

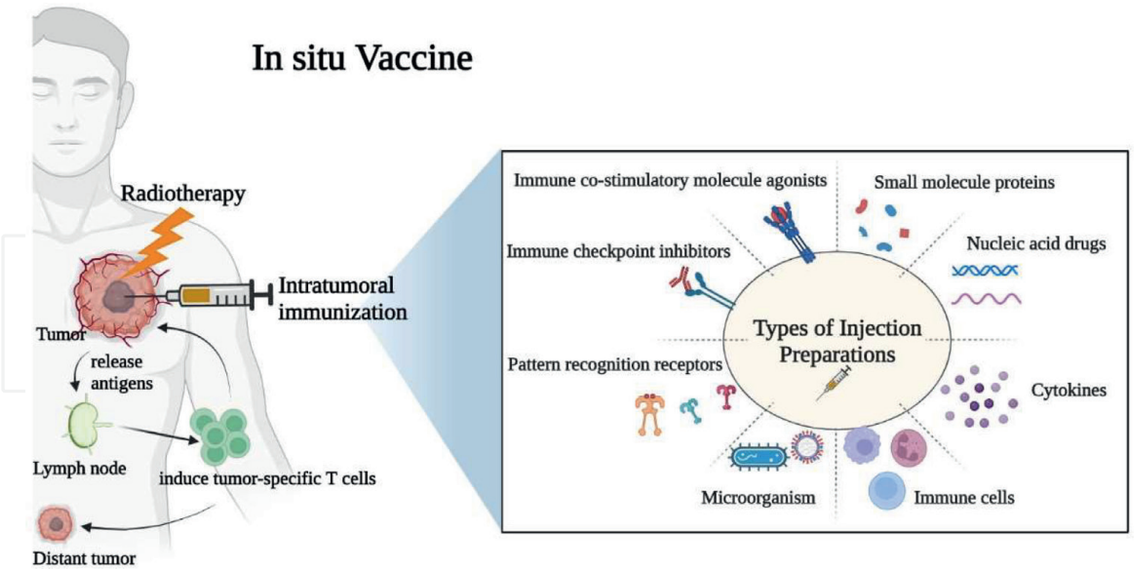

Figure 1.
Specific strategies and immune effects of the in situ vaccine.

immune response. Injecting various biological and chemical agents directly into tumors as a method of cancer treatment has a long history. In the late 19th century, surgeon COLEY pioneered the intratumoral injection of live bacteria and bacterial toxin therapy, observing some therapeutic effects in certain cancer patients [1]. These innovative ideas have since given rise to a new approach to in situ immunotherapy, which has now been successfully applied in the treatment of superficial urothelial bladder cancer.

There are two main approaches to achieving in situ tumor vaccines: radiotherapy and intratumoral immunization (**Figure 1**). For an ideal in situ vaccine, key steps in its establishment include inducing immunogenic cell death (ICD), exposing tumor-reactive antigens to initiate an immune response, activating antigen-presenting cells (APCs) to achieve large-scale antigen presentation, activating T cells, inducing strong and sustained cytotoxic T lymphocyte responses, and ultimately triggering an anti-tumor immune effect [2, 3].

In this chapter, we highlight the specific strategies and promising preclinical results of in situ vaccine, along with ongoing clinical trials. We also discuss the advantages, challenges, and perspectives of this novel approach. Overall, in situ vaccine shows great promise in tumor inhibition and could be a valuable addition to the cancer immunotherapy armamentarium.

2. Specific strategies of in situ vaccines

In situ vaccines themselves are an immunotherapy strategy aimed at achieving immune initiation and immune response, forming a good benign cycle of anti-tumor immunity. Its key factors include triggering antigen release, activating antigen presentation, activating immune effector cells, changing immune negative regulation, inhibiting immune tolerance in the tumor microenvironment, and so on. Therefore, when selecting adjuvants for intra-tumor use, it is necessary to fully consider the above key factors and formulate suitable in situ vaccine strategies.

2.1 Radiotherapy

Radiotherapy, as a local tumor treatment method, can transform tumors into in situ vaccines, inducing systemic anti-tumor immunity. Radiation therapy can induce ICD, which is the initial step in establishing in situ vaccines [4]. During this process, the "ectopic" expression of calreticulin on the cell membrane serves as an "eat me" signal, while the release of damage-associated molecular patterns (DAMPs) such as HMGB1 promotes the activation, maturation, and migration of DCs to tumor-draining lymph nodes [5, 6]. Radiation therapy can also upregulate the expression of MHC class I molecules, pro-inflammatory cytokines, chemokines, death receptors, and NKG2D ligands [4, 5, 7]. In addition, radiation therapy can cause a "distant effect" [6], which means that tumors in untreated areas may also experience regression, but the remote effect caused by radiation therapy alone is very rare. This indicates that radiation therapy alone is not enough to initiate systemic anti-tumor immunity [3], and combining radiation therapy with other immune therapies can produce synergistic effects and enhance anti-tumor immunity [8], better exerting its in situ vaccine effect.

2.2 Intratumoral immunization

Intratumoral immunotherapy directly injects local immunotherapy into the tumor to initiate local and systemic anti-tumor immunity. Compared with systemic immunotherapy, in situ immunization has the following characteristics:

1. Reduced systemic exposure to drugs and alleviated adverse reactions: immunization injections into the tumor can selectively enrich immunotherapeutic drugs within the tumor, achieving a higher initial concentration in local tissues and subsequently entering the systemic circulation. This slow local absorption not only solves the problem of insufficient drug penetration in the lesion area but also reduces the drug dosage and alleviates the immunological adverse reactions caused by systemic exposure while ensuring efficacy. Moreover, direct administration to the tumor or lymph nodes can also increase drug accumulation in the tumor microenvironment and its draining lymph nodes.

2. Enhanced tumor immunogenicity: Immunotherapy drugs injected into the tumor can cause damage or death to tumor cells, releasing specific antigens, activating local antigen presentation and improving the activation and infiltration of effector T cells in the lesion area, thereby triggering effective and persistent systemic anti-tumor immune responses.

3. The release of multi-epitope tumor antigens caused by in situ immunotherapy can help overcome the limitations of tumor heterogeneity and patient leukocyte antigen heterogeneity on tumor immunotherapy. Multiple injections at different sites in the same patient can also enhance the efficiency of multi-clone responses between common antigens of tumor cells.

4. Higher clinical translation value: In situ immunization technology avoids personalized antigen detection, prediction, and antigen peptide synthesis steps, making it more convenient to apply. In addition, the advantages of low toxicity and high biological utilization of the administration method of in situ immunothera-

py provide greater space for its combination with existing treatment methods to exert a synergistic effect.

There are many types of in situ immunotherapy injection preparations (**Table 1**). According to the classification of mechanism of action, they can be divided into: pattern recognition receptor (PRR) agonists, immune checkpoint inhibitors (ICIs), ICD inducers, tumor antigens, cytokines, etc. According to the type of preparation, they can be divided into: pathogens (bacteria, viruses), cells, nucleic acids, proteins (antibodies, small molecule proteins), etc.

2.2.1 Pattern recognition receptors

PRRs are core molecules that initiate and maintain innate immunity, primarily including the Toll-like receptor (TLR) family and the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) system. PRR agonists can stimulate $CD8^+$ T cell responses, induce interferon gamma production by $CD4^+$ T cells, and induce or enhance local inflammation and immunity by mimicking the process of immune and non-immune cell recognition of pathogen-associated molecular patterns (PAMPs). Currently, PRR agonists are mostly used as immune adjuvants in combination with other drugs to exert synergistic anti-tumor effects. The most extensively studied PRR agonists are TLR agonists, including TLR9, TLR4, TLR3, TLR7/TLR8, etc., and many preclinical and clinical studies have been conducted on their use for intratumoral injection.

TLR9 is mainly present in immune cells such as myeloid cells, B cells, and plasmacytoid DCs, and can recognize continuous CpG dinucleotide sequences in unmethylated double-stranded DNA (dsDNA) in bacteria or viruses. Multiple preclinical studies have explored the use of TLR9 agonists as monotherapy or in combination with chemotherapy, radiotherapy, and immunotherapy for systemic or local treatment, confirming their synergistic effects with other therapies, inhibiting mouse tumor growth, and enhancing anti-tumor activity [9]. Clinical trials of TLR9 agonists as monotherapy have shown that their effectiveness in improving immune suppression in patients needs to be improved, and combination therapy is expected to have greater potential. Currently, research on TLR9 combination therapy has been reported in the directions of combination with chemotherapy, radiotherapy, and immunotherapy. In a clinical trial of 22 patients with advanced melanoma (NCT02521870), the combination of SD-101 (a TLR9 agonist) injected into the tumor and systemic pembrolizumab (Pembrolizumab) treatment achieved an ORR rate of 40% and a DCR rate of 64%. Regarding safety, local immune injection mainly caused reactions at the injection site and transient mild to moderate flu-like symptoms. CMP-001 is also a TLR9 agonist that can activate local plasmacytoid dendritic cells in the tumor, promote antigen presentation, induce type I interferon secretion, and systemic anti-tumor T-cell responses [3]. The results of a recent phase Ib clinical trial show that the combination of CMP-001 and pembrolizumab can reverse resistance to PD-1 monoclonal antibodies and induce a lasting therapeutic response in late-stage melanoma (NCT03084640).

TLR4 is primarily expressed on the cell surface of dendritic cells, monocytes, macrophages, T cells, B cells, and some non-immune cells. It can recognize bacterial lipopolysaccharides (LPS). G100 (a TLR4 agonist), as a synthetic LPS analog, can induce local or systemic anti-tumor immune response in preclinical models. Previous clinical trials in Merkel cell carcinoma patients have confirmed its safety

Classification	Injectant	Combination Therapy	Cancer	Phase	NCT number
TLR9 agonist	SD-101	Pembrolizumab	Melanoma	1/2	NCT02521870
TLR9 agonist	CMP-001	Pembrolizumab	Melanoma	1b	NCT03084640
TLR4 agonist	G100	Pembrolizumab/Rituximab	Lymphoma	1/2	NCT02501473
TLR3 agonist	Flt3L + Poly-ICLC	Radiotherapy	Lymphoma	1/2	NCT01976585
STING agonist	MIW815	PDR001	Solid Tumor +Lymphoma	1	NCT03172936
STING agonist	MK-1454	Pembrolizumab	Solid Tumor +Lymphoma	1	NCT03010176
STING agonist	KL340399	—	Solid Tumor	1	NCT05549804
ICIs	Ipilimumab	Nivolumab	Glioblastoma	1	NCT03233152
ICIs	Ipilimumab	Nivolumab	Melanoma	1/2	NCT02857569
ICIs	Nivolumab	—	Kaposi Sarcoma	1	NCT03316274
ICIs	TTI-621	—	Solid Tumor	1	NCT02890368
CD40 agonist	ABBV-927		Solid Tumor	1	NCT02988960
CD40 agonist	APX005M	Pembrolizumab	Melanoma	1/2	NCT02706353
Small molecule protein	LL37	—	Melanoma	1/2	NCT02225366
Cytokine	IL-2 + Ipilimumab	—	Melanoma	1	NCT01672450
Cytokine	NKTR214 + nivolumab	—	Solid Tumor	1/2	NCT02983045
Cytokine	IL-12 plasmid	—	Solid Tumor +Lymphoma	2	NCT01502293 NCT01579318 NCT02345330 NCT 01440816
Cytokine	L19-TNF + L19-IL2		Solid Tumor	2	NCT02076633 NCT05329792 NCT04362722
T cell	CAR-T		Glioblastoma	1	NCT03389230 NCT04077866 NCT04385173 NCT03932565

Classification	Injectant	Combination Therapy	Cancer	Phase	NCT number
NK cell	NK cells		Glioblastoma	1	NCT04254419
Bacteria	*Clostridium novyi*-NT spores		Solid Tumor	1	NCT01924689
Bacteria	*C. novyi*-NT spores	Pembrolizumab	Solid Tumor	1	NCT03435952
Bacteria	SYNB1891	Atezolizumab	Solid Tumor +Lymphoma	1	NCT04167137

Table 1.
Clinical trials related to in situ vaccines.

and efficacy [10]. In addition, a recent phase I/II clinical trial (NCT02501473) on the treatment of follicular non-Hodgkin's lymphoma with G100 combined with low-dose pembrolizumab or rituximab has achieved favorable results. In the G100 20 μg group (n = 18), the overall response rate was 33.3%, with 72.2% of patients showing distant tumor regression. This result provides important reference for the use of TLR4 agonists as vaccine adjuvants in combination with other therapies.

TLR3 mainly expressed on the surface of dendritic cells, fibroblasts, epithelial cells, and tumor cells, is capable of recognizing viral nucleic acids (dsRNA, ssRNA, and ssDNA containing unmethylated CpG motifs). Polyinosinic-polycytidylic acid (polyI:C) is a dsRNA mimetic that can induce blood monocytes to produce IFNα/β and enhance natural killer cell activity in preclinical studies. It has been used as an adjuvant for cancer vaccines to enhance their anti-tumor activity. In 2019, a clinical trial (NCT01976585) using intratumoral injection of FMS-like tyrosine kinase 3 ligand (Flt3L) and Poly-ICLC (TLR3 agonist) in combination with low-dose radiotherapy for the treatment of indolent non-Hodgkin lymphoma showed that 8 out of 11 treated patients achieved complete or partial response, indicating the potential application of this treatment modality.

TLR7 and TLR8 are intracellular receptors that can recognize single-stranded RNA (ssRNA) with viral features. Their agonists can activate immune cells such as DC, monocytes, macrophages, fibroblasts, and human keratinocytes, accompanied by the secretion of pro-inflammatory cytokines and chemokines, thereby altering the tumor microenvironment and promoting effective anti-tumor immune response [11]. Imiquimod is a TLR7 agonist, and its emulsion has been used topically in some superficial tumors, such as basal cell carcinoma of the skin, in clinical applications. In preclinical studies, the combination of TLR7 agonist and OX40 agonist antibody for intratumoral injection showed significantly better efficacy than single-agent treatment, leading to regression of the injection site and distant tumors in mice [12].

The cGAS-STING pathway plays a complex regulatory role in the occurrence and development of tumors. Key factors that induce cGAS-STING pathway activation include pathogen infection, autologous DNA damage, and tumor DNA. DNA receptor cyclic GMP-AMP synthase (cGAS) is activated when it binds to cytoplasmic DNA, catalyzing the synthesis of cyclic GMP-AMP (cGAMP) and binding to downstream stimulator of interferon genes (STING), activating the NF-kB and IRF3 transcription pathways, inducing the secretion of type I interferon, and participating in innate immune responses. Therefore, the activation of the cGAS-STING pathway plays an important role in promoting cancer cell senescence, inducing cancer cell apoptosis, and increasing the cytotoxicity of T cells and natural killer cells, and its activators are worth exploring in cancer treatment. Previous preclinical results have shown that cGAS-STING pathway activation not only induces cytokine production and activates T cells targeted to tumors, but also enhances tumor cell sensitivity to radiotherapy through its activated immune system [13]. In recent years, clinical results of STING agonists such as MIW815 (ADU-S100) and MK-1454 injected into tumors as vaccines have not been ideal, mostly due to lack of observed efficacy and were terminated (such as NCT03172936, NCT03010176), and new STING agonists are also under development. For example, Mn^{2+} is an effective innate immune stimulant that can induce type I IFN and cytokine production without infection. As a new type of STING agonist, it can significantly enhance cGAS sensitivity to double-stranded DNA (dsDNA) and its enzymatic activity, allowing cGAS to produce secondary messenger cGAMP in the presence of low concentrations of dsDNA. In addition, Mn^{2+} can enhance STING activity by increasing cGAMP-STING binding affinity, making it a

potentially ideal component of an in situ vaccine in the future. In addition, the new generation of small molecule STING agonist KL340399 has been approved by the National Medical Products Administration (NMPA) of China and is undergoing clinical trials for the treatment of advanced solid tumors through intratumoral injection (NCT05549804).

2.2.2 Immune checkpoint inhibitors

ICIs work by relieving the immunosuppressive state of T cells to effectively activate the immune system and generate anti-tumor immune responses. Compared to systemic administration, intra-tumor injection of ICIs can better target immune cells in the local tumor microenvironment (TME) and induce systemic immune responses. This increases the therapeutic index while limiting systemic exposure and immune-related adverse events (irAEs). Studies have found that intra-tumoral immunotherapy can generate innate immune responses within the TME to address a large proportion of patients who do not benefit from ICIs and enhance infiltration of local T cells in the tumor. Intra-tumoral injection of ICIs also improves safety and efficacy when combined with other immunotherapies. In a phase I clinical trial (NCT03233152) using intra-tumoral injection of CTLA-4 inhibitor ipilimumab in combination with systemic PD-1 inhibitor nivolumab to treat recurrent glioblastoma patients, the most common grade 1–2 adverse events (AEs) were fatigue (63%), pruritus (26%), rash (15%), hypothyroidism (7%), and nodular reaction (4%), and no grade 3 or higher AEs related to the study treatment were observed. The median overall survival (OS) was 38 weeks (95% CI: 27–49). Many clinical trials investigating ipilimumab are currently ongoing, including the use of ipilimumab in combination with systemic nivolumab in patients with metastatic melanoma (NCT02857569) and the use of intratumoral nivolumab in patients with sarcoma (NCT03316274).

CD47 is a widely expressed cell receptor that interacts with its ligands, including thrombospondin-1 (TSP-1), signal regulatory protein alpha (SIRPα), integrins, and SH2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1), to regulate macrophage phagocytosis, neutrophil migration, and activation of dendritic cells, T cells, and B cells. Blocking CD47 inhibits the anti-phagocytic signal and induces macrophages to phagocytose tumor cells [14]. CD47 blockade also triggers anti-tumor T cell responses by activating antigen-presenting cells (APCs) or inhibiting the interaction between CD47 on tumor cells and thrombospondin-1 on T cells [15]. TTI-621 is a SIRPα fusion protein that targets CD47 and blocks it by inducing the SIRPα-Fc receptor. In a phase I clinical trial (NCT02890368) of intratumoral injection of TTI-621 for patients with relapsed or refractory fungal granulomas or Sézary syndrome, there was no dose-limiting toxicity (DLT) observed, and the most common adverse events were chills (29%), injection site pain (26%), and fatigue (23%). No grade 3 or higher serious adverse events were reported, indicating good tolerability and systemic and regional immunotherapeutic effects.

2.2.3 Immune co-stimulatory molecule agonists

In immune responses, co-stimulatory signals are essential for the activation of T and B lymphocytes. Co-stimulatory molecules are the molecules involved in the transmission of co-stimulatory signals and are currently the focus of much research on intratumoral immunotherapy.

OX40 is a humanized IgG1 agonist monoclonal antibody and a member of the tumor necrosis factor (TNF) receptor superfamily, highly expressed in activated immune cells. Its ligand OX40L can specifically bind to OX40, and the interaction between the two provides important co-stimulatory signals for the activation of T and B cells. The OX40/OX40L signal can initiate downstream signaling pathways through PI3K-PKB/AKT, Nuclear factor of activated T cells (NFAT), and NF-κB, etc. The sustained activation of these pathways by the OX40/OX40L signal can induce the upregulation of some anti-apoptotic B-cell lymphoma-2 (bcl-2) family members and regulatory molecules that control cell division, thereby mediating the activation and proliferation of cytokines and T cells and inducing a long-term T cell response [16]. Studies have shown that in an anti-PD-1 resistant lung cancer mouse model, intratumoral injection of OX40 agonist antibodies after radiation effectively inhibited local tumor growth, restricted lung metastasis, and improved survival rates [17]. In another preclinical study, the intratumoral injection of a combination of immunomodulators targeting anti-CTLA4, anti-CD137, and anti-OX40 resulted in improved local and distant tumor control compared to systemic administration in both lymphoma (A20) and solid tumor (MC38) models [18]. The minimal effective dose was found to be only 10 μg for each immunomodulator. Furthermore, the researchers discovered that injection close to the tumor-draining lymph node (tDLN) exhibited similar efficacy to intratumoral administration and was significantly superior to targeting a non-tDLN, thus supporting the feasibility of tDLN as a viable immunotherapy target. In a Phase I study of OX40 agonist MOXR0916 administered intravenously to patients with advanced solid tumors, most adverse events (AEs) were Grade 1–2 and related to MOXR0916 treatment [19]. The most common treatment-related AEs were fatigue (17%), diarrhea (8%), myalgia (7%), nausea (6%), decreased appetite (6%), and infusion-related reactions (5%). However, the study also found that the single-agent efficacy of OX40 agonists was low, with a disease control rate (DCR) of 33%. Therefore, intratumoral administration of OX40 agonists is a promising way to reduce adverse reactions and improve the effectiveness of combination therapy.

CD40 is a member of the TNF receptor superfamily, which is widely expressed on the cell membranes of antigen-presenting cells (APCs) such as monocytes, dendritic cells, B lymphocytes, and epithelial-origin tumors [20]. ABBV-927 is a CD40-targeting agonistic monoclonal antibody being explored in phase I trials (NCT02988960) for patients with solid tumors, including intratumoral injection as a dosing option. APX005M is another CD40 agonistic monoclonal antibody being tested in a phase Ib clinical trial (NCT02706353) in combination with Nivolumab for patients with metastatic melanoma, using intratumoral immunotherapy.

2.2.4 Small molecule proteins

Small molecule proteins usually refer to proteins with a molecular weight of less than 10,000. Antimicrobial peptides (37-residue cathelicidin antimicrobial peptide, LL-37) are endogenous cationic peptides made up of amino acids and are part of the Cathelicidin family. They have antibacterial activity against bacteria, viruses, fungi, and parasites. However, they can also regulate a wide range of cellular functions, including proliferation, invasion, apoptosis, cell cycle arrest, and cytokine release, and have a dual effect of promoting and inhibiting tumors. Its anti-tumor potential stems from its ability to enhance the recognition and binding of CpG oligodeoxynucleotides by human B lymphocytes and plasmacytoid dendritic cells, as well as its subsequent effect on TLR-9 activation [21]. An I/II phase clinical trial

(NCT02225366) investigated the safety of LL-37 as an intratumoral injection preparation for melanoma patients. A total of 3 patients were enrolled, and no serious adverse reactions were found. Common adverse reactions included bone marrow suppression and bilirubin abnormalities.

2.2.5 Nucleic acid drugs

In recent years, gene therapy using plasmid DNA/RNA vaccines as carriers has broad prospects for the prevention and treatment of tumors. Immunization with plasmid DNA/RNA vaccines can express encoded immunomodulatory cytokines in cells, and induce humoral and cellular immune responses dependent on B cells and T cells.

Several methods of delivering DNA/RNA vaccines to tumors have been preliminarily proven to be safe and effective [22]. A phase I clinical trial showed that the intratumoral introduction of plasmid DNA encoding IL-12 caused complete regression of melanoma in two patients, with minimal systemic toxicity and transient pain and bleeding around the treatment site, both at grade 1–2 [23]. Another phase I clinical trial showed that the dual-functional (BI) shRNA targeting stathmin1, which was directly injected into the tumor, had good tolerability and effectively cleaved stathmin1 messenger RNA (mRNA) [24]. These results suggest that this direction is worth further in-depth research.

2.2.6 Cytokines

Currently, cytokines used in tumor in situ vaccine therapy mainly include interleukin (IL), tumor necrosis factor (TNF), interferon (IFN), and others. These cytokines induce specific changes in cell function and enhance the existing antitumor immune response by binding to high-affinity receptors on the cell membrane. However, intravenous administration often results in dose-dependent adverse reactions such as hypotension, nausea, flu-like symptoms, and capillary leak syndrome (CLS), and in rare cases, life-threatening syndromes can occur. Therefore, local application of cytokines is an effective strategy to reduce adverse reactions and increase clinical feasibility. Currently, cytokines used for intratumoral immune injection mainly include the following categories:

IL-2 mediates the activation of effector T cells and plays a crucial role in antitumor immunity, but it has a short half-life and significant toxicity when administered systemically. On the other hand, low-dose IL-2 preferentially activates Treg cells, which in turn inhibits the immune response. In preclinical studies, the immune cell cytokine recombinant IL2 fused with a humanized monoclonal antibody against disialoganglioside GD2 (hu14.18-IL2), combined with anti-CTLA-4 antibody and targeted radioisotope therapy, induced effective cancer-specific immune responses by releasing antigens from mutated proteins and resulted in shrinking of primary and metastatic tumors as well as increased expression of primary tumor genes in dogs with metastatic melanoma or osteosarcoma. Similarly, in a mouse intracranial melanoma model, the treatment had significant efficacy, with tumor site-specific accumulation lasting up to 72 hours. In clinical studies, intravenous infusion of hu14.18-IL2 was associated with prolonged disease-free survival in high-risk melanoma patients. IL-2 has also been used in combination with intratumoral injection. In a phase I study of ipilimumab/IL-2 combination therapy (NCT01672450), the overall response rate and clinical benefit rate were 40% (95% CI, 10–70%) and

50% (95% CI, 19–81%), respectively, with 89% of melanoma patients (95% CI, 68–100%) showing distant reactions and good tolerance. In addition, in a clinical study (NCT02983045) of 38 patients with advanced solid tumors who had not received prior immunotherapy, treatment with NKTR214 (a polyethylene glycol-modified CD122-preferential IL-2 pathway agonist that drives proliferation and activation of $CD8^+$ T and NK cells via the preferred IL2 pathway signal transduction of IL2βγR, without expanding Tregs) in combination with nivolumab had an ORR rate of 59.5% and a CR rate of 18.9%.

IL-12 is a type I soluble cytokine involved in innate and acquired immunity. It stimulates the activity of T cells and natural killer cells, induces interferon-gamma production, and has anti-angiogenic effects. However, systemic administration of IL-12 often results in serious toxicity due to high drug concentrations, with a narrow therapeutic window. Recombinant IL-12 is also rapidly cleared, leading to a short injection residence time and poor anti-tumor efficacy. Tavokinogene telseplasmid (Tavo), a DNA plasmid encoding the IL-12 subunit, was administered via intratumoral injection with electroporation in clinical trials for patients with malignant melanoma, cutaneous lymphoma, head and neck squamous cell carcinoma, and Merkel cell carcinoma. Tumor regression was observed in primary and distant lesions (NCT01502293, NCT01579318, NCT02345330, NCT01440816), and further exploration is ongoing. Additionally, a phase I clinical trial is currently recruiting patients for intratumoral injection of MEDI1191 (IL-12 mRNA) in combination with durvalumab (PD-L1 monoclonal antibody).

IFN, in addition to its antiviral effects, is also a key factor in anti-tumor immune surveillance. It has direct effects on cancer cells and indirect effects on the immune system. Research on IFN has focused on type I IFN (IFNα/β) and type II IFN (IFNγ). Intra-tumor injection of IFN-γ can significantly reduce tumor volume in mice. In a clinical study, nine melanoma patients who received intra-tumor injections of IFNγ and a vaccine composed of MHCI-restricted melanoma peptides had good tolerance. IFNγ increased the production of chemokines CXCL10, CXCL11, and CCL5 in patients' tumors, inducing secondary immune regulation but not promoting immune cell infiltration or inducing anti-tumor immune gene signals. Combined with other treatments, it may have a synergistic effect [25]. In addition, many clinical studies have shown that treatment with INFα and INFβ can cure skin squamous cell carcinoma and basal cell carcinoma with a cure rate of over 95% and extended disease-free survival [26].

TNFerade is a transgenic human TNF-α protein carried by a defective adenovirus vector. When injected into the tumor, the systemic distribution is minimal, and there is no TNF-α-related systemic toxicity. When combined with radiotherapy and chemotherapy, it shows significant tumor-killing activity and good tolerance to various tumors (such as esophageal cancer, head and neck cancer, and rectal cancer), which can improve disease-free survival and overall survival, and delay tumor progression [27]. However, in a phase III clinical trial of standard treatment for locally advanced pancreatic cancer, it failed to demonstrate a significant increase in patient survival [28]. TNF can also be fused with the antibody fragment (L19) against the extra domain B (ED-B) of the anti-fibronectin protein to form L19-TNF, and mixed with L19-IL2 (recombinant fusion protein of L19 and IL-2) for intratumoral administration. Complete tumor eradication was observed in a mouse fibrosarcoma model, and the mice had good tolerance [29]. Clinical trials combining these immune cytokines are currently in phase II (NCT02076633, NCT05329792, NCT04362722).

2.2.7 Immune cells

Immune cells can be isolated from the patient's own body or from a donor, and can be expanded by genetic engineering techniques or by changing the conditions of in vitro culture before being re-infused into the patient to exert their function. Unlike the commonly used intravenous infusion, immune cells injected into the tumor can directly achieve effective immune responses at the tumor site, which is more favorable for improving the tumor microenvironment. Currently, immune cells mainly used for intratumoral injection include DCs, T cells, and NK cells.

Due to the fact that DC is not the ultimate effector cell of the immune system, the intratumoral injection of DC is more commonly used in combination therapy or with engineered DC. As an important antigen-presenting cell, DC is most commonly used in combination with radiotherapy. The iPSC-DCs derived from pluripotent stem cells combined with local radiotherapy promoted the activation of tumor-specific $CD8^+$ T cells in a mouse model, inhibited the progression of primary and non-radiotherapy tumors, and increased the expression of PD-L1 in tumor-associated macrophages and DCs. This overcomes the resistance to PD-L1 therapy in tumors with poor immunogenicity [30]. BMDCs derived from bone marrow also achieved local tumor control and systemic distant effects in combination with radiotherapy and anti-PD-L1 therapy, significantly increasing T cell proliferation and interferon-gamma release [31]. DCs derived from PBMCs have also made significant progress, and a phase I clinical study of Ad-CCL21-DC vaccine (DCs expressing the CCL21 gene transduced by a defective adenovirus vector) injected intratumorally in patients with advanced NSCLC has yielded similar conclusions to preclinical studies. 25% (4/16) of patients had stable disease on day 56, 37.5% (6/16) of patients had a systemic response to tumor-associated antigens, and 54% (7/13) of patients had infiltration of tumor $CD8^+$ T cells [32]. Meanwhile, when DCs were injected intratumorally with anti-PD-L1 drugs and combined with radiotherapy to treat a patient with squamous cell carcinoma of the skin, more than 10 highly significant T cell-related pathways were found to be upregulated, including leukocyte chemotaxis, T cell proliferation, and activation and differentiation of alpha-beta T cells. After treatment, the maximum standardized uptake value (SUVmax) of the lesions was found to be significantly reduced by about 42% on FDG PET scans [33].

Similar to intratumoral injection of DC, the T cells used for intratumoral injection are mostly genetically engineered. For example, in the CAR-T field, some CAR-Ts have been approved for systemic treatment of leukemia or lymphoma [34]. However, due to the lack of ideal specific targets and the existence of off-target effects and other issues, the development of CAR-T in solid tumors lags behind. Because specific targets expressed in tumors are rare, local application of CAR-T has become one of the directions for the application of solid tumor CAR-T. In a patient with glioblastoma multiforme who received intratumoral injection of CAR-T targeting IL13Rα2, the effect of all intracranial and spinal tumors regressing, cytokines and immune cells increasing in cerebrospinal fluid was observed [35]. Phase I clinical trials of intratumoral injection of CAR-T for the treatment of glioblastoma patients and targeting Nectin4/fibroblast activation protein (FAP) for the treatment of malignant solid tumor patients (NCT03389230, NCT04077866, NCT04385173, NCT03932565) are also recruiting further. There are also relevant reports on TILs modified by intratumoral injection. TILs transiently expressed IL-12 through mRNA electroporation, and their therapeutic effect was further enhanced by co-injection with anti-CD137 monoclonal antibody or transient co-expression of CD137 ligand,

achieving complete eradication of injected and distant tumor lesions in animal experiments [36].

Currently, there are relatively few studies on the intratumoral injection of NK cells for NK cell lymphoma, but NK cells are immune cells that are suitable for intratumoral injection. This is because NK cells generally do not require strict HLA matching and have a wide range of applications. As early as more than 20 years ago, there was clinical experience with intratumoral injection of NK cells [37], that is, intracranial injection of lymphokine-activated killer cells (LAK cells) combined with IL-2 to treat patients with glioblastoma multiforme (GBM), which increased the survival of GBM patients without serious complications. The application of EGFR-CAR-transduced NK-92 and primary NK cells for non-primary intracranial tumors has also been observed to shrink the tumor mass, with NK-92 cells exhibiting stronger cytotoxicity and IFN-γ production, and in combination with oHSV-1 (oncolytic herpes simplex virus-1), remaining EGFR-negative or low EGFR-expressing tumor cells can be further eradicated [38]. Currently, a clinical study (NCT04254419) on the intratumoral injection of NK cells for the treatment of recurrent high-grade gliomas in children is being conducted and is recruiting participants as of July 2022.

2.2.8 Microorganism

Recruiting inflammatory immune cells is a key step in cancer immunotherapy, but due to genetic changes in tumor cells from normal cells, there is generally an issue of immune tolerance. Therefore, inducing a strong immune response as an enhancer of the tumor immune response may partially solve this problem. Microbial vaccines, as an exogenous antigen, can enhance the immune response in the tumor microenvironment. As early as the end of the nineteenth century, Coley's toxin appeared as the beginning of tumor immunotherapy. In recent years, microorganisms represented by oncolytic viruses have played an increasingly important role in the field of tumor immune injection.

Viral agents that selectively cause tumor cell lysis while having limited or no replication in normal cells can play an important role in anti-tumor therapy. Common viruses include adenovirus, poxvirus, herpesvirus, and measles virus, among others. Some have been approved for clinical use or are in phase III clinical trials, demonstrating good anti-tumor activity and the ability to achieve systemic anti-tumor effects, form immune memory and reshape the tumor immune microenvironment. Combining with other anti-tumor treatments can provide greater benefits. A plant virus (CPMV) derived from the leaves of the cowpea plant has also shown potential as an in situ candidate for monotherapy and combination therapy, and is superior to other unrelated plant viruses and virus-like particles (VLPs) [39–41]. Addressing the expensive and time-consuming issue of generating oncolytic viruses that encode one or more tumor antigens, a new direction is to modify and coat them with MHC-I tumor peptides [42]. In 2020, Newman et al. reported a strategy of injecting an unadjuvanted seasonal flu vaccine into the tumor to convert "cold" tumors to "hot" tumors, thereby generating $CD8^+$ T cell-mediated systemic anti-tumor immunity and increasing the sensitivity of resistant tumors to checkpoint blockade therapy. Currently, oncolytic viruses are the most widely used in clinical practice, with many clinical trials underway. Talimogene laherparepvec (T-VEC) is a genetically modified herpes simplex virus that includes GM-CSF and lacks ICP34.5 and ICP47, received FDA approval in 2015 for local treatment of melanoma and is attempting many clinical trials in combination with ICIs [43, 44]. Its design allows selective replication

within tumors, production of GM-CSF, and stimulation of anti-tumor immune responses. In addition, oncolytic molecules have similar characteristics to oncolytic viruses and can infect and destroy tumor cells, transform the tumor microenvironment into a T cell-inflamed phenotype, and achieve anti-tumor effects [45].

The tumor microenvironment often exhibits immune suppression. Traditional tumor antigen release mediated by radiotherapy and chemotherapy cannot fully activate the immune response. Therefore, how to further activate the immune response has become a major hotspot. Bacteria have attracted attention due to their easy amplification, culture, and modification characteristics. Currently, bacteria research can be divided into two categories: conditional pathogenic bacteria (such as *Salmonella*, *C. tetani*, and *Vibrio cholerae*) and non-pathogenic bacteria (such as *Escherichia coli*, *Bifidobacterium*, and *Lactobacillus*). Studies have found that after radiotherapy, injecting Salmonella strains into the tumor can capture tumor antigens using their motility characteristics and release them to the periphery of the tumor, thereby enhancing DC activation and making systemic immune responses relatively easy to initiate. An increasing number of non-pathogenic bacterial strains are also used as immunological adjuvants alone or in combination with other treatment methods for the treatment of tumors. In 2021, Janku et al. injected *C. novyi*-NT (a non-toxic *Novyi* strain lacking α-toxin) spores into the tumor of 24 patients with refractory solid tumors. Nine patients (41%) had a decrease in tumor volume, and 19 patients (86%) had stable disease (NCT01924689) [46]. A clinical trial (NCT03435952) using pembrolizumab combined with intra-tumor injection of *C. novyi*-NT (a clostridial spore) to treat patients with advanced solid tumors and a clinical trial (NCT04167137) evaluating the use of SYNB1891 (a non-pathogenic *E. coli* that can produce cyclic di-AMP) as a single therapy for patients with advanced solid tumors or lymphoma are currently recruiting participants.

3. Progress of in situ vaccination technology

The development of in situ vaccination technology relies mainly on three aspects: First, the emergence of new anti-tumor treatment methods, as well as immunomodulatory drugs and immunotherapy, many of which can be used for in situ immune injection; Second, improvement and development of in situ vaccines to address the deficiencies in clinical applications and meet clinical needs; Third, designing reasonable in situ vaccination treatment models by combining the mechanisms and clinical needs of tumor immunotherapy.

3.1 Application of new tumor treatment technologies in the field of in situ vaccines

In recent years, the emergence of many new local treatment technologies and the development of existing technologies have provided more means for the realization of in situ vaccine technology. The development of radiotherapy techniques has led to a series of new technologies such as the Gamma Knife, TOMO radiotherapy, and proton therapy, which have made radiotherapy a more widely applicable means of in situ vaccination. In addition, there have been continuous developments and improvements in techniques such as radiofrequency ablation, microwave ablation, and electrochemical therapy. Furthermore, the emergence of many new drugs or immunological agents has also provided more possibilities for intratumoral immunization.

3.1.1 Photodynamic therapy

Photodynamic therapy (PDT) for tumors is a method that selectively utilizes photosensitizers to be taken up by tumor tissues, which then undergo a photodynamic reaction under certain wavelengths of light to produce intermediate active substances primarily consisting of reactive oxygen species (ROS) that kill tumor cells [47]. PDT can induce immunogenic cell death (ICD), promote the release of tumor antigens, and further increase the activation, proliferation, and infiltration of antigen-specific T cells. In addition, PDT, as a form of in-situ vaccine, can also be used to enhance the response rate of PD-1/PD-L1 antibodies. There are also reports on the combination of various minimally invasive therapies such as PDT, photothermal therapy (PTT), and sonodynamic therapy (SDT) for anti-tumor treatment [48].

3.1.2 Electrochemotherapy

Electrochemotherapy (ECT) is a local ablation therapy that increases the cytotoxicity of chemotherapy drugs (such as bleomycin or cisplatin) by applying electric pulses to the tumor. Studies have shown that electrochemotherapy can induce immunogenic cell death and can be used as a means of implementing in situ vaccines [49]. Currently, electrochemotherapy is mainly used for superficial tumors such as skin cancer and malignant melanoma.

3.1.3 New types of intratumoral immunotherapeutic agents

Many new types of immunotherapeutic agents can be used for intratumoral immunotherapy injection, such as new CAR-T and TCR-T therapies, and new immunomodulatory drugs. For example, Wagenaar et al. achieved ideal therapeutic effects in animal models by intratumoral injection of mRNA encoding IL-23, IL-36γ, and OX40L. Furthermore, some researchers have proposed bispecific antibodies (BiTEs), antibody prodrugs (probodies), and fusion antibodies, which can be used alone or in combination for intratumoral immunotherapy injection [50].

3.2 New techniques to improve in situ vaccines

3.2.1 Implementation techniques for improving in situ vaccines

Many in situ vaccination strategies, such as PDT and in situ immunization injection, require close-range operation. With the development of various endoscopic and imaging-guided techniques, in situ immunization injection can now be used for most parts of the human body. The development of endoscopic techniques such as digestive endoscopy and bronchoscopy has enabled PDT and in situ injection techniques to be implemented in the digestive tract and bronchus. Various image-guided puncture techniques also enable in situ vaccination techniques to be used for various intra-body lesions. The Comprehensive Cancer Centre of Nanjing Drum Tower Hospital has developed a series of new techniques for percutaneous puncture and injection into tumors under CT guidance, such as the “liquid retreat method” to prevent pneumothorax and the “posture compensation method” to improve needle accuracy. These techniques can be used to puncture many difficult lesions and also improve the feasibility of in situ vaccination techniques. The Comprehensive Cancer Centre of Nanjing Drum Tower Hospital has also invented the “array injection”

technique, which helps to achieve full coverage of injected agents in the lesion and improve the efficacy of in situ vaccines.

3.2.2 Improving the delivery efficiency and retention time of intratumoral injection drugs

One of the significant issues in the practical application of intratumoral injection drugs or cells in situ immunization is the limited penetration and short retention time in tumors. To address this, new drug delivery systems may be used to solve this problem. These mainly include nanodrug delivery systems and in situ immunogels.

Nanoparticle drug delivery system mainly refers to nanoscale particles made of natural or synthetic high molecular materials. Using nanoparticle drug delivery system as an in situ vaccine can not only increase drug solubility and slow down drug release, but also provide opportunities for modulating the tumor microenvironment (TME) due to the tunability of NPs' size, shape, surface charge, biocompatibility, high surface area-to-volume ratio, and physical properties. Studies have shown that NPs can directly stimulate or release immune-stimulating molecules in TME, regulate tumor antigen presentation, and generate tumor-recognizing T cell responses. Nanoparticles used for intratumoral immunization include chemically synthesized NPs and biologically synthesized NPs, among which chemically synthesized NPs can be classified into organic, inorganic, or a combination of both. Liposomes are the most widely used organic NPs. Research has combined anti-tumor drugs and TLR agonists to produce lipid-drug complexes that form mixed nano-aggregates by co-precipitation in water, and used them as a potential in situ anti-tumor vaccine [51]. For example, an in situ vaccine consisting of acid-responsive liposome-coated polydopamine (PDA) nanoparticles, modified with mannose and loaded with resiquimod (R848), was demonstrated that it could actively target the tumor site, promote photothermal therapy, capture tumor-associated antigens, activate DCs, enhance cytotoxic T lymphocyte response, and inhibit distant tumor recurrence and metastasis [52]. In addition, polymers and chitosan NP-ISVs have also been widely used in recent years in immunotherapy research. Inorganic NPs are composed of non-carbon elements such as gold and silicon, and can be used alone for in situ immunization. Gold nanoparticles are often used as photosensitizers in photothermal in situ vaccines. For example, Nam et al. developed a photothermally stable and efficient gold NP-ISV that, when injected into the tumor and exposed to light, regulated the infiltration and effector function of $CD8^+$ T cells and NK cells in the tumor, showing strong anti-tumor efficacy against both locally and distantly untreated tumors [53]. Bio-synthesized NP-ISVs can be composed of virus NPs or bacterial NPs. Plant virus NPs are non-lytic and can locally stimulate anti-tumor responses by interacting with pattern recognition receptors (PRRs). Another type of bio-synthesized NPs comes from bacteria, which can secrete nano-sized spherical protein-lipid vesicles called microvesicles. Since bacterial components are PAMPs, which are conserved small molecular motifs in microorganisms, they can be recognized by toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in plants and animals. In summary, both bacterial and virus NPs not only can load on-site vaccine formulations but also have on-site vaccine effects themselves.

Hydrogels are widely used in drug delivery systems because of their good biocompatibility, high drug-loading capacity, and ability to sustain drug concentration for a long time. Hydrogels used for therapeutic tumor vaccines can be roughly divided into several categories: low molecular weight hydrogels derived from peptides or

DNA, thermosensitive hydrogels, and low-temperature gels. Peptide hydrogels and thermosensitive hydrogels are commonly used for in-situ vaccine injections. In-situ hydrogel vaccines can also be combined with local photothermal therapy to significantly activate the anti-tumor immune effect. Zhang et al. functionalized hyaluronic acid-polydopamine nanoparticles (HA-PDA NPs) with adjuvants (Imiquimod, IQ) and doxorubicin (DOX) and integrated them into a thermosensitive hydrogel to prepare a new type of near-infrared in-situ tumor vaccine (HA-PDA@IQ/DOX HG) [54]. The vaccine was injected into breast cancer mice under near-infrared irradiation at 808 nm (2 W/cm^2, 5 min). Firstly, DOX was quickly released and killed some tumor cells in the TME and high temperature, then IQ was released from the gel, and polydopamine nanoparticles activated DC through the TLR7 signaling pathway in acidic environment. The results showed that the experimental group with the gel-encapsulated drug had the highest DC maturation rate, and the proportion of memory T cells in the inguinal lymph nodes of mice also significantly increased. The Comprehensive Cancer Centre of Nanjing Drum Tower Hospital also constructed a DC activation hydrogel based on dual-functional fusion membrane nanoparticles (FM-NPs) composed of autologous tumor cell membranes and grass branch bacillus membranes. FM-NPs not only provide tumor antigens but also activate DC. Loading FM-NP with granulocyte-macrophage colony-stimulating factor (GM-CSF) in an injectable alginate hydrogel can promote DC maturation in tumor-draining lymph nodes and induce sufficient effector memory T cells to migrate to the tumor microenvironment, converting "cold" tumors into "hot" tumors [55].

3.3 Establishment of a new paradigm for in-situ vaccine therapy

Due to the complexity of the immune response in tumors, immune combination therapy has become a recognized development direction for immunotherapy. For in-situ vaccines, combining them with systemic anti-tumor therapy based on the immune characteristics of the tumor, or combining different in-situ vaccine methods in a rational manner, is a development direction that meets clinical needs. In recent years, various composite in-situ vaccine treatment modes have been reported. The more common ones include combining in-situ vaccine methods with systemic immune checkpoint inhibitors, injecting multiple immunomodulators into the tumor, and combining in-situ immunotherapy with radiotherapy. For example, Hammerich L et al. used radiotherapy combined with TLR3 agonist Flt3L as an in-situ vaccine, and found that it can effectively promote antigen cross-presentation within the tumor and increase the response of $CD8^+$ T cells. In clinical studies (NCT01976585), this in-situ vaccine combined with PD-1 monoclonal antibody has achieved good therapeutic effects for lymphoma patients.

The Comprehensive Cancer Centre of Nanjing Drum Tower Hospital also found in previous work that an in situ vaccine composed of TLR7 agonist and agonistic OX-40 antibody can produce significant synergistic anti-tumor effects [12]. After TLR7 binds with its agonist, it can produce inflammatory factors, chemokines, and type I interferons, and can promote the maturation and activation of DC cells. Furthermore, TLR7 agonist R837 can significantly up-regulate the expression of OX-40 on T cells, increasing the proportion of antigen-specific T cells and memory T cells. The team applied the RO formulation in animal models and obtained the desired local anti-tumor effect, which also induced a distant effect, resulting in shrinkage of tumors not treated with the in situ vaccine. The Comprehensive Cancer Centre of Nanjing Drum Tower Hospital combined this in situ vaccine formulation with large-segmented

radiotherapy to create the "R-ISV-RO" in situ vaccine technology, and designed a self-initiated clinical study (ChiCTR2100053870) to apply this technology to advanced cancer patients who had failed conventional treatment, achieving good results.

The Comprehensive Cancer Centre of Nanjing Drum Tower Hospital has also constructed a FLT3L-OX40 fusion protein and used probiotics, such as Lactobacillus, to express the protein. Flt3L, OX40 agonist, and three bacterial components were used to form the "FOLACTIS" in situ vaccine system. Preclinical studies have shown that this system can effectively activate NK, DC, and T cells, and effectively improve the efficacy of immunotherapy for tumors [56]. Relevant clinical studies (ChiCTR2200060660) are also underway.

4. Challenges and prospects of in situ vaccination technology

With the development of tumor immunotherapy, new drugs and technologies continue to emerge. Developing novel immunotherapy techniques with clinical potential is currently the most urgent need. A clinically valuable technology needs to consider various factors such as efficacy, treatment cost, and operability. In situ vaccination for tumors has spanned three centuries and has become a technology platform that has been given new meaning in the rapidly developing field of immunotherapy. Its technical basis is mainly based on radiotherapy and intratumoral injection techniques, both of which can be carried out in most tertiary hospitals in China, and because radiotherapy, ultrasound, CT and other technologies are covered by medical insurance, the economic burden on patients is relatively low. On the other hand, there is ample room for development in how to use the technology platform of in situ vaccination, how to choose and develop intratumoral injection drugs, and how to apply the treatment mode reasonably.

However, as a therapy technique that has been given new meaning, the development of in situ vaccines also faces deficiencies and challenges, mainly reflected in the following aspects: First, how to establish an effective in situ vaccine? The development of in situ vaccine technology embodies the characteristics of "technological explosion". In recent years, many new drugs and treatment methods have emerged. How to organically combine these methods, integrate the principles of tumor immunology with the clinical needs of oncology, and establish an effective, practical, and promotable paradigm requires more preclinical research and clinical practice. Second, how to improve the efficacy evaluation criteria of in situ vaccines? For patients with advanced solid tumors, the difference between the efficacy of "treatment lesions" and "non-treatment lesions" will be brought about by intratumoral immunotherapy, which is a problem that has not been encountered in the previous evaluation of immunotherapy efficacy. The existing immunotherapy efficacy evaluation criteria (iRECIST) are not applicable to the objective evaluation of intratumoral immunotherapy efficacy. In 2020, Goldmacher et al. proposed a consensus on the evaluation criteria for intratumoral immunotherapy efficacy for solid tumors (itRECIST), which included the measurement of lesion sites, selection of lesion classification, order of injection, and evaluation methods and cycles during treatment into the scope of investigation, and made a series of preliminary regulations based on this [57]. However, due to the complexity of the treatment method of in situ vaccines, the efficacy evaluation method still needs to be further improved. Third, how to standardize the specific implementation process? Although in situ vaccines have distinct clinical feasibility, they also face some problems in actual application. The

first is the formulation of treatment plans: due to the significant individual specificity of the number, site, depth, overall disease status, and physical condition of each patient's tumor, it is necessary to develop individualized treatment plans for patients through multidisciplinary discussions; secondly, the specific implementation of intratumoral injection technology. Early intratumoral injections were mostly used for superficial tumors. With the development of imaging-guided techniques, endoscopic techniques, surgical techniques, and related anesthesia techniques, the challenges in the operational techniques of intratumoral immunotherapy are gradually decreasing, but promotion and training for related treatment teams are still required. This not only requires long-term team collaboration but also requires sufficient manpower and technical resources, configuration of professional personnel and perfect facilities, obviously building such a team requires a lot of time and accumulation of technical experience.

5. Conclusions

In summary, the in situ vaccine technology has both clinical value and potential for frontier development. It is a technology system that is worth clinical translation and exploration. In the future, it will undoubtedly be increasingly applied to modern cancer immunotherapy through continuous development.

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Conflict of interest

The authors declare no conflict of interest.

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