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Review article

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Enhancing in situ cancer vaccines using delivery technologies

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Abstract

In situ cancer vaccination refers to any approach that exploits tumour antigens available at a tumour site to induce tumour-specific adaptive immune responses. These approaches hold great promise for the treatment of many solid tumours, with numerous candidate drugs under preclinical or clinical evaluation and several products already approved. However, there are challenges in the development of effective in situ cancer vaccines. For example, inadequate release of tumour antigens from tumour cells limits antigen uptake by immune cells; insufficient antigen processing by antigen-presenting cells restricts the generation of antigen-specific T cell responses; and the suppressive immune microenvironment of the tumour leads to exhaustion and death of effector cells. Rationally designed delivery technologies such as lipid nanoparticles, hydrogels, scaffolds and polymeric nanoparticles are uniquely suited to overcome these challenges through the targeted delivery of therapeutics to tumour cells, immune cells or the extracellular matrix. Here, we discuss delivery technologies that have the potential to reduce various clinical barriers for in situ cancer vaccines. We also provide our perspective on this emerging field that lies at the interface of cancer vaccine biology and delivery technologies.

Sections

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Introduction

Cancer immunotherapy harnesses a patient's immune system to combat tumours[1]. Although many cancer immunotherapy reagents such as immune checkpoint antibodies and chimeric antigen receptor (CAR) T cell therapies have demonstrated great success in treating blood cancers, treatment of solid tumours remains challenging. A successful cancer immunotherapy requires the activation of the cancer-immunity cycle[2] (Fig. 1). This cycle includes the release of tumour antigens from cancer cells (Box 1), antigen processing and presentation by antigen-presenting cells (APCs) such as dendritic cells, the generation of tumour-specific cellular and humoral immune responses (Box 2) and the lysis of tumour cells, which increases tumour antigen release and amplifies the cycle. However, the cancer-immunity cycle is usually deficient in most tumours[2], which leads to tumour cells escaping from immune surveillance and to the failure of immunotherapy (immune escape). In situ cancer vaccination aims to restore the cancer-immunity cycle[3] by using agents that improve tumour antigen release (mainly by inducing tumour cell death), improve processing and presentation of antigens by dendritic cells, and therefore generate tumour-specific T cell responses[4,5]. These agents are directly injected into the tumour or its microenvironment. Successful activation of the cancer-immunity cycle can also induce an abscopal effect[6,7], whereby shrinkage of untreated tumours occurs concurrently with shrinkage of tumours within the area of the localized treatment.

In situ cancer vaccines were first described in the 1890s by William Coley, who found that intratumoural injection of heat-killed bacteria led to potent antitumour immune responses[8] (Fig. 2). More than 70 years later, the bacillus Calmette–Guérin (BCG) vaccine[9], the live attenuated vaccine form of *Mycobacterium bovis*[10], was approved for the treatment of bladder cancer. These inactivated bacteria are now known to activate pattern recognition receptors (PRRs) including Toll-like receptor 2 (TLR2) and TLR4, and to serve as an adjuvant that improves tumour antigen processing and presentation. In another landmark development, a synthetic TLR7 agonist imiquimod[11] was approved for the treatment of superficial basal cell carcinoma[12]. Additionally, oncolytic viruses that preferentially infect tumour cells and lead to tumour cell lysis were introduced into the clinic in 2015 as in situ vaccine agents[13,14]. Oncolytic viruses improve the activation of PRRs to facilitate the processing and presentation of tumour antigens by dendritic cells[15]. Another advancement was the nanoparticle-based radiosensitizer Hensify[16], which enhances radiotherapy efficiency by inducing an abscopal effect. This received European Union (EU) approval in 2019 for the treatment of locally advanced soft tissue sarcoma. These clinical studies[17,18] (Fig. 2) point to the great promise of in situ vaccines for treating cancer.

However, despite these successes, there are several challenges to achieving safe and effective in situ cancer vaccines. For example, inadequate tumour antigen release limits antigen uptake by APCs and thus restricts tumour-specific immune responses. Also, the low immunogenicity[19] of tumour antigens means that they are inefficiently processed by APCs to generate antigen-specific T cell responses[20]. Moreover, the suppressive immune microenvironment of the tumour causes T cell exhaustion and death of antigen-specific T cells[21]. In addition, limiting systemic toxicity by implementing in vivo tumour-targeting approaches is also challenging. These obstacles restrict the broader application of in situ cancer vaccines, and thus new delivery technologies that enable improved tumour antigen release, enhanced tumour antigen processing and tumour microenvironment modulation are urgently needed (Fig. 3).

The past few decades have witnessed the rapid development of successful drug and gene delivery technologies[22,23] including liposomes[24], polymeric nanoparticles[25] and lipid nanoparticles (LNPs)[26]. Drug and gene delivery carriers can be constructed using materials with different physicochemical properties such as size[27], shape[28] and surface characteristics[29]. These technologies enable the design of on-demand drug delivery systems[30] and can alter the biodistribution, metabolism, clearance and toxicity of therapeutic molecules[31,32]. Some technologies can also target specific tissues and organs, either actively or passively[33,34], which decreases off-target effects[35]. Moreover, delivery systems can be constructed with stimuli-responsive materials[36] to achieve cargo release under conditions such as a specific pH or temperature[37]. These features make delivery technologies uniquely suited to overcome the clinical challenges that face in situ cancer vaccines.

In this Review, we describe drug delivery technologies that are being developed for solid tumours to enhance the effects of in situ cancer vaccines at several stages of the cancer-immunity cycle. For enhancement of tumour antigen release, we discuss the delivery of chemotherapeutics, nanosensitizers and biomolecules. For enhancement of tumour antigen processing and presentation, we focus on the delivery of PRR agonists and virus- and bacteria-derived materials, as well as the delivery of agents to activate immunogenic cell death (ICD) or endogenous retroviral genes. For approaches to overcome the immunosuppressive tumour microenvironment, we discuss the delivery of immune checkpoint inhibitors, cytokines and agents to deplete suppressive immune cells. Finally, we consider promising clinical studies and provide perspectives on the future of this emerging field.

Enhancing tumour antigen release

Tumour antigens are confined within the cell membranes of intact cancer cells, so induction of tumour cell lysis and death is an effective strategy to release tumour antigens[38]. Several cell death pathways such as apoptosis, necrosis, autophagy, pyroptosis and ferroptosis can induce the leakage of cytosolic constituents containing tumour antigens into the extracellular space[39]. Cell death can be induced by chemotherapeutic drugs[40], physical methods (such as X-ray radiation[41], cryoablation[42], microwave ablation[43] and photothermal and photodynamic[44] therapeutic modalities) and biomolecules (such as oncolytic viruses[45] or cytolytic peptides[46]) (Table 1). However, these methods of inducing tumour cell lysis have several limitations. For example, chemotherapeutics are toxic not only to tumour cells but also to normal cells[47] and their nonspecific biodistribution in normal tissues and cells can induce severe adverse effects[48]. Therapies based on physical methods are also limited by their lack of tumour specificity. For example, a high dose of X-ray irradiation induces both tumour cell death and substantial killing of adjacent normal cells[49]. Biomolecules such as oncolytic viruses are limited by the potential safety risk of the live virus[50] and the effect of a virus-specific immune response on the therapeutic outcome[51]. In this section, we discuss how delivery technologies can solve these problems and improve tumour antigen release by in situ cancer vaccines.

Delivery of chemotherapeutics

Chemotherapeutics have been used for decades in cancer treatment[52], inducing tumour cell lysis and antigen release through apoptosis, necrosis or autophagy[53]. Moreover, chemotherapeutics also induce pro-inflammatory immune responses by inducing ICD, whereby dying cells release immune signals such as damage-associated molecular patterns (DAMPs)[54,55]. Although these features are ideal for in situ

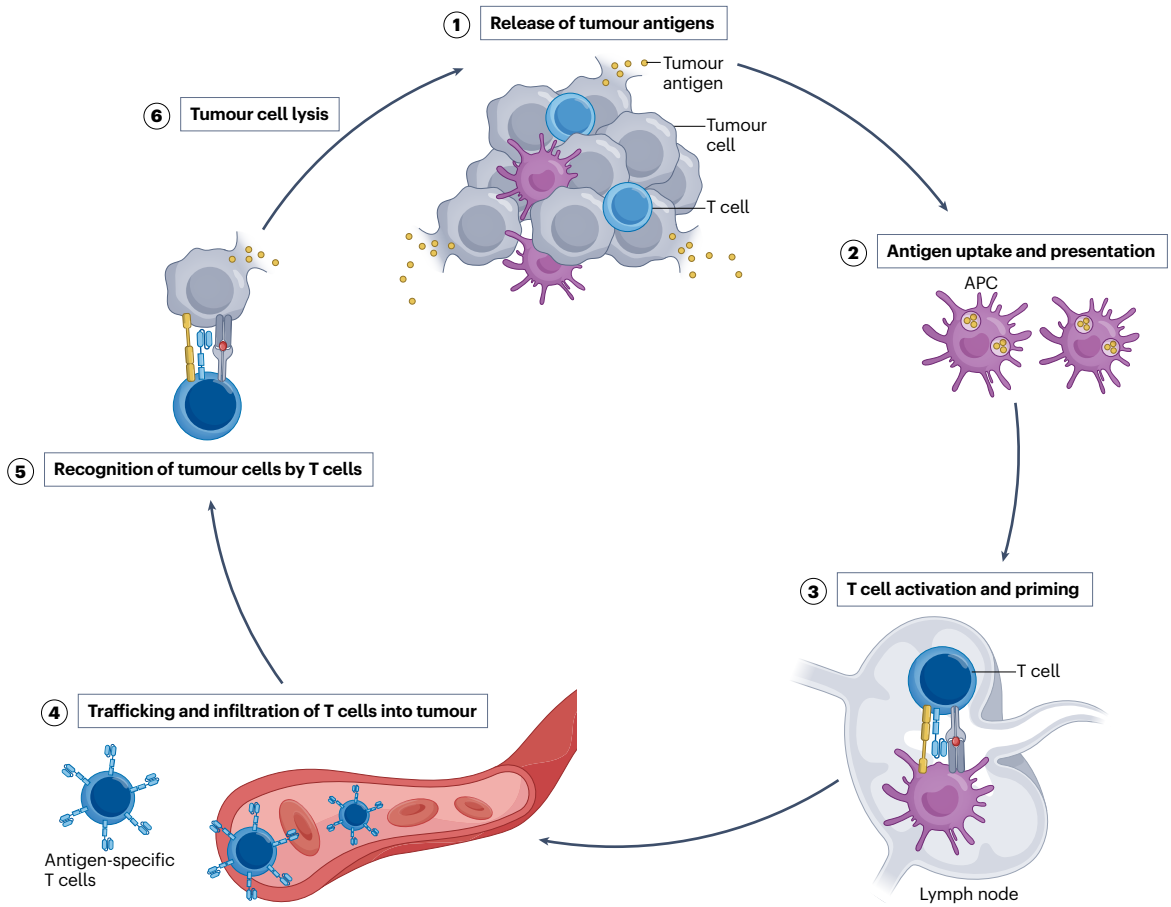

Fig. 1 | The cancer-immunity cycle. The cancer-immunity cycle is a multistep framework used to describe how the immune system recognizes and kills cancer cells. The main steps are: the release of cancer antigens by dying tumour cells (step 1); antigen uptake and presentation by antigen-presenting cells (APCs) such as dendritic cells (step 2); the priming and activation of T cells in the lymph nodes (step 3); the trafficking of antigen-specific T cells into tumours (step 4); recognition of tumour cells by T cells (step 5); and tumour cell lysis by T cells (step 6).

vaccine applications, the in situ vaccine effect of chemotherapeutics is usually limited owing to lack of tumour specificity[56] and toxicity to normal tissues[57].

Nanoparticle-based delivery systems are well suited to improve the accumulation of chemotherapeutics in tumour tissues through the enhanced permeability and retention effect (EPR effect)[58,59]. The decoration of nanoparticles with poly(ethylene glycol) (PEG) enhances their blood circulation time and thus improves tumour accumulation[60,61], which ultimately enhances tumour cell lysis[62]. For example, nanoparticles were designed with an anisamide-tagged PEG–poly lactic-co-glycolic acid (PLGA) block copolymer to deliver the chemotherapeutics doxorubicin and icaritin[63]. The nanoparticles triggered substantial release of tumour antigens and DAMPs such as ATP and calreticulin (CRT), which stimulated APCs to process tumour antigens and elicited improved tumour-specific T cell responses in vivo. Another strategy involves the conjugation of active targeting ligands (such as tumour-specific antibodies, peptides or aptamers) on nanoparticles to improve tumour tissue-specific delivery and tumour antigen release[64–66]. Furthermore, delivery systems for in situ vaccines have been designed to be responsive to various characteristics of the tumour microenvironment[67–70]. For example, a pH-responsive nanoparticle was designed for delivery of both doxorubicin and the immune adjuvant resiquimod[71]. In the acidic tumour microenvironment (pH 6.5), doxorubicin is released to induce tumour cell lysis and antigen release, and when combined with R848 a strong antitumour immune response was seen in a mammary carcinoma 4T1 tumour-bearing mouse model. In another study[72], conjugation of doxorubicin to phospholipid-based nanodiscs containing a pH-responsive imine bond led to substantial tumour cell killing, thereby improving the release of the DAMP molecule high mobility group protein B1 (HMGB1) and resulting in an in situ vaccination effect in a mouse model of metastatic breast cancer. In addition to stimuli-responsive delivery systems, another strategy[73] involved a local chemotherapeutic delivery system to decrease off-target toxicity. Intratumoural injection of an alginate-based gel encapsulating oxaliplatin induced substantial ICD and tumour antigen release. This formulation acted synergistically with the FDA-approved TLR7 agonist and immune adjuvant imiquimod plus the immune checkpoint blockade antibody to programmed cell death 1 ligand 1 (anti-PDL1) to elicit potent immune responses in mouse breast cancer and colon cancer models[72].

Box 1 | Types of tumour antigen

Tumour-associated antigens

Tumour-associated antigens (TAAs) are self-antigens that are preferentially expressed on tumour cells[217]. Examples of TAAs include cancer testis antigens, such as NY-ESO1, members of the MAGE, GAGE, XAGE, BAGE and PAGE families, SSX1, SSX2; differentiation antigens such as gp100, tyrosinase, melan-A/MART1, PSA; overexpressed antigens such as HER2, hTERT, CEA; and oncofetal antigens such as PSA, AFP, WT1. Some TAAs are also expressed in normal tissues and these TAAs are not usually highly immunogenic owing to central and peripheral tolerance, which limits the use of TAA-based cancer vaccines.

Viral antigens

Viral antigens are of foreign origin and are highly immunogenic[218]. Several viruses, such as human papillomavirus (HPV), Epstein–Barr virus (EBV) and Merkel cell polyomavirus (MCV), cause tumour formation. Therefore, virus-derived proteins have been used as antigens to design vaccines for cancer. These vaccines are mostly effective in preventing viral infection-related cancers but fail to treat late-stage tumours because not all tumour cells express viral antigens. Moreover, only limited numbers of tumours are correlated with viral infections. The mechanism of viral antigen-based cancer vaccines is not applicable to other cancer vaccines.

Tumour neoantigens

Tumour neoantigens are the result of several types of tumour-specific genomic aberration — including single nucleotide variants, indels, gene fusions, aberrant splicing events and the integration of oncogenic viruses — that can introduce novel chimeric transcripts[211]. In comparison with TAAs and viral antigens, tumour neoantigens are highly tumour specific and broadly abundant in cancer cells[219]. They can be recognized as foreign by the immune system and are highly immunogenic. They are often unique to each patient. Significant progress has been made in developing neoantigen-based vaccines[220]. However, there are still concerns around the neoantigen-based traditional vaccine strategy owing to the high heterogeneity of neoantigens in solid tumours, as antigen loss might occur after neoantigen-based traditional vaccine treatment. Creating vaccines in situ in tumour tissue could generate a polyclonal immune response towards different neoantigen subtypes and thus holds great promise for cancer treatment.

Although nanoparticle-mediated chemotherapeutic delivery can improve drug accumulation in tumours and lead to improved tumour antigen release, a large proportion of administered chemotherapeutics accumulate in the liver[71] and other organs, which can cause tissue damage. Future studies should further improve the tumour-targeting specificity of nanoparticle-based delivery systems. Additionally, given the demonstrations that cells such as red blood cells, neutrophils and T cells can be used as carriers for targeted delivery of chemotherapeutics to tumours[74], future studies should explore the use of such cells as carriers for chemotherapeutic delivery to induce effective in situ vaccination.

Delivery of nanosensitizers for inducing antigen release using physical methods

Tumour cell death and antigen release can be induced using physical methods such as radiation therapy, photodynamic therapy (PDT) and sonodynamic therapy (SDT), which act through the generation of reactive oxygen species (ROS). Alternative approaches such as photothermal therapy, microwave ablation therapy and focused ultrasound therapy induce tumour cell death by causing extreme temperature increases in the area of the tumour. In addition, cryoablation damages cell membranes and induces cell death via the generation of ultra-low temperatures in the tumour area. Although all of these treatments can induce remission of treated tumours, only a limited in situ vaccination effect is generally observed[75]. Reasons for this include the low efficiency of tumour cell lysis and the off-tumour toxicity related to these strategies. The development of new delivery technologies has provided solutions to overcome these obstacles.

Nanomaterials can be designed with specific physicochemical properties that act as sensitizers to tumour cell death mediated by these physical method-based therapies. To improve radiotherapy, many metallic nanomaterials have been designed as radio-sensitizers, as they can increase secondary electron and ROS production in the tumour microenvironment and thus amplify the radiobiological effects on DNA damage. For example, gold nanoclusters modified with glutathione can accumulate in tumours via the EPR effect. Upon X-ray irradiation, the nanocluster significantly enhanced radiotherapy-induced tumour cell death in a mouse model of cervical cancer[76]. In another study[77], an Hf-based porous nanoscale metal–organic framework (nMOF) acted as a radiosensitizer and helped to achieve low-dose X-ray-mediated tumour cell lysis. The in situ vaccination effect induced by the local radiotherapy made possible using nMOFs synergistically acted with anti-PDL1 or an indoleamine 2,3-dioxygenase 1 (IDO1) inhibitor to inhibit the growth of distant tumours through the abscopal effect[77,78]. Whereas these materials relied on the EPR effect for nanoparticle accumulation in tumours, another study used gold nanoclusters with cyclic RGD peptide shells (which promote cell attachment) to target α5β3 integrin-positive cancer[79]. The nanoclusters combined with radiation improved tumour growth inhibition in a mouse breast tumour model. Collectively, these studies demonstrate the potential for the use of nanomaterials as radiotherapy sensitizers to improve tumour antigen release.

In addition to nanomaterials being used as radiotherapy sensitizers, many nanomaterials have been designed to act as sensitizers to improve PDT or SDT for enhanced tumour antigen release. The self-assembly of a porphyrin derivative (TAPP-GCP), meso-tetra(4-carboxyphenyl) porphyrin (TCPP) and bovine serum albumin (BSA) formed a TAPP-GCP@TCPP@BSA nano-photosensitizer[80]. This nanoparticle accumulated in tumour tissue and led to significant tumour cell death and antigen release after laser irradiation, thereby promoting the generation of tumour-specific immune responses in a breast cancer model. Because the hypoxic tumour microenvironment restricts ROS generation during PDT, another study designed oxygen-carrying nanoparticles[81] that delivered oxygen to the hypoxic tumour

microenvironment and improved ROS generation when the tumour area was irradiated with near-infrared (NIR) light[81]. This treatment thereby led to the release of greater amounts of antigens and DAMPs, and induced an enhanced in situ vaccination effect[81]. Similarly[82], use of an iron-based MOF[82] to trigger the decomposition of H_2O_2 into O_2 in the tumour tissue was found to increase cancer cell CRT exposure, leading to improved ICD[82]. Metallic or polymeric nanomaterials delivering sonosensitizers such as porphyrins, hypericin and curcumins have shown great promise in improving SDT-induced tumour antigen release and in situ vaccines[80,83]. A nano-sonosensitizer termed HMME/R837@Lip[84] was constructed by encapsulating an ultrasound-responsive sonosensitizer (haematoporphyrin monomethyl ether, HMME) and the imiquimod adjuvant into liposomes. Ultrasound irradiation at the tumour area led to the generation of ROS and significantly increased the release of tumour antigens. This treatment resulted in a potent antitumour immune response that not only inhibited the treated tumours, but exhibited a significant abscopal effect and protected mice from tumour cell re-challenge[84]. In addition, various types of micro- and nano-bubbles[85], as well as other particles[86], have been proposed to sensitize focused ultrasound for improved cancer cell killing in mouse breast and colon cancer models.

Tumour antigen release can also be achieved by photothermal therapy. For example, a study showed that photothermal therapy reagent gold nanorods covalently coupled with amphiphilic polyTLR7/8a and matrix metalloproteinase 2 (MMP2)-sensitive R9–PEG conjugate (AuNRs-IMQD-R9–PEG)[87] markedly increased the infiltration of effector $CD8^+$ T and natural killer T cells into tumours, and promoted long-term animal survival. In another study[88], polydopamine nanoparticles functionalized with hyaluronic acid encapsulating imiquimod and doxorubicin promoted dendritic cell maturation and cytotoxic T lymphocytes in the spleen. Moreover, black phosphorus–Au nanoparticles[89] carrying CpG oligodeoxynucleotide triggered tumour-specific immunity in vivo in a mouse model of breast cancer.

Nanosensitizers have also been used to enhance microwave ablation and cryoablation therapies for improved cancer cell lysis. Liposomes loaded with ethyl formate, a microwave ablation sensitizer[90], were shown to enhance tumour cell lysis[90]. Moreover, biodegradable MgO (ref. 91) and Fe_3O_4 (ref. 92) nanoparticles increased the probability of intracellular ice formation and cellular dehydration following freeze–thaw cycles during cryoablation therapy.

Altogether, enhanced tumour cell lysis can be achieved by using nanosensitizers for radiotherapy, PDT, SDT, microwave ablation, focused ultrasound or cryoablation. Although some of the materials used in these studies were biodegradable, others were not and some even contained heavy metals. Future studies should focus on using biodegradable materials to improve antigen release. Even though nanosensitizers substantially improved therapeutic efficacy, a challenge is that using the various physical lysis methods on tumours adjacent to important organs and tissues such as brain, kidney and aorta might allow nanosensitizer leakage into normal tissues and cause unwanted damage. Future studies should design nanosensitizers with high retention in the tumour tissue to decrease nonspecific biodistribution and mitigate damage to normal tissue.

Delivery of biomolecules

Tumour cell lysis can also be achieved by the delivery of biomolecules that disrupt cell membranes[93,94] or modulate cell death pathways[95]. For example, an α-melittin–nanoparticle (α-melittin-NP)[93] substantially promoted tumour antigen release through the disruption of cell membranes by the α-melittin peptide. Because of their ultrasmall size (10–20 nm), the nanoparticles drained into lymph nodes and activated both macrophages and dendritic cells, thereby generating tumour antigen-specific T cells in mice. Compared with free α-melittin, the α-melittin-NPs showed remarkably enhanced lymph node accumulation and activation of APCs, leading to a 3.6-fold increase in antigen-specific $CD8^+$ T cell responses and an abscopal effect that inhibited distant tumours.

Engineered protein nanoparticles have been designed that fuse oncolytic viral particles to tumour-targeting agents[94] (see later). Moreover, a study[95] showed that the burst release of the annexin protein A5 from mesoporous nanoparticles blocked immunosuppressive apoptosis and promoted immunostimulatory necrosis to enhance in situ vaccination in breast cancer models[95]. Collectively, these studies demonstrate the promise of biomolecule delivery for in situ vaccination.

Despite promising results, the immune system will typically mount antibodies to protein-based materials and reduce the effectiveness of such therapies, especially when repeated dosing is needed. The development of mRNA delivery systems encapsulating mRNAs encoding tumour-lysing peptides or proteins could potentially solve this problem. Moreover, these nanoparticles could be designed to target tumour cells to enhance specificity.

Enhancing antigen processing by activating innate immune responses

Although delivery technologies can enhance in situ cancer vaccination and improve tumour antigen release, the antitumour immunity generated following primary tumour destruction is relatively weak[96].

Box 2 | Cellular and humoral immune responses

Cellular immune response

The cellular immune response, or cell-mediated immunity, is an immune response that does not involve antibodies, but relies on the following cellular mechanisms.

- Activated antigen-specific cytotoxic T cells induce apoptosis of cells displaying foreign antigen epitopes on their surface (such as cells infected with viruses or bacteria, and cancer cells displaying tumour antigens).
- Macrophages destroy pathogens via recognition and phagocytosis, and natural killer cells destroy pathogens via the secretion of cytotoxic cues.
- Activated immune cells secrete cytokines and chemokines to influence the function of other cells involved in innate and adaptive immune responses.

Humoral immune response

The humoral immune response, or humoral immunity, is mediated by macromolecules that are located in extracellular fluids. These molecules include secreted antibodies, complement proteins and certain antimicrobial peptides. The main purpose of the humoral immune response is to protect the extracellular spaces of the body when intracellular pathogens spread from one host cell to another via the extracellular fluids.

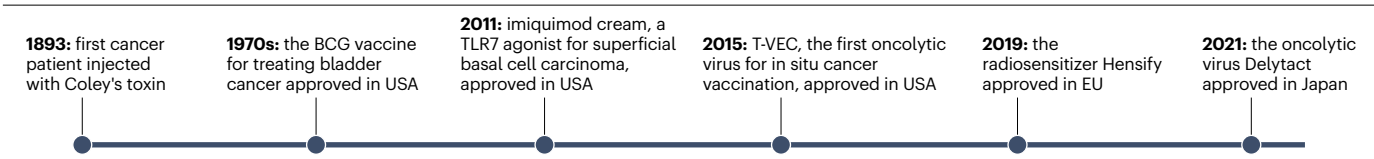

Fig. 2 | The history of in situ cancer vaccines. Major breakthroughs in the field are noted. BCG, bacillus Calmette–Guérin; EU, European Union; TLR7, Toll-like receptor 7; T-VEC, talimogene laherparepvec.

APC-mediated tumour antigen processing and presentation can be improved by the activation of PRRs such as TLRs, RIG-I-like receptors, NOD-like receptors (NLRs) and C-type lectin receptors[97]. PRR activation leads to the stimulation of intracellular signal transduction pathways and increased expression of inflammatory genes to improve antigen processing. There are two main strategies to enhance innate immune system activation during in situ vaccination. First, innate immunity can be directly activated using agonists for PRRs[98]. Second, innate immunity can be indirectly activated using reagents that target intrinsic stress pathways[99,100] to induce the release of DAMPs from tumour cells. Improved tumour antigen release in combination with activation of dendritic cells (particularly conventional type 1 dendritic cells) leads to trafficking of dendritic cells to lymph nodes and the generation of tumour-specific T cells. In this section, we discuss the use of delivery technologies to improve antigen processing and presentation by dendritic cells through direct and indirect PRR activation (Fig. 4).

Activation of pattern recognition receptors

Many studies have shown that synthetic PRR agonists and virus- or bacteria-derived materials can improve antigen processing and presentation in dendritic cells through PRR activation[101]. However, the nonspecific, systemic distribution of these reagents induces toxicity[102]. Delivery systems offer the opportunity for improved tumour targeting of synthetic PRR agonists, as well as virus- and bacteria-derived materials, to enhance in situ cancer vaccination (Fig. 4a).

Delivery of synthetic PRR agonists. Synthetic agonists for TLRs[103], NLRs[104] and stimulator of interferon genes (STING)[105] can potently activate PRRs in cells and improve antigen processing and presentation. The delivery of PRR agonists into tumours is difficult because of the dense extracellular matrix, solid stress and abnormal vascular structures inside tumours. In pioneering work, administration of CpG oligodeoxynucleotides (which act as TLR9 agonists) after cryoablation led to enhanced inhibition of tumour growth in a melanoma mouse model[106]. Moreover, the treatment protected mice that were cleared of tumours from tumour cell re-challenge, indicating that an immune memory effect was induced[106]. However, free immunological adjuvants can easily diffuse into healthy tissues and induce systemic toxicity. This problem can be solved by using ultrasound-responsive nanocarriers. For example, a liposome encapsulating the TLR7 agonist imiquimod[84] showed substantial accumulation in the liver. To avoid liver toxicity, ultrasound was administered only to the tumour area, which allowed imiquimod to be released from the liposome only in the tumour tissue. The tumour antigens can thus be taken up together with the TLR7 agonist by dendritic cells. Combining this nanoparticle with anti-PDL1 antibody treatment led to increased dendritic cell maturation and strong antitumour immune responses. There are many other reports of the use of stimuli-responsive drug delivery systems to improve in situ vaccination while decreasing toxicity[107]. These include NIR light- and ROS-responsive black phosphorus quantum dot nanovesicles encapsulating CpG oligodeoxynucleotides[107] for PDT-based in situ vaccination[107] and a wolfram-based cationic nMOF for PDT-responsive CpG delivery[107]. These promising results in preclinical models demonstrate the potential of PRR agonist delivery for in situ vaccines.

The chemical instability of certain PRR agonists can be attributed to their susceptibility to enzyme degradation[108]. This often restricts their efficacy as components of vaccines. For example, cyclic dinucleotides (CDNs) are a class of STING agonists that elicit strong immune responses[109]. However, natural CDNs are small hydrophilic molecules that cannot cross cell membranes and can easily be degraded by enzymes, leading to low bioavailability in target tissues[110]. Therefore, CDNs were conjugated to PEGylated lipids via a cleavable linker and the conjugate was anchored to a lipid nanodisc (LND)[111].The LNDs efficiently penetrated into tumours and tumour debris, allowing CDNs to be taken up simultaneously by dendritic cells. A single dose of LND–CDNs not only led to tumour growth inhibition but also protected the mice from tumour cell re-challenge, indicating induction of an immune memory effect[111]. These results demonstrate that drug delivery systems improve the penetration of PRR agonists into the tumour and protect them from degradation.

Even though substantial advances have been made in the delivery of synthetic PRR agonists for improved in situ vaccines, there are still many challenges. First, nonspecific biodistribution of nanocarriers encapsulating PRR agonists could lead to toxicity to normal tissues. This problem could be solved by the design of an activity-controllable PRR agonist[112]. Second, for delivery systems that use PEG as a component, a potential concern is that, after repeat dosing, a humoral response against PEG could be generated and result in rapid clearance of the delivery carrier. Future delivery system design should consider replacing PEG with zwitterionic polymers[113] or polysarcosine[114] to avoid unwanted humoral responses.

Oncolytic nanomaterials. Oncolytic virus infection can induce the release of tumour antigens. Virus or virus-derived materials can be recognized by cancer cell PRRs and elicit strong antiviral immune responses, which promote the internalization and cross-presentation of tumour antigens by APCs[115]. Although oncolytic viruses are showing promise as antitumour agents, with several in clinical trials[13,45,116], broader application of oncolytic virus therapy is limited by humoral immune responses to the virus and by biosafety issues. Delivery technologies could potentially solve these problems.

The host immune system is highly evolved to neutralize pathogens[117], especially after repeat dosing[118]. Therefore, to shield oncolytic viruses from immune recognition and destruction, the use of cells as systemic delivery vehicles has been explored[117]. Several cell carriers[119,120] have been shown to protect oncolytic viruses following intravenous delivery, which then allows for repeat dosing and improves therapeutic outcomes. In a different approach, synthetic RNA viruses have

been designed that consist of a viral RNA genome formulated within LNPs[51]. LNPs delivering the viral RNA of Seneca Valley virus (SVV) and Coxsackievirus A21 produced these viruses in vivo, which were able to replicate, spread, lyse tumour cells, promote immune cell infiltration and induce a potent antitumour response, even in the presence of neutralizing antibodies in the bloodstream[51]. Therefore, nanoparticles delivering viral RNA genome to produce viruses in vivo can avoid the rapid clearance of virus that occurs after repeat dosing and achieve an improved in situ vaccination effect.

Clinical application of oncolytic viruses is also restricted by biosafety issues[121]. Many attempts have been made to engineer virus-derived proteins into nanoparticles to improve safety[94,122–125]. For example, an oncolytic protein derived from chicken anaemia virus (apoptin) that induces tumour cell lysis was fused with an EGFR-specific repebody for targeting tumours[94]. This repebody–apoptin fusion protein self-assembles into a supramolecular nanoparticle. After administration, this nanoparticle induced antitumour immune responses with negligible side effects in a xenograft mouse model of breast cancer[94]. In another study[124], inspired by the key role of vesicular stomatitis virus (VSV) matrix protein in VSV-induced apoptosis, a tumour-targeting nanoparticle delivering a plasmid encoding a neutral VSV matrix gene was designed for cancer therapy[124]. The formulation efficiently accumulated in tumour tissues, inhibited melanoma growth and metastasis and prolonged the survival of tumour-bearing mice without inducing obvious systemic toxicity.

Altogether, overcoming the humoral immune response can be achieved through the design of nanoparticles that deliver mRNAs encoding virus, and the biosafety problem of oncolytic virus can be solved through the design of noninfectious virus-like particles. Other challenges in this field include the difficulty of penetrating solid tumours and overall systemic toxicity risks. Future studies should examine the integration of permeation enhancers into nanomaterials to improve tumour penetration. Moreover, local and stimuli-responsive delivery systems should be developed to decrease off-tumour toxicity. As more computational techniques emerge for protein structure prediction and engineering, the use of engineered virus-derived proteins[126] might improve the specificity of these therapies and decrease potential toxicity. Increasing viral particle specificity for tumour cells could be an important way to increase the proportion of tumour-associated antigens presented by local APCs.

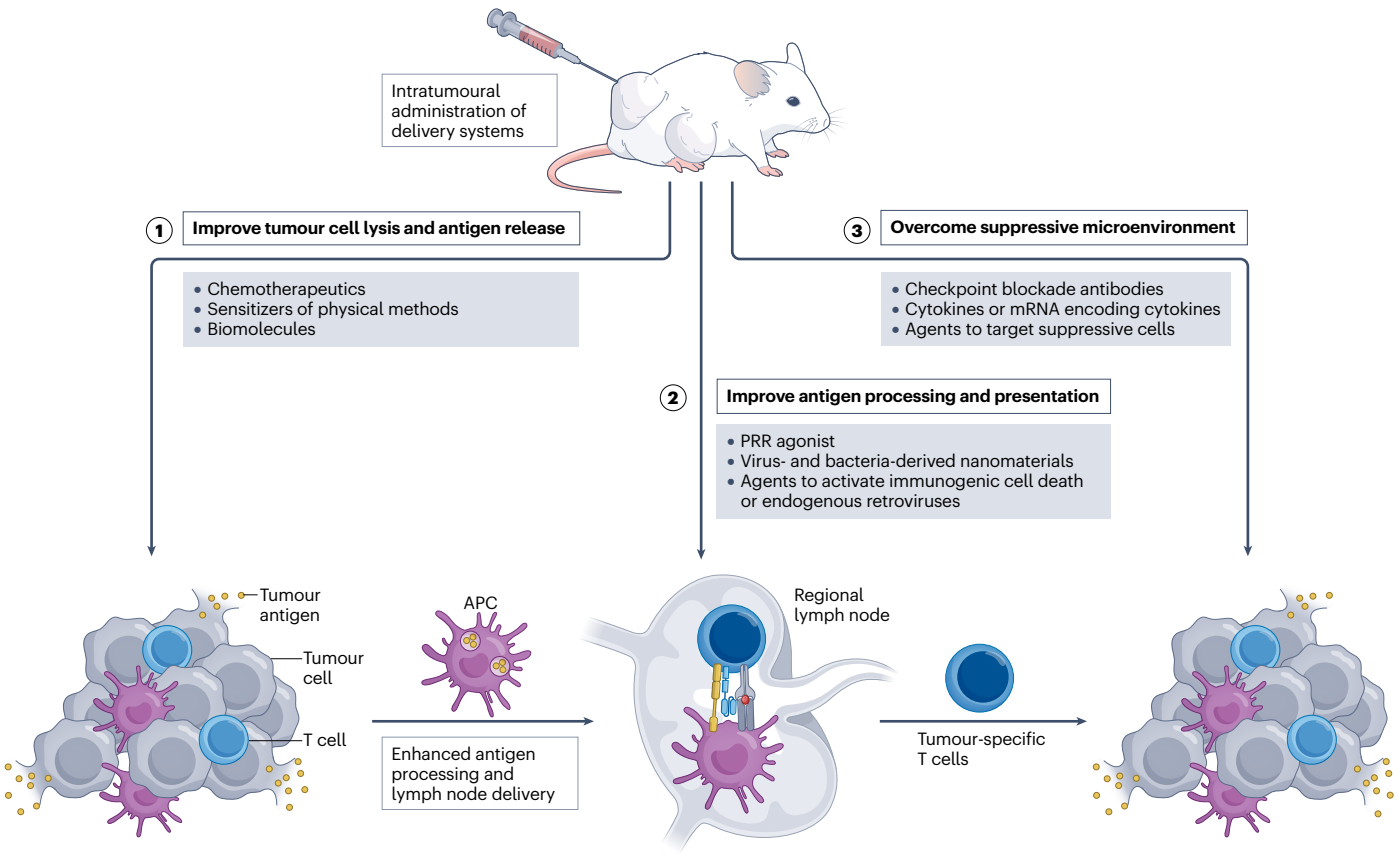

Fig. 3 | Improving in situ cancer vaccines using delivery technologies. Delivery technologies can be used to enhance the action of in situ cancer vaccines at several stages of the cancer-immunity cycle. To improve tumour cell lysis and release of tumour antigens, methods include local or systemic delivery of chemotherapeutics, physical therapy sensitizers or biomolecules that lead to tumour cell lysis (step 1). To improve antigen processing and presentation, methods include delivery of pattern recognition receptor (PRR) agonists or virus- or bacteria-derived materials, or induction of immunogenic cell death by methods that include the delivery of agents that activate endogenous retroviral genes. Improved tumour antigen release and activation of antigen-presenting cells (APCs) leads to trafficking of APCs to lymph nodes and the generation of tumour-specific T cells (step 2). Methods to overcome the suppressive immune microenvironment include the delivery of reagents that target immune checkpoints and the delivery of cytokines or agents that aim to reprogramme immunosuppressive cells (step 3).

Table 1 | Selected delivery technologies for enhanced tumour antigen release

Delivery technology	Therapeutic agent	Mechanism of cell death	Status (clinical trial number)	Refs.
Delivery of chemotherapeutics				
INT230-6 (cell penetration enhancer)	Cisplatin, vinblastine	Apoptosis and necrosis	Phase I/II (NCT04781725, NCT03058289)	200
Poly(L-histidine) and hyaluronic acid-based nanoparticle	Doxorubicin	Apoptosis and necrosis	Preclinical	71
Alginate-based gel	Oxaliplatin	Apoptosis and necrosis	Preclinical	73
Delivery of sensitizers for physical methods				
Hensify (HfO_2-containing nanoparticles)	Radiation	Heat and ROS	Phase I/II (NCT02379845, NCT04484909, NCT04505267, NCT04862455, NCT04615013, NCT05039632)	201
Gold nanoclusters	Radiation	Heat and ROS	Preclinical	76,79
Hf-based metal–organic framework	Radiation	Heat and ROS	Preclinical	77
Liposomes loading indocyanine green	Photodynamic therapy	ROS generation	Preclinical	80
TAPP-GCP@TCPP@BSA	Sonodynamic therapy	ROS generation	Preclinical	80
Gold nanorods	Photothermal therapy	High-temperature-induced necrosis; cell membrane damage	Preclinical	87
HMME/R837@Lip	Focused ultrasound	High-temperature-induced necrosis; mechanical disruption; cell membrane damage	Preclinical	84
Liposomes loaded with ethyl formate	Microwave ablation	High-temperature-induced necrosis; cell membrane damage	Preclinical	90
MgO and Fe_3O_4 nanoparticles	Cyroablation therapy	Cell membrane damage; necrosis	Preclinical	91,92
Delivery of biomolecules				
Lipid nanoparticles	α-Melittin	Cell membrane damage; cell apoptosis; necrosis	Preclinical	93
Nanoparticles	Repebody–apoptin	Cell membrane damage; cell apoptosis; necrosis	Preclinical	94
Mesoporous nanoparticles	Annexin A5	Necrosis	Preclinical	95

BSA, bovine serum albumin; HMME, haematoporphyrin monomethyl ether; ROS, reactive oxygen species; TCPP, meso-tetra(4-carboxyphenyl) porphyrin.

Delivery of bacteria-derived materials. Similar to virus-induced innate immune activation, bacterial or bacteria-derived materials can also be recognized by PRRs and enhance antigen processing and presentation for in situ vaccines[127]. Because of the high infection risk of live bacteria, many studies use bacterial outer membrane vesicles (OMVs) for in situ vaccines[128–131]. For example, eukaryotic–prokaryotic vesicles[128] prepared by encapsulating the *Salmonella* OMVs and indocyanine green-based polymeric nanoparticles into melanoma cytomembrane vesicles were administered to mouse tumours, which were then irradiated with NIR light. The PDT effect led to tumour cell lysis and tumour antigen release[128]. The eukaryotic–prokaryotic vesicles enhanced antigen processing and presentation to generate potent antitumour T cell responses. Another study[131] showed that hybrid membranes from bacterial OMVs and B16F10 melanoma cancer cells coated onto hollow polydopamine nanoparticles also achieved a strong in situ vaccination effect. Moreover, combining bacteria-derived nanoparticles with radiation therapy[130] and photothermal therapy[129] to enhance tumour antigen release could further enhance the in situ vaccination effect. These successes in preclinical models demonstrate the potential of bacteria-derived materials for in situ cancer vaccines. However, these materials could induce a systemic inflammatory response and lead to toxicity, so future studies should systematically evaluate the biodistribution, metabolism and clearance of these materials, and some well-established controlled-release delivery systems[132,133] should be used.

Targeting intrinsic stress pathways

The delivery of reagents that target intrinsic stress pathways, such as ICD pathways[134] and endogenous retroviral gene activation[135], is also effective in activating PRRs (Fig. 4b). Compared with PRR agonists, these reagents do not directly activate PRRs but they lead to the release of DAMPs from dying tumour cells that can be recognized by PRRs to activate innate immune responses.

Targeting immunogenic cell death. Depending on the initial stimulus, the death of cancer cells can be immunogenic (ICD) or non-immunogenic[38]. ICD is characterized by dying or injured cells secreting, or releasing, DAMPs such as CRT, ATP, HMGB1, heat shock proteins, uric acid, histones and extracellular DNA or RNA[134]. These DAMPs, together with tumour antigens, activate dendritic cells to generate cytotoxic

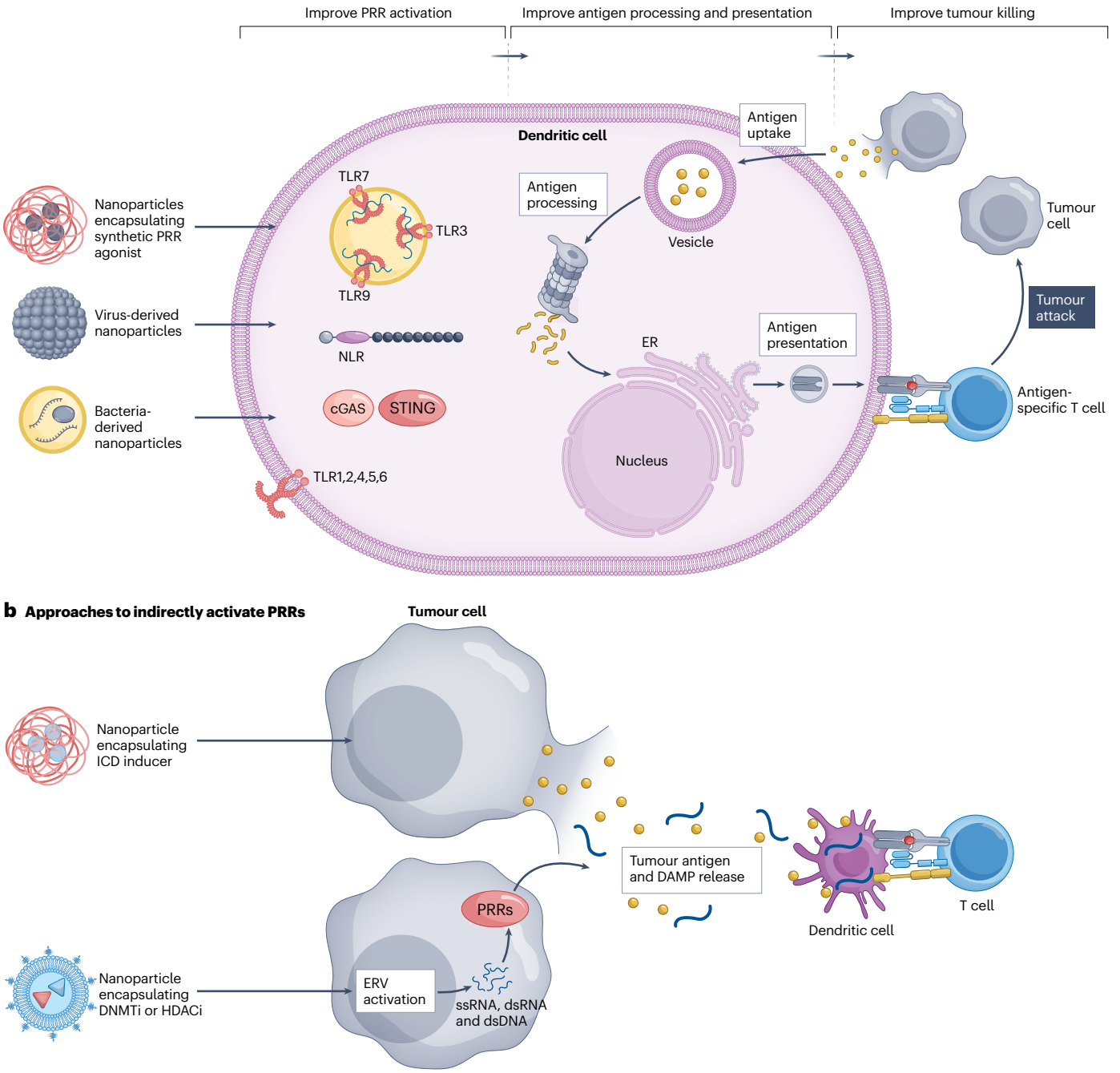

Fig. 4 | Delivery technologies to improve tumour antigen processing and presentation. a, Delivery technologies that directly activate pattern recognition receptors (PRRs) include nanoparticles that deliver synthetic PRR agonists, virus-derived materials or bacteria-derived materials. These agents are recognized by PRRs such as Toll-like receptors (TLRs) expressed on the plasma membrane or endosomes, the NOD-like receptor (NLR) or cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS–STING). Active PRRs stimulate antigen processing and presentation to improve tumour killing. **b**, Approaches to indirectly activate PRRs include nanoparticles that deliver inducers of immunogenic cell death (ICD) resulting in tumour cell lysis, release of tumour antigens and damage-associated molecular patterns (DAMPs), and the subsequent activation of PRRs in dendritic cells. Also, nanoparticles that deliver DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis) can activate endogenous retroviral (ERV) genes in cancer cells and lead to the formation of single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) and double-stranded DNA (dsDNA) in the cytoplasm. These nucleic acids can be recognized by PRRs and lead to the expression of type I interferons and pro-inflammatory cytokines in the tumour tissue that trigger innate immune responses. ER, endoplasmic reticulum.

T cells for tumour cell killing. ICD can be induced by chemotherapeutics or by physical therapies that kill cancer cells through the generation of ROS.

Many chemotherapeutics such as doxorubicin, epirubicin, idarubicin, mitoxantrone, bleomycin, bortezomib, cyclophosphamide and oxaliplatin induce ICD, but most of these drugs are hydrophobic and lack tumour specificity[136]. As discussed earlier, their bioavailability and tumour specificity can be improved through the use of various delivery technologies. The improved delivery of chemotherapeutics to tumour cells enhances the induction of ICD in tumour cells.

High levels of ROS induce ICD in tumour cells[137] by damaging subcellular organelles and plasma membranes, leading to the release of DAMPs, tumour-associated antigens and pro-inflammatory cytokines and chemokines, and thereby stimulating tumour-specific T cell responses[138]. Radiotherapy, PDT and SDT cause cell death mainly by inducing high levels of ROS in cells[139]. However, the in situ vaccination efficacy of these technologies is hindered because innate immunity mechanisms are not activated and ICD induction is low. Nanosensitizers have the potential to solve this problem by improving ROS generation and the activation of innate immunity during radiotherapy, PDT and SDT. However, it is important that nanosensitizers have a well-defined mechanism for inducing ICD, a stable structure and function, controllable preparation and satisfactory biological safety.

Targeting endogenous retroviral genes. Endogenous retroviruses (ERVs) are ancient deactivated retroviral elements that account for almost 8% of the human genome[140]. Under normal circumstances, they are mostly dormant and kept silent by heterochromatin maintenance factors such as DNA methyltransferases and histone methyltransferases[141]. Activation of ERVs by a therapeutic can potentially give rise to the presence of neoantigens in cancer cells, thus increasing the visibility of cancer cells to immune surveillance by the host[142]. ERV activation can also activate other transposable elements such as Alu elements[143] and long interspersed elements[144] to induce viral mimicry, whereby a cell responds as if it were infected by an exogenous virus[145] and mounts an innate immune response. The ensuing production of type I and III interferons and other cytokines promotes an in situ vaccination effect. Activation of ERVs also generates double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and double-stranded DNA (dsDNA) in the cytoplasm, which are detected by cytoplasmic sensors such as MDA5 to activate the MDA5–MAVS–TBK1 pathway[146]. This pathway promotes type I interferon activation, antigen processing and presentation, and neoantigen-specific T cell generation, resulting in control of tumour growth. Therefore, the ERV genes are a promising target for in situ vaccination applications.

The activation of ERVs in cancer cells has been achieved by using epigenetic drugs[147] such as DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis). Several of these inhibitors have been approved by the FDA[148] but they have low solubility, low bioavailability and lack tumour specificity. Therefore, delivery systems are required to achieve their potential for in situ cancer vaccination. For example, a PLGA–PEG di-block copolymer[149] has been formulated to stabilize the DNMTi azacitidine. In mouse breast cancer models, these nanoparticles showed increased drug solubility and bioavailability, enrichment in cancer cells, pH-responsive drug release and a greater antiproliferative effect[149], which eventually led to enhanced therapeutic efficacy compared with treatment with azacitidine alone. In another study, nanoparticles delivering pH-responsive prodrugs were designed to protect HDACis from external metabolism[150]. This stimuli-responsive delivery system dramatically improved the efficacy of HDACis, specifically in solid tumour therapies, by decreasing HDACi release in unwanted tissues. In addition, targeted delivery of an epigenetic inhibitor to cancer cells was achieved by using T cell membrane-derived vesicles[151]. ORY-1001, an inhibitor of lysine-specific histone demethylase 1 (LSD1; also known as KDM1A), was encapsulated in programmed cell death protein 1 (PD1)-displaying nanovesicles called OPEN[151]. The OPEN nanovesicles bind to and block PDL1 on cancer cells and are internalized to enable cell-specific delivery of ORY-1001, thereby inducing the accumulation of the Lys4 mono- and dimethylated forms of histone H3, which are associated with gene activation. ORY-1001 was shown to activate retroviral genes and upregulate the expression of interferons and downstream interferon-stimulated genes such as major histocompatibility complex class I (MHC I) and PDL1. Upregulated MHC I improves antigen presentation, thus facilitating the generation of tumour antigen-specific T cells[151]. Given that all materials used for the construction of OPEN are biocompatible and that the preparatory technologies are achievable in industrial settings, this platform has great potential for clinical translation.

Altogether, these studies demonstrate that delivery systems can be used to target epigenetic modifiers of ERV gene activation to cancer cells and tumour-infiltrating immune cells and therefore improve in situ cancer vaccination. However, although delivery technologies can improve the specificity of these inhibitors at the tissue or cell level, adverse effects are also likely because the entire genome is being exposed to the drug.

Overcoming the immunosuppressive tumour microenvironment

The generation of antigen-specific immune responses against tumours is one of the goals of a successful in situ cancer vaccine[152]. Ideally, the antigen-specific T cells that are generated should not only kill cancer cells in the treated tumour, but should also eradicate tumour cells in distant foci. Solid tumours have a highly immunosuppressive microenvironment[21], with activation of various immune checkpoints[153] and an abundance of immunosuppressive cells and cytokines. Antigen-specific T cells can become exhausted, inactivated and/or dead in such microenvironments, and therefore the tumour escapes elimination by immune cells. Here, we discuss the use of drug delivery technologies that target immune checkpoints, cytokines and suppressive immune cells for improved in situ cancer vaccination (Fig. 5).

Targeting immune checkpoints

T cell proliferation and activation are tightly regulated by several co-stimulatory and inhibitory signalling molecules[154]. The inhibitory signals are mediated by several immune checkpoint proteins, such as PD1 (ref. 155), cytotoxic T lymphocyte associated protein 4 (CTLA4)[156], B and T lymphocyte attenuator (BTLA)[157], IDO1 (ref. 158) and CD47 (ref. 159). Within the tumour microenvironment, cancer cells often evade antitumour immune responses by activating these immune checkpoints. Combinations of in situ vaccines and immune checkpoint inhibitors improve tumour growth inhibition in many cancer models[160–162]. However, immune checkpoint blockade can be cleared easily, is difficult to retain in the tumour tissue and can cause toxicity. These issues restrict further improvement of the antitumour vaccination effect.

Local delivery of immune checkpoint inhibitors that synergize with in situ vaccines is a potential way to improve vaccination effects

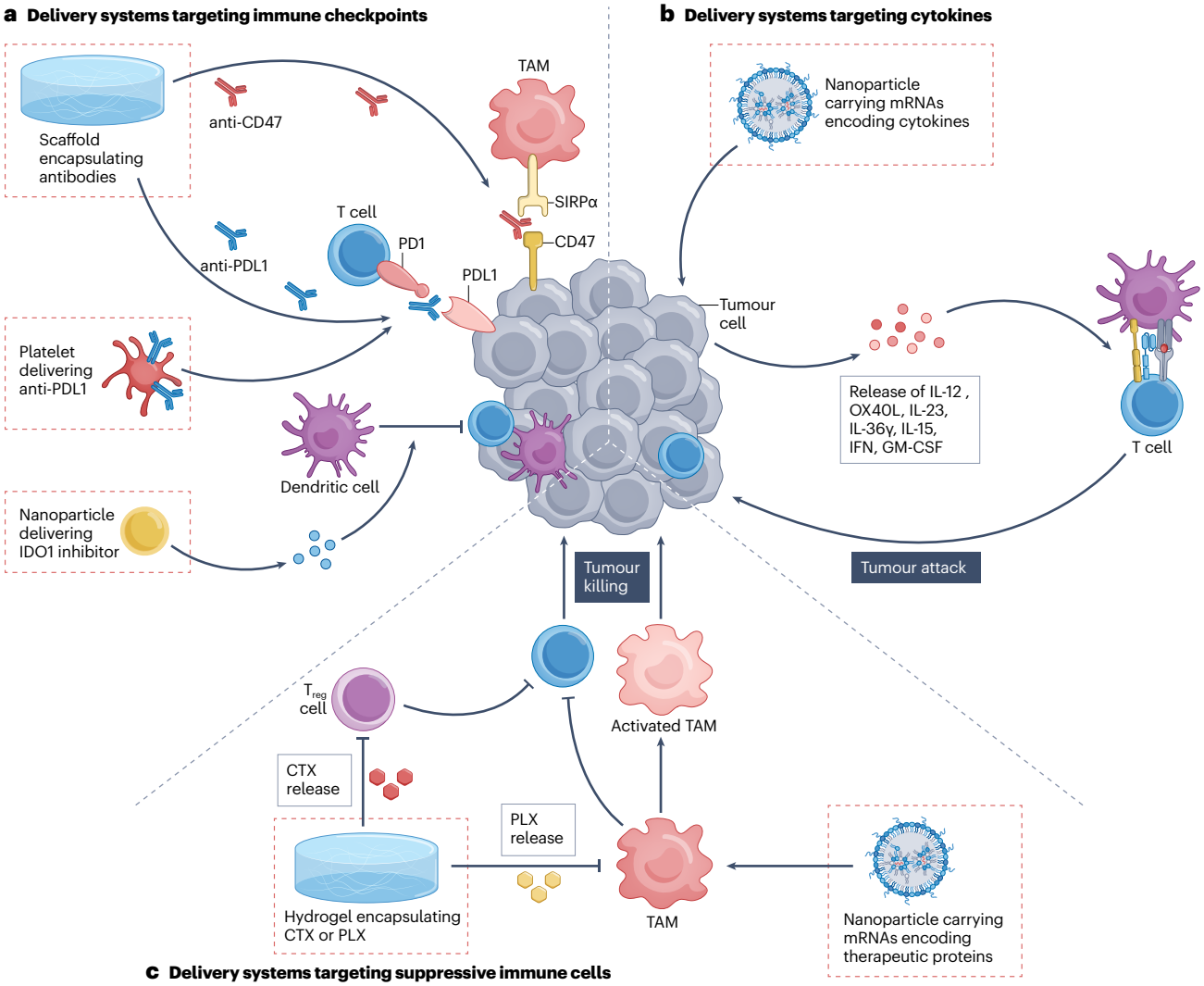

Fig. 5 | Delivery technologies to overcome the suppressive immune microenvironment. a, Delivery systems for immune checkpoint inhibitors are used in combination with in situ vaccines to enhance the vaccination effect. For example, scaffolds encapsulating anti-CD47 or anti-programmed cell death 1 ligand 1 (PDL1) antibodies; platelets that deliver an anti-PDL1 antibody; or nanoparticles that deliver an indoleamine 2,3-dioxygenase 1 (IDO1) inhibitor. **b**, For localized delivery of cytokines, nanoparticles encapsulating mRNAs that encode various combinations of cytokines are used to stimulate dendritic cells for antigen processing and presentation. **c**, Delivery systems to target suppressive immune cells include hydrogels that deliver cyclophosphamide (CTX) or pexidartinib (PLX) to induce the depletion of regulatory T (T_{reg}) cells or tumour-associated macrophages (TAMs), respectively. Also TAM-targeted nanoparticles deliver mRNAs encoding IRF5 and IKKB to activate the macrophages and promote tumour inhibition. GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; PD1, programmed cell death protein 1; SIRPα, signal regulatory protein-α.

in tumours[163] (Fig. 5a). For example, a polydopamine nanoparticle (PN)-based in situ vaccination strategy was designed. Imiquimod was loaded into nanoparticles that were then surface-modified with anti-PDL1 antibody (PDL1Ab–IQ/PNs)[164]. NIR light irradiation induced a photothermal effect, which led to tumour cell lysis and the generation of antitumour immune responses. The anti-PDL1 antibodies increased the binding of nanoparticles to a colorectal cancer cell line overexpressing PDL1. In vivo, PDL1Ab–IQ/PNs completely prevented the growth of a secondary challenged tumour at a distant site, which greatly improved mouse survival[164]. Another study used a designer scaffold loaded with doxorubicin, the TLR7/8 agonist resiquimod and an anti-PDL1 antibody for post-surgical in situ vaccination[165]. The high levels of anti-PDL1 antibody bound to tumour cells served to inhibit PDL1–PD1-mediated T cell suppression in the tumour tissue, and doxorubicin and resiquimod induced tumour cell ICD and APC activation, respectively. This treatment led to substantial inhibition of tumour recurrence and a prolonged mouse survival rate of 100% over a month-long period[165]. In a further study, anti-PDL1 antibody was chemically conjugated to the surface of platelets[166]. In mice bearing partially removed primary melanomas or triple negative breast carcinomas, anti-PDL1 antibody was effectively targeted to the post-surgical cavity and released from platelet-derived microparticles upon platelet activation[166].

For some patients, immune checkpoint blockade therapy induces toxicity in normal tissues[167]. A therapeutic scaffold[168] was designed to solve this problem. This scaffold, when formed in situ, allows for the local release of gemcitabine and an anti-PDL1 antibody in response to high ROS levels in the tumour microenvironment. The gemcitabine and anti-PDL1-co-loaded scaffold greatly improved the antitumour immune response compared with the untreated control group[168]. This stimuli-responsive delivery system restricted the release of anti-PDL1 to the tumour area, which suppressed tumour growth and simultaneously decreased toxicities related to anti-PDL1 therapy. In another study, a MMP-responsive delivery system was designed to improve tumour penetration of an immune checkpoint inhibitor[169]. A prodrug nanoplatform was developed by integrating a PEGylated IDO1 inhibitor (epacadostat) and a photosensitizer (indocyanine green) into nanoparticles[169]. These nanoparticles transformed into smaller nanoparticles (<40 nm) in response to the MMP in the tumour microenvironment and penetrated deep into tumour tissues. NIR light irradiation induced rapid tumour antigen release. Moreover, epacadostat significantly inhibited IDO1-mediated immunosuppression in the tumour microenvironment[169]. A strong antitumour immune response was observed in a mouse model of melanoma. In addition to ROS and MMP being used as stimuli for the local delivery of immune checkpoint inhibitors, other studies have shown that low pH[170] and the hypoxic tumour environment can also be used as triggers for stimuli-responsive delivery.

Another strategy for cancer immunotherapy is to boost the phagocytosis of tumour cells by targeting the CD47–signal regulatory protein-α (SIRPα) axis. Tumour cells avoid phagocytosis by upregulating the expression of the cell surface molecule CD47, which inhibits the SIRPα receptor expressed on macrophages and represses phagocytosis. A fibrin gel encapsulating an anti-CD47 antibody (aCD47@$CaCO_3$) was developed, which gradually released anti-CD47 into tumours in a pH-responsive manner[171]. The antibody disrupted CD47–SIRPα signalling, improved the activation of M1-type macrophages, induced the phagocytosis of cancer cells by macrophages, boosted antitumour immune responses and inhibited local tumour recurrence and metastasis. In another study[172], a mesoporous silica nanoparticle was designed to co-deliver anti-CD47 antibodies and doxorubicin. While the anti-CD47 antibody disabled the 'don't eat me' phagocytic signal, doxorubicin induced ICD and led to CRT exposure to the surface of tumour cells, which constitutes an 'eat me' signal. This design enhanced antigen cross-presentation by APCs and elicited efficient T cell-mediated immune responses in mouse models of breast cancer and melanoma without inducing toxicity.

Altogether, through local delivery and stimuli-responsive delivery technologies, in situ vaccination efficacy can be improved and the adverse effects caused by systemic immune checkpoint blockade can be minimized. The usefulness of these therapies will depend on how quickly and strongly tumour-specific immune responses can be generated. Immune checkpoint blockade antibody-based therapies show low responses to many types of tumour because of the low expression of immune checkpoint proteins on tumour cells, so future studies should combine immune checkpoint blockade with therapeutics to enhance the expression of immune checkpoints in tumour tissues and further improve vaccination efficacy.

Glossary

Adjuvant
A substance that enhances the immune response to an antigen with which it is mixed.

Antigen-presenting cells
(APCs). Immune cells that process antigens and display their peptide fragments on the cell surface together with molecules required for T cell activation. The main APCs for T cells are dendritic cells, macrophages and B cells.

Cytokines
Secreted proteins that act on specific cytokine receptors to affect cellular behaviour. Cytokines made by lymphocytes are often called lymphokines or interleukins.

Enhanced permeability and retention effect
(EPR effect). An effect defined by the heightened build-up of macromolecules, including liposomes, drugs and nanoparticles, in tumours compared with normal tissues. This phenomenon occurs because the blood vessels in tumour areas are permeable and the lymphatic system is impaired.

Immune checkpoint
Inhibitory regulator of the immune system that is crucial to maintain self-tolerance, prevent autoimmunity and control the duration and extent of immune response to minimize collateral tissue damage. Immune checkpoint proteins are often overexpressed on tumour cells and compromise the antitumour immune response.

Immune escape
The growth and metastasis of tumours by avoiding recognition and attack by the immune system through various mechanisms.

Immune surveillance
The detection and elimination of tumours by lymphocytes specific for tumour antigens.

Pattern recognition receptors
Proteins capable of recognizing molecules frequently found in pathogens (pathogen-associated molecular patterns; PAMPs) or molecules released by damaged cells (damage-associated molecular patterns; DAMPs).

Stimuli-responsive materials
Materials that are capable of altering their chemical and/or physical properties upon exposure to external stimuli.

T cell exhaustion
The gradual loss of T cell effector function and memory characteristics that occurs under continuous antigen exposure.

Immune modulation via cytokines

Cytotoxic T lymphocytes have a key role in cancer immunotherapy[173]. A potent tumour immunotherapy not only requires activation of antitumour effector cells but also relies on cytokines to enable the development of antitumour T cells[174]. As immune regulators, cytokines have a major role in modulating the tumour microenvironment. Indeed, IL-2, IL-12 and IL-15 have shown great promise in modulating the immunosuppressive microenvironment for in situ cancer vaccination applications[175–177]. However, the clinical use of cytokines has been limited because they can induce severe systemic toxicity and have a short half-life after administration.

In comparison with traditional cytokine administration routes such as intravenous injection, local delivery of cytokines might increase therapeutic efficacy as well as decrease systemic toxicity (Fig. 5b). For example, IL-12 was modified onto the surface of liposomes and the nanoparticles were further coated with poly-L-arginine and poly-L-glutamic acid to form PLE-IL-12-NPs[176]. After intratumoural injection, the PLE-IL-12-NPs preferentially localized to the outer surface of tumour cells and acted to deposit IL-12. The slow release of IL-12 led to a decreased number of regulatory T (T_{reg}) cells in the tumour. Furthermore, the PLE-IL-12-NPs inhibited tumour growth in mouse models of colon cancer

and ovarian cancer without inducing systemic toxicity. Another study designed an IL-12-loaded PLGA nanoparticle that was surface-modified with anti-CD8 and anti-glypican 3 antibodies[177]. These nanoparticles function as bispecific T cell engagers that bind to $CD8^+$ T cells and glypican 3^+ HepG2 tumour cells to induce tumour cell lysis. Moreover, the release of IL-12 in the tumour microenvironment enhanced the expansion, activation and cytotoxic activity of antigen-specific T cells and eventually led to greater inhibition of tumour growth[177].

Although direct delivery of stimulatory cytokines can improve in situ vaccination, typically cytokines are cleared easily and have short durability. Using delivery systems that contain mRNAs that encode cytokines could promote the persistence of cytokines and improve the in situ vaccine effect. For example, intratumoural administration of LNPs that deliver mRNAs that encode the cytokines IL-23 and IL-36γ and the T cell co-stimulator OX40L (mRNA-2752) led to in situ vaccination and $CD8^+$ T cell-dependent tumour regression in mouse models of melanoma and colon cancer[178]. Also, local administration of LNPs encapsulating mRNAs encoding IL-12 single chain (IL-12sc), IL-15 with the sushi domain of the IL15Rα, interferon-α (IFNα) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (SAR44100) mediated successful antitumour immunity[179]. A combination of SAR44100 and immune checkpoint inhibitors improved overall mouse survival[179]. Although these strategies are effective, the duration of expression of the cytokine-encoding mRNAs is low, which restricts the production of long-lasting antitumour immune responses. To solve this problem, a self-replicating RNA encapsulated in LNPs[180] was designed with three key elements: first, an LNP composition called TT3 that promotes ICD; second, RNA that stimulates danger sensors in transfected cells; and third, RNA that encodes IL-12 for modulation of immune cells. Intratumoural administration of LNPs encapsulating RNA replicons led to high expression of IL-12 and cancer cell ICD[180] as well as a type I interferon response. These effects eventually resulted in a highly inflamed tumour microenvironment and primed systemic antitumour immunity[180].

In all, delivery of cytokines or mRNAs encoding cytokines can overcome the suppressive immune microenvironment and improve in situ cancer vaccination. Clinical translation of cytokine therapy is hindered by limitations in preclinical animal models. For example, the immune systems of mice and humans have important differences in cellular make-up, receptor expression and cytokine responses[181]. Mice seem to have altered IL-2R biology[182], which could be a major contributor to the increased toxicity observed when IL-2 was tested in humans compared with mouse models[183]. Therefore, more clinically relevant models such as humanized mouse models and patient-derived 3D organoids should be developed for the evaluation of cytokine-based therapeutics.

Modulating suppressive immune cells

Distorted blood vessels in the tumour microenvironment and the rapid growth of tumour cells directly result in hypoxia[184], which leads to the accumulation of immunosuppressive cells such as T_{reg} cells, myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages (TAMs)[185]. M1-like TAMs are pro-inflammatory and tumoricidal, whereas M2-like TAMs are anti-inflammatory and pro-tumoural. The immune inhibitory cells secrete immunosuppressive cytokines such as TGFβ, VEGF and IL-10 (ref. 186). To enhance the efficacy of in situ cancer vaccination, these immunosuppressive cells can be targeted by nanoparticles to either deplete them or transform them into a more immunosupportive state (Fig. 5c).

Depletion of suppressive immune cells can be achieved by chemotherapeutics such as docetaxel and cyclophosphamide (CTX), which induce apoptosis of these cells[187,188]. An injectable fibrin hydrogel[189] that can be administered locally and that enables the sequential release of CTX and an anti-PDL1 antibody was designed. CTX acted to deplete T_{reg} cells in the tumour tissue and synergized with the delayed release of the anti-PDL1 antibody to induce a strong in situ vaccination effect[189]. In mouse models, the hydrogel formulation exhibited promising inhibition of post-surgery tumour recurrence and metastasis. Another study[190] reported a biocompatible alginate-based hydrogel loaded with pexidartinib-encapsulated nanoparticles. The hydrogel gradually released pexidartinib, leading to inhibition of colony-stimulating factor 1 (CSF1) receptors and TAM depletion at the tumour site. The controlled depletion of TAMs creates a favourable milieu for local and systemic delivery of anti-PD1 antibody-conjugated platelets to inhibit post-surgery tumour recurrence in mouse models[190]. These studies confirm that suppressive immune cell depletion or modulation by the delivery of certain chemotherapeutics might be useful in eliciting a strong in situ vaccination effect.

The delivery of therapeutic RNAs to reprogramme immunosuppressive cells is another approach to enhance in situ vaccination. Specifically, charge-altering releasable transporters (CARTs) have been developed[191] to deliver mRNA to TAMs. CARTs were used to deliver a combination of mRNAs encoding the immunomodulatory ligands OX40L, CD80 and CD86 to various subcutaneous bilateral tumour models[192], in which only one tumour was treated[192]. Upon intratumoural administration, CARTs transfected 28% of TAMs, reprogrammed the immune cells to produce immunostimulatory cytokines and eliminated the treated tumour and induced systemic antitumour immunity. Others have used nanoparticles modified with motifs that target TAMs to improve the specificity of nanoparticle delivery to these cells. For example, biodegradable polymeric nanoparticles were functionalized with di-mannose moieties on their surface[193] to target TAMs and were used to encapsulate mRNAs that reprogramme these cells. An mRNA encoding the transcription factor IRF5 was combined with another encoding the IRF5-activating kinase IKKB, which together downregulated the expression of M2 genes, such as *Serpinb2* and *CCL11*, and upregulated the M1 gene *Ccl5*. In a mouse ovarian tumour model, intraperitoneal injections of these nanoparticles effectively reprogrammed TAMs towards the M1-like state and increased infiltration of T cells and neutrophils into the tumours[193]. The same treatment was also effective in murine models of ovarian cancer, glioma and lung cancer[193].

Altogether, strategies can be designed to deliver small molecules or RNAs to inhibit immunosuppressive cells for improved in situ cancer vaccination. A challenge to the use of chemotherapeutics to deplete immunosuppressive cells is that these molecules are also toxic to several types of immune cells. Future studies should develop precision targeting systems to enable better targeting of immunosuppressive cells. Because the optimal synergistic effect of cytokines might be achieved when they are expressed in a sequential manner, design of delivery systems that enable sequential expression of several cytokines could further enhance the vaccination outcome.

Targeting metabolism

Cancer cells undergo modifications in their cellular metabolism to facilitate their growth and evade immune cell detection and elimination. One prominent metabolic alteration observed in cancer cells is the upregulation of aerobic glycolysis, leading to heightened glucose consumption and the accumulation of lactic acid, known as the Warburg effect[194]. This increased lactic acid production can hinder T cell activation, impede macrophage activity and diminish the antigen

presentation capacity of dendritic cells. Tumour cells also undergo changes in tryptophan and glutamine catabolism, macromolecular synthesis and redox homeostasis, all of which collectively contribute to the establishment of a suppressive immune microenvironment.

Delivery of metabolism-modulating compounds into tumours has emerged as a potential strategy for in situ cancer vaccination. For instance, one study[195] designed a liposomal nanoparticle to co-deliver the mTOR inhibitor rapamycin and an anti-angiogenic agent, regorafenib. Inhibition of mTOR reduced glycolytic metabolism and led to diminished lactic acid production. This nanoparticle effectively repolarized macrophages towards the M1 subtype and reversed the immunosuppressive tumour microenvironment. In vivo, the nanoparticle enhanced antitumour immune responses in a colon cancer mouse model. In another study[196], a redox-responsive nanoparticle was developed to co-deliver doxorubicin and lactate dehydrogenase A (*LDHA*) small interfering RNA. Doxorubicin triggered ICD and DAMP release, while inhibition of *LDHA* reduced lactic acid generation, resulting in decreased recruitment of MDSCs in the tumour tissue. These improvements ultimately enhanced in situ tumour vaccination in a mouse model of breast cancer. In addition to modulation of lactate metabolism, alteration of kynurenine metabolism is another potential avenue for altering the tumour microenvironment. A semiconducting polymer conjugated with kynureninase (SPNK) was designed to intervene in the kynurenine metabolism pathway[197]. Under NIR light irradiation, SPNK generated singlet oxygen, inducing cancer cell ICD and activating kynureninase to degrade the immunosuppressive kynurenine. This synergistic effect mediated by the nanoparticle led to systemic antitumour immunity that inhibited the growth of mouse melanoma in vivo.

Nanotherapeutics such as those described above aim to enhance antitumour immunity by manipulating the metabolism of cancer cells and immune cells that infiltrate the tumour. However, these approaches often neglect the impact on cancer stem cells (CSCs), which have a role in tumour recurrence, metastasis and antitumour immune responses[198]. Unlike highly glycolytic cancer cells, CSCs mainly rely on oxidative phosphorylation for their energy requirements. Thus, a promising strategy involves developing delivery systems that simultaneously regulate the metabolic characteristics of CSCs and other cancer cells. Additionally, cancer cells dynamically adjust their metabolism to support metastasis, leading to variations in cellular metabolism between primary tumours and metastatic sites[199]. Therefore, nanomedicines capable of modulating cancer metabolism in both primary tumours and micrometastases could potentially enhance antitumour immunity against both primary and metastatic cancer.

Clinical studies

Many drug delivery systems are under clinical evaluation for in situ cancer vaccine applications (Table 2). To improve tumour antigen release, a formulation INT230-6 was designed that consists of the cytotoxic agents cisplatin and vinblastine, combined with the cell penetration enhancer 8-((2-hydroxybenzoyl)amino) octanoate (IT-006). This enhancer greatly improved the transport of the hydrophilic chemotherapeutics across lipid-based cell membranes and thus improved tumour cell lysis and killing by the chemotherapies[200]. INT230-6 alone or in combination with immune checkpoint blockade therapies (anti-PD1 or anti-CTLA4 antibodies) is currently under investigation in clinical trials for treatment of multiple malignant cancers (NCT04781725, NCT03058289).

As another method for improving antigen release, the hafnium oxide nanoparticle-based radiosensitizer Hensify in combination with radiation therapy is approved in the EU for the treatment of locally advanced soft tissue sarcoma[201]. After activation by ionizing radiation, Hensify yields a localized high-energy deposit and increases tumour cell killing and tumour-specific immune responses. In a phase II/III clinical trial, Hensify doubled the percentage of patients with a pathologically complete response rate compared with patients who received radiation therapy alone (NCT02379845). Multiple clinical trials are investigating Hensify, alone or in combination with immunotherapies, for the treatment of various types of cancer (Table 2).

To improve antigen processing and presentation, a poly-lysine-stabilized poly(I:C) termed Hiltonol is being tested in multiple clinical studies. These studies include combining Hiltonol with radiation therapy for the treatment of recurrent B and T cell lymphomas (NCT00880867; NCT02061449), and combining it with anti-PD1 or anti-PDL1 antibodies for the treatment of head and neck cancer, sarcoma and skin cancers (NCT02423863). Similarly, poly(I:C) formulated with the cationic carrier polyethylenimine (BO-112)[202] in combination with radiotherapy and anti-PD1 antibodies is under clinical evaluation for the treatment of metastatic non-small-cell lung cancer, soft tissue sarcoma and melanoma (Table 2). Moreover, other delivery systems such as glucopyranosyl lipid-A (GLA) in a stable, oil-in-water emulsion (G100) and glycated *N*-acetylglucosamine polymer (IP-001) are under evaluation in clinical trials (Table 2). Additionally, a noninfectious virus-like particle encapsulating a TLR9 agonist (CMP-001)[203] has shown promise for inducing tumour cell lysis in early clinical trials[203], and combinations of CMP-001 with an anti-PD1 antibody, stereotactic body radiation therapy or an anti-OX40 monoclonal antibody are under clinical evaluation for the treatment of various cancers (Table 2).

To improve in situ vaccination by overcoming the suppressive immune microenvironment, clinical trials are underway for delivery systems of mRNAs encoding cytokines. LNPs delivering mRNA encoding IL-12 (MEDI1191)[204] have been tested systemically in combination with the anti-PDL1 antibody durvalumab (NCT03946800). Also, LNPs encapsulating OX40L mRNA (mRNA-2416) are being evaluated for treatment of refractory solid tumour malignancies or lymphoma (NCT03323398). Furthermore, LNPs that encapsulate mRNAs encoding several synergistic cytokines are being tested in patients. For example, an LNP delivering mRNAs encoding IL-23, IL-36γ and OX40L (mRNA-2752)[178] is in phase I trials (NCT03739931). Another LNP encapsulating mRNAs encoding IL-12 single chain, IL-15 with the sushi domain of the IL15Rα, IFNα and GM-CSF (SAR44100; BNT131) has also been tested in patients (NCT03871348)[179].

Overall, a growing number of clinical studies are exploring delivery technology-based in situ vaccines. It is important to note that tumours with higher mutational burden might release a greater diversity of tumour neoantigens[205], potentially resulting in more robust antigen-specific immune responses. Therefore, assessing the mutation level in a patient's tumour is crucial to predict the potential benefits of an in situ vaccine. Additionally, considering that some immunotherapies have been linked to immune system overactivation and severe toxicities[206], it is essential for upcoming studies to systematically evaluate these risks before administering in situ vaccines.

Perspective and outlook

In situ cancer vaccination has had a major impact on cancer treatments in the clinic[8]. The identification of tumour neoantigens is difficult, and manufacturing ex vivo cancer vaccine products is resource intensive and time consuming[207]. However, in situ tumour vaccines avoid the need for antigen identification and isolation, which reduces delays

Table 2 | Delivery technology-based in situ vaccines in clinical trials

Name	Combinations	Conditions	Clinical trial stage (number)
INT230-6	NA	Breast cancer	Phase II (NCT04781725)
INT230-6	Anti-PD1 antibody, anti-CTLA4 antibody	Breast cancer, head and neck cancer, squamous cell carcinoma, lymphoma, pancreatic cancer, liver cancer, colon cancer, lung cancer, bile duct cancer, chordoma of sacrum, sarcoma	Phase I/II (NCT03058289)
Hensify	Radiation therapy, pembrolizumab	Head and neck squamous cell cancer	Phase II (NCT04862455)
Hensify	Radiation therapy, ipilimumab, nivolumab	Lung and/or liver metastases from solid malignancy	Phase I/II (NCT05039632)
Hensify	Radiation therapy, chemotherapy	Oesophageal cancer	Phase I (NCT04615013)
Hensify	Radiation therapy	Pancreatic cancer, NSCLC	Phase I (NCT04484909; NCT04505267)
Hiltonol (poly-lysine-stabilized poly(I:C))	Anti-PD1, anti-PDL1	Melanoma, head and neck cancer, sarcoma, non-melanoma skin cancers	Phase II, completed (NCT02423863)
Hiltonol	Low-dose radiation	B and T cell lymphoma	Phase I, completed (NCT00880867)
BO-112 (poly(I:C) formulated with the cationic carrier polyethylenimine)	Pembrolizumab	Malignant melanoma	Phase II (NCT04570332)
BO-112	Radiotherapy and nivolumab	NSCLC	Phase I (NCT05265650)
BO-112	Nivolumab	Soft tissue sarcoma	Phase I (NCT04420975)
G100 (GLA in emulsion)	Pembrolizumab, rituximab	Merkel cell carcinoma	Terminated (NCT02501473)
G100	MK3475, metronomic CTX	Advanced sarcomas	Phase II (NCT02406781)
CMP-001 (virus-like particle containing CpG-A)	Pembrolizumab	Head and neck squamous cell carcinoma	Completed (NCT04633278)
CMP-001	Nivolumab	Melanoma; Merkel cell carcinoma, cutaneous squamous cell carcinoma, triple negative breast cancer, metastatic cancer	Phase II (NCT04698187; NCT04916002)
CMP-001	Stereotactic body radiation therapy	Triple negative breast cancer	Phase II (NCT04807192)
CMP-001	Anti-OX40 monoclonal antibody	Pancreatic cancer, other cancers	Phase Ib/II (NCT04387071)
IP-001 (glycated *N*-acetylglucosamine polymer)	Thermal ablation	Colon cancer, NSCLC,soft tissue sarcoma, advanced solid tumours	Phase Ib/IIa (NCT05688280; NCT03993678)
MEDI1191 (LNP encapsulating mRNA encoding IL-12)	Durvalumab	Advanced solid tumours	Phase I (NCT03946800)
mRNA-2416 (LNP encapsulating OX40L mRNA)	Durvalumab	Solid tumour malignancies or lymphoma	Terminated (NCT03323398)
mRNA-2752 (LNP encapsulating mRNAs encoding OX40L, IL-23, IL-36γ)	NA	Solid tumour malignancies, lymphoma	Phase I (NCT03739931)
SAR44100 (LNP encapsulating mRNAs encoding IL-12 single chain, IL-15 with IL15Rα sushi domain e, IFNα and GM-CSF)	NA	Metastatic neoplasm	Phase I (NCT03871348)

CpG, unmethylated cytosine-guanine dinucleotide-containing oligodeoxynucleotides; CTLA4, cytotoxic T lymphocyte associated protein 4; CTX, cyclophosphamide; GLA, glucopyranosyl lipid-A; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFNα, interferon-α; IL15Rα, interleukin 15 receptor-α; LNP, lipid nanoparticle; NA, not applicable; NSCLC, non-small-cell lung cancer; PD1, programmed cell death protein 1.

and costs associated with exogenous production of a personalized vaccine[208] and thus provides an 'off-the-shelf' strategy for cancer vaccine manufacture[117]. Moreover, whereas tumour cells can easily escape from traditional single-antigen vaccine-mediated immune responses, in situ cancer vaccines lead to the generation of a polyclonal immune response against antigens expressed across different cancer cell subclones[209], providing a potential means to address tumour heterogeneity[210]. Because the antigens released from tumour cells are tumour mutation-derived peptides[211], in situ vaccination is patient specific and results in a personalized therapy[5]. Delivery approaches can improve in situ cancer vaccination by inducing tumour antigen release, facilitating antigen presentation to generate tumour-specific T cells and dampening the

suppressive microenvironment to improve T cell activity. Moreover, delivery technologies often reduce systemic leakage of the therapeutic agents and thus prevent off-target toxicity[22]. Although there are numerous clinical trials ongoing, some with encouraging results (Table 2), several considerations must be addressed.

The success of in situ vaccination hinges on the release of tumour antigens, but lysing tumour cells also releases self-antigens[212]. The processing of self-antigens by APCs can induce immune tolerance[213], which is undesirable for effective in situ vaccination in which the aim is to generate high-intensity and specific antitumour immune responses. To address this challenge, delivery technologies that can specifically capture tumour antigens and deliver them to APCs to induce tumour antigen-specific immune responses are urgently needed[214].

The ideal dose and schedule for in situ vaccines has not been established and is likely to vary according to the safety profiles of the immunomodulatory agents. Although systemic immunotherapy is generally dosed by patient weight, in situ vaccination can be dosed according to the volume of a specific tumour, the size of the overall tumour burden or patient weight. The dosing algorithm that is most appropriate can also vary based on whether the treatment regimen is more likely to cause a local reaction or systemic toxicity. In addition, the dose should also vary depending on the delivery technology as different delivery technologies have different bioavailability and toxicity characteristics. Furthermore, several in situ vaccination strategies require repeat dosing, so future studies should aim to develop sustained release strategies for more persistent in situ cancer vaccines.

The accessibility of tumour lesions is another challenge for in situ vaccines. Most in situ vaccine clinical studies have been performed in easily accessible lesions such as breast or skin cancers. However, less-accessible lesions could be accessed with the assistance of imaging modalities such as ultrasound[215] or CT[216]. The administration of therapeutics into tumours is not uniform, and some very dense tumours are difficult to inject. Ongoing and future clinical investigations will have to address these points to define the recommendations for in situ vaccinations and to optimize the efficacy of such local immunotherapies or combinatorial regimens.

In all, delivery technologies are increasingly being used to enhance in situ vaccines, showing effectiveness in enhancing antigen release, antigen processing and countering the suppressive tumour microenvironment to boost antitumour immune responses. These encouraging, immunomodulatory delivery technologies hold promise for broader application in both basic immunology research and clinical settings for in situ vaccination. With ongoing improvements, these delivery systems could significantly enhance the effectiveness of existing in situ vaccine-based cancer treatments and contribute to the creation of more advanced cancer immunotherapies.

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Author contributions

N.G., M.-G.A., D.W. and M.J.M. developed the concept, researched data and wrote the article. R.E.-M. and L.X. contributed substantially to discussion of the content. R.E.-M. helped with language and figure modifications. All authors reviewed and edited the manuscript before submission.

Competing interests

D.W. is named on patents that describe the use of nucleoside modified as a platform to deliver therapeutic proteins and vaccines. M.J.M, N.G., D.W. and M.-G.A. are named on patents describing the use of lipid nanoparticles and lipid compositions for nucleic acid delivery. The other authors declare no competing interests.

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